

TetraThymosin β Is Required for Actin Dynamics in *Caenorhabditis elegans* and Acts via Functionally Different Actin-binding Repeats

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Generating specific actin structures via controlled actin polymerization is a prerequisite for eukaryote development and reproduction. We here report on an essential *Caenorhabditis elegans* protein tetraThymosin β expressed in developing neurons and crucial during oocyte maturation in adults. TetraThymosin β has four repeats, each related to the actin monomer-sequestering protein thymosin β 4 and assists in actin filament elongation. For homologues with similar multirepeat structures, a profilin-like mechanism of ushering actin onto filament barbed ends, based on the formation of a 1:1 complex, is proposed to underlie this activity. We, however, demonstrate that tetraThymosin β binds multiple actin monomers via different repeats and in addition also interacts with filamentous actin. All repeats need to be functional for attaining full activity in various *in vitro* assays. The activities on actin are thus a direct consequence of the repeated structure. In containing both G- and F-actin interaction sites, tetraThymosin β may be reminiscent of nonhomologous multimodular actin regulatory proteins implicated in actin filament dynamics. A mutation that suppresses expression of tetraThymosin β is homozygous lethal. Mutant organisms develop into adults but display a dumpy phenotype and fail to reproduce as their oocytes lack essential actin structures. This strongly suggests that the activity of tetraThymosin β is of crucial importance at specific developmental stages requiring actin polymerization.

INTRODUCTION

Actin filament nucleation, elongation, and turnover form the basis of the dynamic remodelling of the actin cytoskeleton that takes place during cellular migration and cytokinesis. Multicellular organisms critically depend on these actin-based motile processes for development, reproduction, host defense, and survival. Many actin-binding proteins affect, in a regulated manner, either actin filament organization or the dynamics of the actin polymerization cycle (Ono *et al.*, 1999; Ono, 2001; Condeelis, 2001; Small *et al.*, 2002; Pollard and Borisy, 2003). The high dynamics of the actin system ultimately results from the interplay between differently functioning actin-binding proteins. Many of these exert their function via a single actin interaction site. However, the activity of a number of actin modulators is generated through combined activity of multiple actin-binding modules within the same molecule. This has evolved via different ways: either by multiplication of a specific module leading

to a repeated structure or by domain swapping, *i.e.*, by bringing together different actin-binding modules (Van Troys *et al.*, 1999). Gelsolin is a well-known example where homologous segments have different actin-binding activities and their combined activity is responsible for severing and subsequent capping (Pope *et al.*, 1991; Van Troys *et al.*, 1996a). Multiple differential actin-binding units have also been reported in Ena/VASP proteins (Bachmann *et al.*, 1999; Walders-Harbeck *et al.*, 2002), Calponin Homology-domain containing proteins (Gimona *et al.*, 2002), twinfilin (Ojala *et al.*, 2002), MARCKS (Yarmola *et al.*, 2001), and MIM (Mattila *et al.*, 2003; Yamagishi *et al.*, 2004).

Also the β -thymosin or WH2 module (Van Troys *et al.*, 1996b; Paunola *et al.*, 2002) is found as repeats. In Van Troys *et al.* (1999), we first reported the sequence of a hypothetical *Caenorhabditis elegans* protein tetraThymosin β with the striking feature that it consists of a four times-repeated thymosin β -module. The actin-binding determinants of this module are clearly delineated: a hexapeptide motif (¹⁷LKKTET²² in thymosin β 4) and a hydrophobic cluster in a preceding α -helix (Vancompernelle *et al.*, 1992; Van Troys *et al.*, 1996b; Rossenu *et al.*, 1997; Simenel *et al.*, 2000; Rossenu *et al.*, 2003; Domanski *et al.*, 2004). Given their repeated structures, tetraThymosin β , *Drosophila* ciboulot, and the amoeba protein actobindin (harboring four, three, and two repeats, respectively; Lambooy and Korn, 1986; Van Troys *et al.*, 1999; Boquet *et al.*, 2000) form a novel subset of β -thymosins. Both ciboulot and actobindin have been demonstrated to display a similar promotive effect on filament growth as is observed

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Abbreviations used: CMC, critical monomer concentration; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; RU, response unit; SPR, surface plasmon resonance; T β 4, thymosin β 4; tT β , tetraThymosin β ; WH, WASP homology; WT, wild type.

Table 1. Mutations introduced into tetraThymosin β

tT β R ₁ : LHST62-65AAAA (in R ₂), ^a LKKT100-103AAAA (in R ₃), LHHV134-137AAAA (in R ₄)
tT β R ₂ : LKKV23-26AAAA (in R ₁), LKKT100-103AAAA (in R ₃), LHHV134-137AAAA (in R ₄)
tT β R ₃ : LKKV23-26AAAA (in R ₁), LHST 62-65 AAAA (in R ₂), LHHV134-137AAAA (in R ₄)
tT β R ₄ : LKKV23-26AAAA (in R ₁), LHST 62-65 AAAA (in R ₂), LKKT100-103AAAA (in R ₃)
tT β R _{2,3,4} : LKKV 23-26 AAAA (in R ₁)
tT β R _{1,3,4} : LHST 62-65 AAAA (in R ₂)
tT β R _{1,2,4} : LKKT100-103AAAA (in R ₃)
tT β R _{1,2,3} : LHHV134-137AAAA (in R ₄)

^a R₁, R₂, R₃, and R₄ refer to the repeats 1 to 4; in the mutant nomenclature the repeat(s) indicated in the name are unchanged.

for profilin (Boquet *et al.*, 2000; Hertzog *et al.*, 2002). Indeed, profilin serves as a polymerization catalyst by capturing an actin monomer and ushering the actin onto the growing filament barbed end as a 1:1 profilin:actin complex, whereupon profilin itself is released (Pantaloni and Carlier, 1993; Kang *et al.*, 1999). Ciboulot is suggested to act on elongation via the same mechanism as profilin by forming a 1:1 complex with an actin monomer (Boquet *et al.*, 2000). The complex of the first domain of ciboulot and monomeric actin have very recently been crystallized (Hertzog *et al.*, 2004).

The activity of the larger β -thymosins contrasts that of single repeat β -thymosins that, by forming a 1:1 complex, only function in monomer sequestering. We postulated that the newly acquired functions (e.g., promoting polymerization) are a consequence of the repeated structure. Indeed, in contrast to the reported 1:1 stoichiometry for its homolog ciboulot, we observe formation of tetraThymosin β complexes with multiple actin monomers. All four tetraThymosin β repeats are functional actin-binding units and, moreover, have different preferences for monomeric and filamentous actin. The combined action of the repeats is absolutely required for the observed effects on actin polymerization. We provide evidence that tetraThymosin β is, among other, enriched in developing neuronal structures and maturing oocytes. The observed defects present in tetraThymosin $\beta^{-/-}$ organisms demonstrate its essential role in the generation of specific actin structures in vivo.

MATERIALS AND METHODS

TetraThymosin β Wild-Type and Mutant Proteins—tetraThymosin β Peptides

The tetraThymosin β cDNA was amplified via PCR from a tetraThymosin β EST containing phagemid y326d7, obtained from Y. Kohara (Genome Biology Lab, National Institute of Genetics, Mishima, Japan) and *Nco*I and *Bam*HI cloned into the bacterial expression vector pET11d (Promega, Madison, WI). From this wild-type (WT) construct, we generated tetraThymosin β variants carrying one or multiple mutated repeats via one or several consecutive rounds of mutagenesis using the PCR-based QuikChange method (Stratagene, La Jolla, CA). The generated mutations result in substitution of the first four amino acids of the conserved motif of the targeted repeat into four alanines (Table 1). For the N-Cys tetraThymosin β -construct, needed for surface plasmon resonance (SPR) measurements (see below), a variant 5'-PCR-primer with an additional Cys-codon was used, resulting at the amino acid level in MACAV instead of MAAV as amino terminal sequence. All constructs were checked by sequencing.

Recombinant tetraThymosin β WT and mutant proteins were obtained after isopropyl- β -thiogalactoside induction of *Escherichia coli* MC1061 cells harboring the pET11d constructs and plasmid pT7POL26 (Mertens *et al.*, 1995). We purified tetraThymosin β WT and mutants from bacterial lysates by DEAE anion exchange chromatography in 25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA,

and 1 mM dithiothreitol (DTT), followed by hydrophobic interaction chromatography (phenyl sepharose) using 25% saturated (NH₄)₂SO₄, 25 mM Tris-HCl, pH 8.0, as starting conditions. The purified proteins were concentrated via ultrafiltration (Amicon, Beverly, MA), dialyzed against 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.02 mM EDTA, and stored on ice.

TetraThymosin β peptides were chemically synthesized on a model 431A peptide synthesizer (Applied Biosystems, Foster City, CA), purified, and checked for correct mass. Their concentration was determined as in Van Troys *et al.* (1996b).

Immunofluorescence Microscopy

Wild-type strain N2 was obtained from *Caenorhabditis* Genetics Center (Minneapolis, MN). Adult worms were stained as described (Finney and Ruvkun, 1990). Worm embryos (obtained by cutting gravid adults; Epstein *et al.*, 1993) and dissected gonads (prepared as in Rose *et al.*, 1997) were attached on poly-lysine-coated slides by freeze-cracking, fixed with methanol at -20°C for 5 min, and stained with antibodies. Rabbit polyclonal anti-tetraThymosin β antibody raised against full-length recombinant protein at the Centre d'Economie rurale, Laboratoire d'Hormonologie (Marloie, Belgium) was enriched by affinity purification over tetraThymosin β coupled to CNBr-Sepharose 4B (Pharmacia, Piscataway, NJ). The specificity of the tetraThymosin β stainings is based on loss of signal in a control experiment using antibody preincubated with resin-coupled recombinant tetraThymosin β . Mouse monoclonal anti-actin (C4) and mouse monoclonal anti- α -tubulin antibody were from ICN Biomedicals (Costa Mesa, CA) and Amersham Biosciences (Piscataway, NJ), respectively. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Sigma, St. Louis, MO) was used at 0.1 μ g/ml as DNA-stain. Samples were viewed using a Nikon Eclipse TE2000 (Garden City, NY) inverted microscope with a 40 \times CFI Plan Fluor objective. Images were captured by a SPOT RT Monochrome CCD camera (Diagnostic Instruments, Sterling Heights, MI) and processed by the IPLab imaging software (Scanalytics, Billerica, MA).

RNA Interference Experiments and Gene Knockout

For RNAi we either microinjected dsRNA (0.5 μ l 1.0 mg/ml) of the entire coding region of tetraThymosin β generated by aMEGAScript T7-kit (Ambion, Austin, TX) in adult *C. elegans* or used soaking or feeding. The latter was performed as described in Ono and Ono (2002) using *E. coli* HT115(DE3) harboring plasmid L4440 (provided by Dr. A. Fire, Carnegie Institution of Washington, Baltimore, MD). The *tth-1* null allele *gk43* was generated by the *C. elegans* Reverse Genetics Core Facility (University of British Columbia, Canada). This allele was maintained with the *sz11* balancer in the strain VC115 (+/*sz11* [*lon-2* (*e678*)] I; *tth-1* (*gk43*)/*sz11* X). *tth-1* (*gk43*) carries a deletion of 715 base pairs in F08F1.8 (cosmid F08F1 coordinates 18199–18914 inclusive). This strain was outcrossed three times with no attempt of recombination within X chromosome. Genomic DNA fragments of the *tth-1* gene were amplified from a single worm as described (Barstead and Waterston, 1991) by two rounds of PCR using nested sets of primers (with 5'-GCCCAT-TATTGTTTTTCACC and 5'-CCTCGCGACATCTGAAATG as first round, IR (5'-CCACTTATCGGTGACTAACA) and IL (5'-GACTCCGAACATC-GAAAACA) as second round primers). To determine the tetraThymosin β protein levels, the lysate of 50 worms, obtained by incubating at 97°C for 2 min in 2% SDS, 80 mM Tris-HCl, 5% β -mercaptoethanol, 15% glycerol, 0.05% bromophenol blue, pH 6.8, was analyzed by SDS-PAGE (15%) and subjected to Western blot as described previously (Ono and Ono, 2002) with anti-tetraThymosin β antibody. Anti-actin antibody was used to monitor equal loading levels of the lysates.

Reverse Transcription-PCR

Total RNA (1 μ g) from WT worms was used as template for amplifying RNA for *act-1* (a muscle actin gene), F08F1.3 and *tth-1* using the SuperScript III One-Step RT-PCR System with Platinum *Taq*DNA polymerase (Invitrogen, Carlsbad, CA). As sense and antisense primers we used for *act-1*: 5'-GATC-GAATTCTTCTATGTGTGACGACGAGGTTGC and 5'-GATCGCTAGCT-TAGAAGCACTTGGGTGAACGATG, for F08F1.3: 5'-TGCGTTTTGTATACATCTTCTGG and 5'-ACTTTTCAAATCTAATGATGGCAGGT, and for *tth-1*: 5'-TGGCTGCCGTACCCGAAGCT and 5'-ATTGAGCTTCAGTGACG-CGGACAT.

Northern Blot

Embryo, four larval stages (L1 to L4) and adult N2 *C. elegans* were collected according to Sulston and Hodgkin (1988). Ten micrograms total RNA of each of these stages, prepared using RNeasy (Qiagen, Valencia, CA), was separated on a 1.5% agarose gel with 5% formaldehyde, transferred to nylon membrane (ZetaProbe, Bio-Rad, Richmond, CA) and UV-cross-linked. Northern blotting was performed according to Eyckerman *et al.* (1999).

G- and F-actin-binding Assays

Ca-ATP-G-actin was prepared from rabbit skeletal muscle (Spudich and Watt, 1971) and further purified via Sephacryl S-300 (Pharmacia) in G-buffer (5 mM Tris-HCl, pH 7.7, 0.1 mM CaCl₂, 0.2 mM ATP, 0.2 mM DTT, 0.01% Na₂S₂O₃). We

Figure 1. Primary structure alignment of *C. elegans* tetraThymosin β and family members. The four *C. elegans* tetraThymosin β (NM_077029) repeats (R₁ to R₄) are aligned to human thymosin β 4 (*Hs* T β 4, NP_004193) and to the repeats of *Acanthamoeba castellanii* actobindin (*Ac* actob, P18281) and *Drosophila melanogaster* ciboulot (*Dm* cib, NP_525065). The actin-binding determinants, as defined for thymosin β 4 (Van Troys *et al.*, 1996b), are in bold (motif [underlined]), hydrophobic triplet [*]). The hydrophobic triplet indicated in ciboulot domain 1 (R1) is a subset of the hydrophobic residues involved in the contact with actin (Hertzog *et al.*, 2004). The residues boxed in this sequence are the proposed determinants in the barbed end elongation promoting activity (Hertzog *et al.*, 2004). The sequences highlighted in gray in tetraThymosin β correspond to the synthetic peptides (r1, r2, r3, and r4), used as individual repeat mimics (see Figure 8A). Together the four peptides comprise 100 of the 151 tetraThymosin β residues.

Ce Ttetra R ₁	1-MAAVTELPKMNQELAGAVR-EGLELKKVETTEKNVLPTEKEDVAEEK-45
Ttetra R ₂	46-QHVERIHEIEHFDSTK LHSTP VKEKIVLPSADDIKQEK-83
Ttetra R ₃	84-QHLELTDKINNFPS ENLKKTTET IEKNVLPSPDVAEREK-121
Ttetra R ₄	122-TLQMAASFDK SALH HVETIVSTDRVTEAQ-151
<i>Hs</i> T β 4	1-MSDKPDMAEIEKFDK SKLKKTTET QEKNPSPKETIEQEKQAGES-44 * * *
<i>Ac</i> actob. R ₁	1-MNPQLQSAIGQGAAL LKHAET VDKSAPQI-ENVTVKKVDRSS-40
actob. R ₂	41-FLEEVAK PHL LKHAETVDKSGPAIPEDVHVKKVDRGA-77
<i>Dm</i> cib. R ₁	1-MAAPAPALKDLPKVAENLKSQLEGFNQDK LKN QEKIILPTAEDVAEKTQ-53
cib. R ₂	54-QSIFEGITAFNQNN LKHTET NEKNPLPDKEAIEQEK-91
cib. R ₃	92-NQFIAGIENFD AKK LKHTETNEKNVLPTEKVEIEAEKQA-129

assayed for monomer binding of tetraThymosin β using band shift in non-denaturing PAGE (Safer, 1989). Actin monomer binding was also assayed using G-actin labeled with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) after *N*-ethylmaleimide treatment according to Detmers *et al.* (1981). Emission spectra of 1.5 μ M NBD-actin in the presence of increasing concentrations of tetraThymosin β WT in G-buffer were recorded in a Hitachi F4500 spectrophotometer (Woodbury, NY; using 470 nm as excitation wavelength). The fluorescence change at the emission maximum (535 nm) induced by binding of tetraThymosin β to actin is Δ (delta)-fluorescence: the measured fluorescence—the fluorescence of 1.5 μ M NBD-actin in the absence of ligand. Alternatively, Δ -fluorescence at 535 nm was measured for a set of samples in which the NBD-actin concentration was changed so that the ratio of NBD-actin to tetraThymosin β WT was increased up to 4:1. In this experiment, tetraThymosin β was constant at 4 μ M.

F-actin binding of tetraThymosin β protein was monitored using cosedimentation: samples containing varying concentrations, as indicated, of pre-polymerized actin were incubated for 1 h at RT with WT (40 μ M) or mutant (60 μ M) tetraThymosin β and subsequently spun at 100,000 \times g (Beckman airfuge, Berkeley, CA) for 20 min at RT. A control sample with only tetraThymosin β was used to correct for the small amount of self-aggregated and hence self-sedimented tetraThymosin β .

For binding of peptides (that mimic the tetraThymosin β repeats) to monomeric or filamentous actin, zero-length cross-linking reagents (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [EDC, Sigma] and sulfo-*N*-hydroxysuccinimide [sulfo-NHS, Pierce, Rockford, IL]) were added to incubated mixtures of the peptides (increasing concentrations as indicated in figure) and a constant amount of G-actin or pre-polymerized F-actin (10 μ M), respectively (as in Van Troys *et al.*, 1996a, 1996b). For the F-actin-peptide interactions the cross-linking step was followed by sedimentation (100,000 \times g, 10 min, at RT). The cross-linking step is included given the potentially high off rates of the peptides (based on data for WT tetraThymosin β ; see SPR measurements). In addition, using this strategy for F-actin interaction, we prevented having to correct the amount of F-actin bound peptide for the free peptide that is specifically captured in the actin pellet upon sedimentation. The concentration of actin and tetraThymosin β proteins or peptides used in these assays is shown in figures or figure legends. Binding was evaluated by analyzing the samples of sedimentation and/or cross-linking on SDS-mini slab gels (Bio-Rad) followed by Coomassie staining and densitometry.

Surface Plasmon Resonance

The N-Cys-tetraThymosin β variant, carrying a unique cysteine residue, was labeled using LC-biotin-iodoacetamide (Pierce) following instructions of the manufacturer. After removal of free label by desalting, the biotinylated tetraThymosin β was coupled in HBS buffer to a streptavidine-coated chip (SA) mounted in a BiacoreX (Pharmacia). The system was washed with G-buffer supplemented with 150 mM NaCl. G-actin in G-buffer, to which we added 150 mM NaCl immediately before injection, was passed in different concentrations over the chip using a flow of 5 μ l/min. The salt was added to prevent nonspecific binding (probed on a nontetraThymosin β coated chip [blank] analyzed in parallel) and did not induce actin polymerization within the time span of the measurement. The blank corrected increase in response units (RU) is proportional to the amount of complex formed and the stoichiometry (n) of the tetraThymosin β -G-actin interaction under these conditions can be calculated using the equation:

$$R_{\max} = nR_c(MW_{\text{actin}}/MW_{\text{tTB}})$$

with MW the molecular weights of the respective proteins and R_c the RU-signal corresponding to the amount of coupled tetraThymosin β , in casu 118 RU. Using this value, R_{max} will equal either 310, 620, 929, or 1240 RU, depending on a stoichiometry n of 1, 2, 3, or 4, respectively. The observation that the RU values measured for the highest G-actin concentrations converge to a maximal read-out (see Figure 7B) indicates that the experimental RU increase of 864 RU for 60 μ M (curve a, Figure 7B, corresponding to n = 2.8) approaches R_{max} and that n equals three, i.e., three actin monomers vs. one tetraThymosin β . The overall affinity for the formation of this complex (~15 μ M) is derived from the G-actin concentration yielding R_{max}/2. This corresponds to the G-actin concentration for half fractional occupancy (C_{50%}).

Actin Polymerization Assays

Actin was labeled on Cys 374 using *N*-pyrenyl-iodoacetamide (Kouyama and Michashi, 1981). Pyrene fluorescence was measured on a Hitachi F4500 spectrophotometer using 365 and 388 nm as excitation and emission wavelength, respectively. We induced polymerization of G-ATP-actin (10 μ M, 10% labeled) in absence or presence of tetraThymosin β WT (2 μ M) or variants (2–25 μ M as indicated) by the addition of 100 mM KCl, 1 mM MgCl₂ and followed fluorescence increase as a function of time.

We assessed the sequestering capacity of tetraThymosin β WT, mutants, or peptides based on steady state measurements of F-actin (2.5 μ M, 10% labeled) as described in Van Troys *et al.* (1996b) but using gelsolin capped actin filaments (molar ratio gelsolin:actin, 1/200). The decrease in fluorescence, correlated with the amount of actin complexed by tetraThymosin β , is used to calculate a K_{d,seq} as in Van Troys *et al.* (1996b) but taking into account the different possible stoichiometries of the sequestered complex tetraThymosin β -(actin)_n-complex with n between 1 and 3 (i.e., using the equation that the slope of the decrease in F-actin vs. the total tetraThymosin β concentration is given by n CMC/(K_d + CMC) with CMC the critical monomer concentration). We performed similar measurements using uncapped filaments at final actin concentrations of 2.5 μ M. The hypothetical data corresponding to pure sequestering in the presence of actin filaments with free barbed ends were derived as in Hertzog *et al.* (2002).

The putative promotive activity of WT or mutant tetraThymosin β on filament elongation is measured from induced CMC-shifts at equilibrium in the presence and absence of thymosin β as in Pantaloni and Carlier (1993).

RESULTS

A Thymosin β Homologue with Repeated Structure in *C. elegans*

In search of a thymosin β homologue in *C. elegans*, we discovered a predicted protein of 151 amino acids (accession number NM_077029, gi:17551491, www.wormbase.org; Van Troys *et al.*, 1999) that consists of four stretches of 30–47 amino acids each with similarity to thymosin β 4. We named this protein tetraThymosin β and the gene *tth-1*. Figure 1 shows the four repeats aligned to human thymosin β 4 and to the repeats of the analogous proteins actobindin from *A. castellanii* and ciboulot from *Drosophila melanogaster*. This alignment shows that the two regions important for actin interaction in thymosin β 4, a hexapeptide motif and a pre-

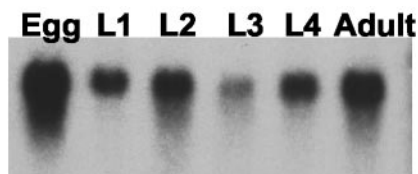


Figure 2. TetraThymosin β mRNA is present throughout development. Signals obtained by Northern blot using a tetraThymosin β specific probe for the different developmental stages of *C. elegans*: embryo, Egg; four larval stages, L1–L4; and adult. A representative experiment is shown.

ceding hydrophobic triplet (Van Troys *et al.*, 1996b), are well conserved in the tetraThymosin β repeats. All four tetraThymosin β motifs, even the most diverse motif LHSTPV in the second repeat, could in principle allow actin-binding based upon an extensive mutagenesis study of phage displayed thymosin β 4 (Rossenu *et al.*, 2003) yielding insight in the plasticity of the hexapeptide motif.

TetraThymosin β Is Present in Specific Actin-rich Structures at Various Stages during Development

Expression of the predicted tetraThymosin β protein was confirmed by a Western Blot of total lysate of adult *C. elegans* using antibodies raised against purified recombinant protein (see *Materials and Methods*); this revealed a single band of similar size as the recombinant protein (our unpublished

data). A Northern Blot of different developmental stages (Figure 2) shows that tetraThymosin β mRNA is present throughout the worm's lifespan. To gain insight in the physiological processes tetraThymosin β may participate in, we studied tissue distribution and intracellular localization using immunofluorescence microscopy. The localization of tetraThymosin β is shown in oocytes (in dissected gonads of adult worms, Figure 3), in embryos (Figure 4A) and in whole adult organisms (Figure 4B). We tested several different fixation conditions and found that the optimal conditions to preserve the signals for tetraThymosin β were cold methanol (-20 C for 5 min) for embryos and dissected gonads and a mixture of 4% formaldehyde and 50% methanol (on ice for 60 min) for whole-mount staining of adult worms. Preincubation of the antibody with tetraThymosin β coupled to Sepharose beads eliminated immunofluorescent signals (unpublished results), indicating that the signals were specific for tetraThymosin β . TetraThymosin β was maternally expressed and in the adult gonad (Figure 3) diffusely present in the cytoplasm of the oocytes, with strong staining at the cell cortex (Figure 3A) where also actin is present (Figure 3, B and C). In the distal arm of the gonad, tetraThymosin β localized to the inside edges of the membrane cubicles surrounding germ cell nuclei (Figure 3A). Here, tetraThymosin β did not localize to the lateral side of the membrane cubicles where actin is arranged in a "honeycomb" structure (Strome, 1986). Staining of actin at the inside edges of the cubicle was weak (Figure 3B), but this is likely due to fixation with methanol that does not preserve microfilaments

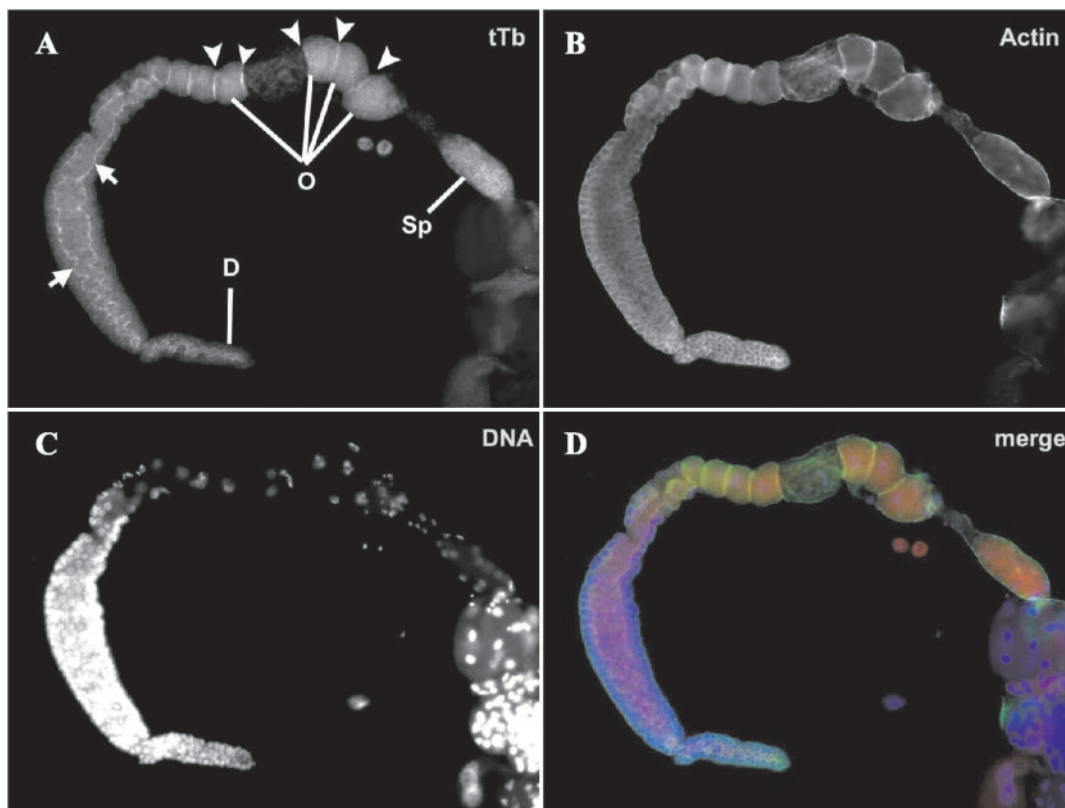


Figure 3. Localization of tetraThymosin β in dissected adult gonad of *C. elegans*. Immunolocalization of tetraThymosin β (A) and actin (B) in the dissected adult gonad (D, distal tip; Sp, spermatheca; O, oocytes). A merge of both images with DAPI staining (C) is shown in D (red, tetraThymosin β ; green, actin; and blue, DNA; scale bar, 50 μ m). TetraThymosin β localizes to the cytoplasm and the cortex (arrowheads in A) of the oocytes and to the inside edges of the membrane cubicles (arrows in A) surrounding germ cell nuclei.

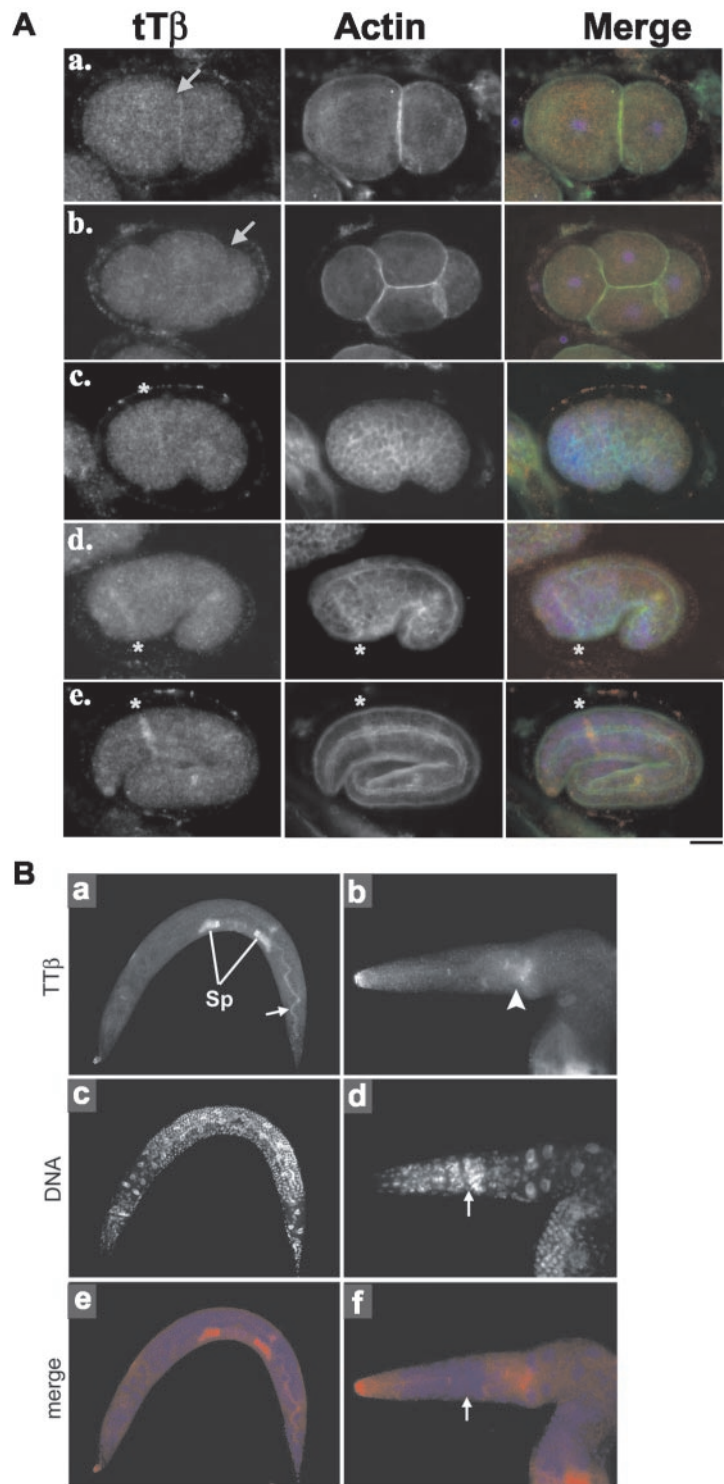


Figure 4. Localization of tetraThymosin β in *C. elegans* embryo, larvae, and adults. (A) (a–e) Immunolocalization of tetraThymosin β (left) and actin (middle) at different phases of embryonic development (scale bar, 10 μ m). In the right panel, colocalization is visualized (red, tetraThymosin β ; green, actin; blue, DAPI staining). Arrows and stars indicate the tetraThymosin β staining at cell-cell contacts and at the site of the nerve ring, respectively. (B) Localization of tetraThymosin β in WT young adults; anti-tetraThymosin β antibody (a and b) and DAPI for DNA (c and d); scale bar, 50 μ m. (e and f) Merged images of tetraThymosin β (red) and DNA (blue). The entire worm is shown in a, c, and e; the head region is enlarged in b, d, and f. TetraThymosin β is diffusely detected in the entire body (a) and enriched in the spermatheca (a, Sp), the lumen of the intestine (a, arrow), and the posterior bulb of the pharynx (b, arrowhead). Only a part of the intestine was stained because of incomplete penetration of the antibody. Adult nerve ring was identified as a region where DAPI staining was weak (d, arrow), but it was not stained by anti-tetraThymosin β antibody (f, arrow).

very well. In two-cell stage embryos (Figure 4Aa), tetraThymosin β was enriched in the cell-cell contact where actin was also concentrated. The tetraThymosin β signal was clearly apparent until the four-cell stage (Figure 4Ab) and became weaker later on (unpublished data). At the comma stage (~290 min after the first cell division) (Figure 4A, c–e), tetraThymosin β staining was observed at the developing nerve ring that is the largest axonal bundle in the nematode body and that positions around the isthmus of the pharynx.

TetraThymosin β was evident in this region even before a clear actin signal was apparent (Figure 4Ac). In larvae and adults (Figure 4B), immunofluorescence yielded a diffuse but specific staining of the entire worm body with enrichment at the intestinal tract and spermatheca (Figure 4Ba). At these stages, in contrast to what is observed in developing embryo (see above), no prominent signal was observed in the head region where the nerve ring is located (Figure 4B, b, d, and f). These immunostaining patterns were reproduc-

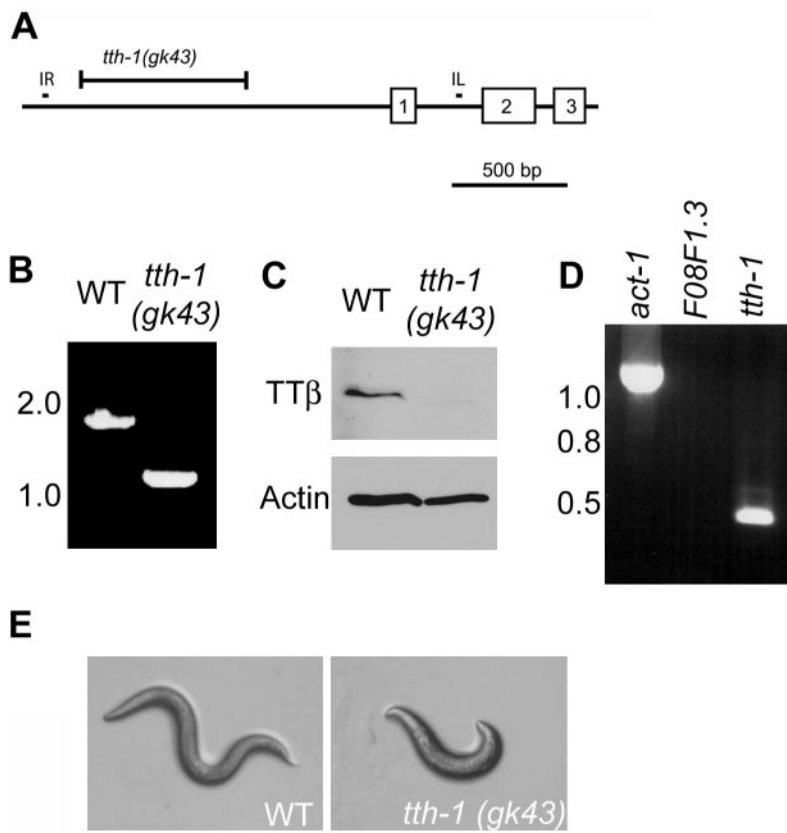


Figure 5. Characterization of the *tth-1* mutant. (A) Genomic structure of the *tth-1* gene. Exons 1–3 are indicated by open boxes. The *tth-1(gk43)* deletion is located in the upstream region of the gene. IR and IL indicate the location of PCR primers used in B. (B) Genomic DNA fragments of the *tth-1* gene from a WT or *tth-1(gk43)* homozygote were amplified by PCR using primers IR and IL. DNA size markers in kb are shown on the left. (C) Protein levels of tetraThymosin β and actin in WT or *tth-1(gk43)* homozygotes were examined by Western blot using anti-tetraThymosin β and anti-actin antibody. (D) Reverse transcription-PCR of mRNAs for *act-1* (1.1 kb), F08F1.3 (predicted size, 650 base pairs), and *tth-1* (450 base pairs) in WT worms. DNA size markers in kb are shown on the left. (E) Morphology of WT or *tth-1(gk43)* homozygous adult worms (scalebar, 0.2 mm). The *tth-1(gk43)* homozygotes have a dumpy phenotype.

ible in three or more experiments and in 20 or more worms or embryos.

TetraThymosin β ^{-/-} Organisms Show Defects in Oocyte Maturation

To further assess the importance of tetraThymosin β *in vivo*, we first used RNA interference, by feeding, soaking, or microinjection, to inhibit protein expression in live animals. However, this technique did not result in reduced tetraThymosin β levels as judged by Western blot, and consequently we did not observe an altered phenotype in the treated worms or in their progeny. This is consistent with the result of a large-scale RNA-interference project (Kamath *et al.*, 2003).

We however obtained a tetraThymosin β mutant, *tth-1(gk43)*, that has a deletion of 715 base pairs in the 5'-upstream region (Figure 5, A and B). By Western Blot, the tetraThymosin β protein was undetectable in the *tth-1(gk43)* homozygotes (Figure 5C), indicating that the deletion removes an essential part of the tetraThymosin β promoter sequence. Analogously, staining of mutant worm with anti tetraThymosin β antibody was negative. It should be noted that this deletion is located within 4-kb upstream of the adjacent predicted gene F08F1.3 with unknown function. Therefore, we could not a priori exclude the possibility that the deletion also affected expression of this neighboring gene. However, the mRNA of F08F1.3 (expected size: 650 base pairs) is not present in WT adult worms as demonstrated in Figure 5D showing a reverse transcription-PCR analysis on total RNA of WT worms. In accordance, reports by others also suggest that F08F1.3 may not be a functional gene: i) no Expressed Sequence Tag clones have been iso-

lated for this gene (www.wormbase.org), ii) the ORFeome project was not able to amplify cDNA for this gene (Reboul *et al.*, 2003) and (iii) RNAi of this gene causes no detectable phenotype (Kamath *et al.*, 2003). Together, these observations strongly suggest that the deletion in *tth-1(gk43)* specifically affects expression of *tth-1*. The *tth-1(gk43)* homozygotes died as late larvae (L3 or L4) or young adults displaying a dumpy phenotype (Figure 5E). Staining of mutant worms with rhodamine-phalloidin revealed no remarkable abnormalities in actin-rich tissues including pharynx, body wall muscle, vulva, and spermatheca (our unpublished results). Therefore, the specific cause of the dumpy phenotype is not clear. In addition, the *tth-1* mutants showed a maternal-effect lethal phenotype. The F1 *tth-1* homozygotes from the *tth-1/+* heterozygous parents survived to late larvae or adults but produced only dead eggs or arrested embryos. Mating the F1 *tth-1* homozygous hermaphrodites with WT males did not rescue lethality (unpublished data). A more detailed phenotypic analysis of dissected gonads (Figure 6) revealed that mutant eggs that were ovulated from the ovary were significantly deformed in the spermatheca and uterus (Figure 6, D and F, arrow). In WT worms, actin (Figure 6A, arrows) and tetraThymosin β (Figure 3A, arrows) are concentrated at the cortex of oocytes in the ovary. However, in the mutant oocytes, actin is uniformly distributed in the cytoplasm and not concentrated at the cortex (Figure 6B, arrows). Because methanol fixation, used for the stainings in Figure 6 is not optimal to preserve microfilaments, we also fixed dissected gonads with 4% formaldehyde, stained with rhodamine-phalloidin and confirmed that 0% of WT (n = 23) and 74% of *tth-1* mutants (n = 23) showed reduced actin filaments at the cortices of the

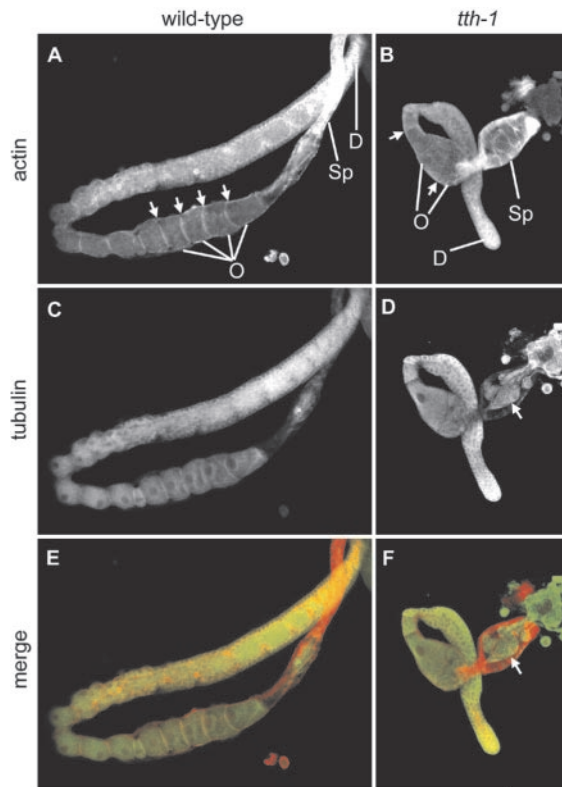


Figure 6. Morphology and cytoskeletal organization of gonads from WT and *tth-1*-mutant adult. Dissected adult gonads (D, distal tip; Sp, spermatheca; O, oocytes) from a WT (A, C, and E) or *tth-1* (*gk43*) homozygote (B, D, and F) were stained for actin (A and B) and tubulin (C and D) (scalebar, 50 μm). Merged images of actin (red) and tubulin (green) are shown in E and F. Actin is accumulated at the cortex of the oocytes (A, arrows) in WT but absent in the *tth-1* mutant (B, arrows). The fine filamentous actin network observed in B is derived from the myoepithelial cells of the ovary, not from the oocytes. In the *tth-1* mutant, deformed oocytes are found in the spermatheca (D and F, arrow).

oocytes. Localization of tetraThymosin β to the oocyte cortex (Figure 3A) in WT organisms and the *tth-1* mutant phenotype suggest that tetraThymosin β is required in assembly of the cortical actin network necessary for oocyte rigidity. As a result, mutant oocytes do not endure the ovulation process that involves vigorous contraction of the ovarian myoepithelial cells (Hubbard and Greenstein, 2000).

TetraThymosin β Binds Multiple Actin Monomers and Has Both Sequestering and Filament Binding Capacity

In view of its important role in the nematode, it is essential to gain insight in the effect of tetraThymosin β on actin and actin dynamics using *in vitro* analysis. In line with its homology with thymosin β 4, it is expected that tetraThymosin β interacts with actin monomers but because tetraThymosin β consists of a fourfold repeat of the β -thymosin module, complexes containing multiple actins could potentially be formed. The purified protein partly shifts monomeric α -skeletal muscle actin in native PAGE and intriguingly, several extra bands are already apparent in the mixture (arrowheads, Figure 7A) indicative of complexes with different stoichiometries. TetraThymosin β can be chemically cross-linked to actin monomers using a zero-length cross-linker and it retains *C. elegans* G-actin from total worm lysates

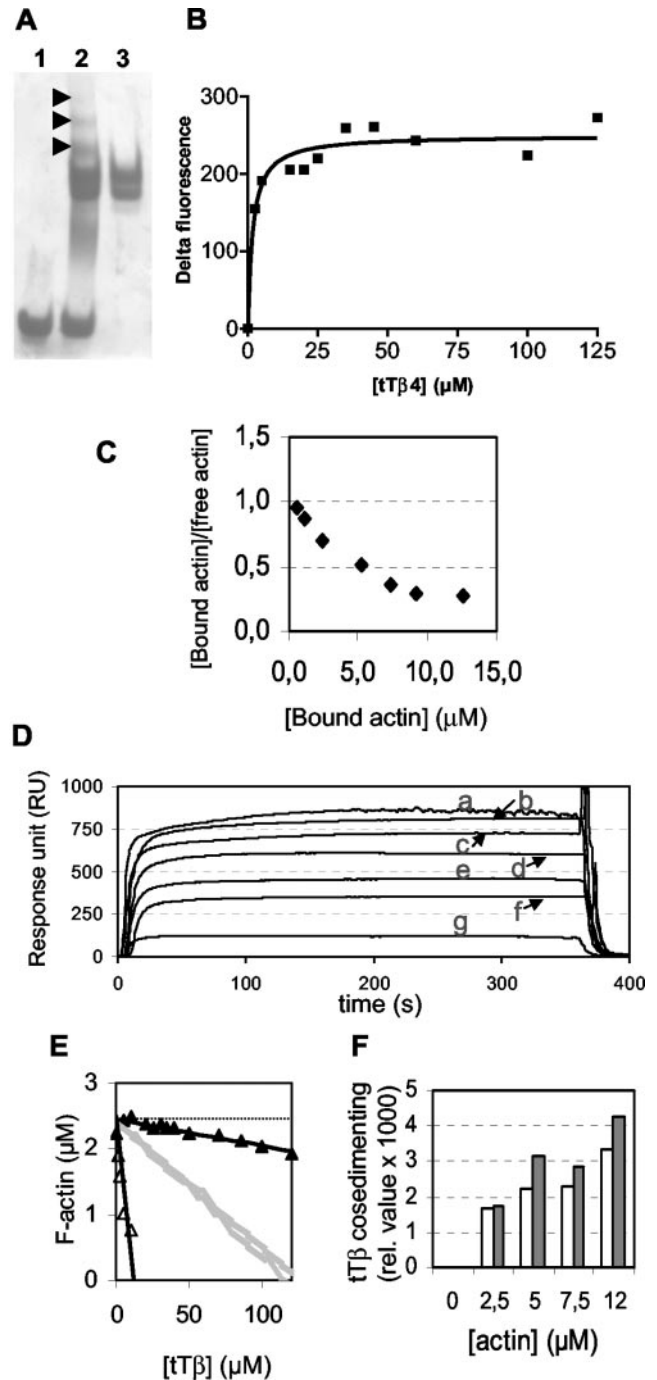


Figure 7. TetraThymosin β binds multiple actin monomers and has both sequestering and F-actin-binding capacity. (A) Native gel electrophoresis of 12 μM G-actin without (lane 1) or with 24 μM tetraThymosin β (lane 2), only 24 μM tetraThymosin β (lane 3). Arrowheads point at additional bands observed in the mixture as a result of complex formation. (B) Fluorescence change induced by binding of tetraThymosin β to NBD-labeled actin monomers measured at the emission maximum (535 nm). The Δ -fluorescence is calculated as the fluorescence of the NBD-actin (1.5 μM)-tetraThymosin β mixture minus the fluorescence of NBD-actin (1.5 μM). Nonlinear fitting is performed using GraphPad Prism software. (C) Scatchard analysis of fluorescence measurements of NBD-actin-tetraThymosin β complexes (excitation 470 nm, emission 535 nm) obtained by incubating 4 μM tetraThymosin β with increasing concentrations of NBD-actin (up to 16 μM the actin-tetraThymosin β ratio is maximally 4). This fluorescence was for each sample cor-

using affinity chromatography (our unpublished results). TetraThymosin β binding to NBD-labeled monomeric actin (1.5 μM) induces a dose-dependent increase in fluorescence at the emission maximum of the fluorophore (Figure 7B). To compare this tetraThymosin β activity with that reported for its homologues (ciboulot, actobindin), we used nonlinear fitting of the data assuming a 1:1 stoichiometry. The thus obtained value 1.72 μM (SD 0.51 μM) is comparable; however, below we present several experiments indicating that the stoichiometry is not one to one. Indeed, doing an inverse experiment, i.e., increasing the concentration of NBD-actin over tetraThymosin β , yields data that the actin-tetraThymosin β interaction is not a simple binding event. In a Scatchard plot the data obtained under these conditions cannot be fitted by linear regression (Figure 7C). This nonlinearity is caused by the presence of multiple binding sites (either of different affinity or showing cooperativity) and hence indicative of a stoichiometry larger than 1:1. For this reason, we additionally performed SPR measurements (Biacore) in which monomeric actin is allowed to interact with tetraThymosin β that is coupled to a sensor chip (Figure 7D) as a means to determine the stoichiometry of complex formation. The observed increase in response units (ΔRU) is a measure for the tetraThymosin β -actin complex formed and this signal increases at higher actin concentrations (Figure 7D). As outlined in detail in *Materials and Methods*, this SPR analysis demonstrates that at least three actin monomers can simultaneously interact with one tetraThymosin β molecule. This is clearly in contrast with conclusions made for the homologous protein ciboulot where a 1:1 stoichiometry is proposed (Boquet *et al.*, 2000; Hertzog *et al.*, 2002, 2004). The steepness of the slopes of the binding curves suggests that on and off rates are fast. In this assay the overall affinity of tetraThymosin β for actin monomers over the different contributing binding sites is $\sim 15 \mu\text{M}$ ($C_{50\%}$ -value based on the theoretical maximal response (R_{max}) most closely corresponding to the highest measured ΔRU and *in casu* corresponding with an 1:3 complex). This is consequently an overall K_d for three G-actin molecules binding to tetraThymosin β and this, together with buffer differences (note that

the NaCl concentration is higher than in the NBD-actin interaction assay, see *Materials and Methods*), may account for the higher K_d -value compared with the one described above. Given the multimodular structure of tetraThymosin β , the results on stoichiometry (presented in Figure 7, C and D) are consistent with the concept that more than one repeat interacts with actin.

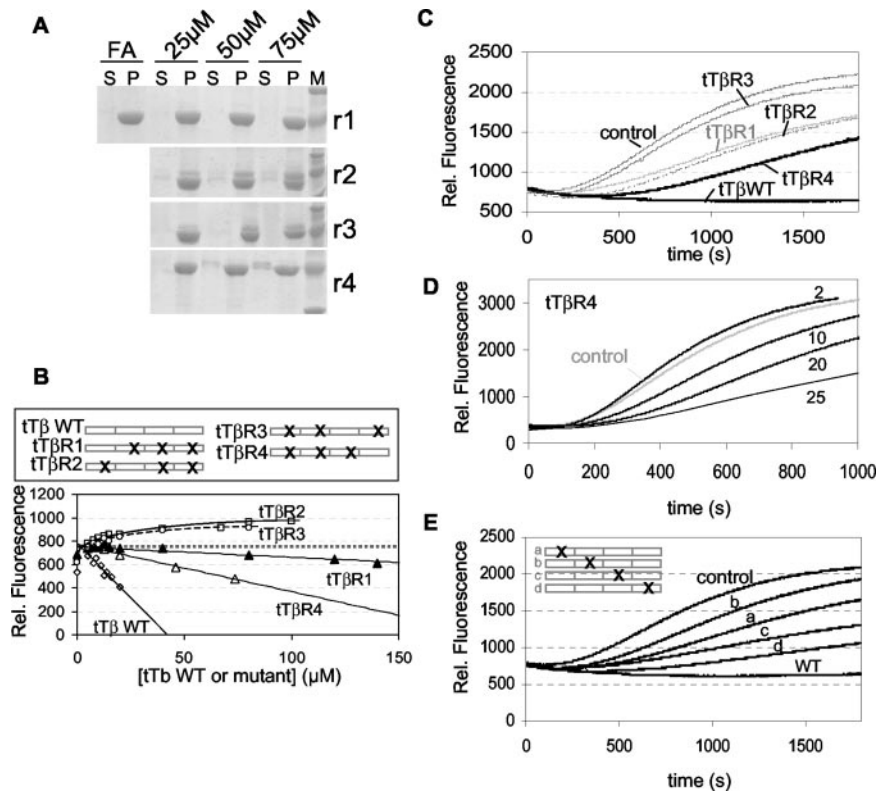
We analyzed whether the observed interaction of tetraThymosin β with actin monomers results in a monomer sequestering effect. When actin filaments are capped at their barbed ends, the addition of increasing amounts of tetraThymosin β leads to a strong decrease in filamentous actin (Figure 7E, Δ). This indicates that, under these conditions, tetraThymosin β binds actin monomers and shifts the equilibrium to the monomeric form, similarly as observed for thymosin $\beta 4$. From the slope of the decrease in F-actin plotted vs. tetraThymosin β concentration (Figure 7E), we calculated an overall affinity that varies from 10 to 28 μM , depending on the possible stoichiometry of the sequestered (actin) $_n$ -tetraThymosin β complex ($n = 1, 2$ or 3 , see above). At present it is unknown whether these values are the resultant of independent or cooperative binding of the repeats. This value thus forms an indication of the overall sequestering capacity of this protein.

The single repeat protein thymosin $\beta 4$ is a pure sequestering agent, independent whether the actin filaments with which the actin-thymosin $\beta 4$ pool is in equilibrium, have capped or free barbed ends (Pantaloni and Carlier, 1993). In contrast, profilin only effectively sequesters actin monomers in the presence of barbed end capped filaments (Pantaloni and Carlier, 1993; Kang *et al.*, 1999), a property used to demonstrate its ability to deliver actin monomers to free barbed ends and hence promote barbed end elongation. A same effect has been described for tetraThymosin β homologues ciboulot and actobindin (Boquet *et al.*, 2000; Herzog *et al.*, 2002). Similar as in Hertzog *et al.* (2002), we performed the same assay as above but now using filaments with free barbed ends in order to determine whether the sequestering activity of tetraThymosin β is dependent on the status of the filament ends. Figure 7E (\blacktriangle) shows that using filaments with free ends, the equilibrium amount of F-actin decreases much less as a function of tetraThymosin β concentration. If, under this condition, tetraThymosin β were a pure sequestering agent (with a K_d of 10 μM ($n = 1$) to 28 μM ($n = 3$), see above), a decrease in F-actin should be observed that only differs from the one when barbed ends are capped as a result of the different critical monomer concentration when barbed ends are free. We theoretically calculated this decrease that should accompany pure sequestering activity in the presence of free barbed ends (Figure 7E, gray lines, n varies from 1 [top gray curve] to 3 [bottom gray curve]). It is evident that this is significantly different from the experimental decrease observed (\blacktriangle ; note this conclusion is independent of the assumed stoichiometry for the sequestered complex). In line with Herzog *et al.* (2002), this may be indicative of a barbed end elongation promoting activity for tetraThymosin β (see *Discussion*).

In cosedimentation experiments, tetraThymosin β also interacts with actin filaments. In function of increasing F-actin concentration more tetraThymosin β is cosedimenting (Figure 7F, white bars). We calculated that 1.2, 2.3, 4.0, and 13.0% of the total tetraThymosin β is cosedimenting in the presence of 2.5, 5.0, 7.5, and 12 μM F-actin, respectively, whereas only 0.7% sediments in the absence of actin. An estimate, based on relative intensities of the actin and tetraThymosin β bands, as derived from densitometry of the Coomassie-stained gel on which the samples were analyzed (taking size differences into account), suggests that tetraThymosin β binds to the filament in a molar ratio varying from 1:5–1:10

Figure 7 (cont). rected for the fluorescence of NBD-actin in the absence of tetraThymosin β . Delta fluorescence is translated into μM bound actin using the plateau value obtained in A. A nonlinear correlation is observed in this Scatchard Plot. (D) Response unit increases obtained in SPR measurements using tetraThymosin β coupled to the sensor chip over which different monomeric actin concentrations were passed: 60 μM (a), 40 μM (b), 30 μM (c), 20 μM (d), 15 μM (e), 10 μM (f), 5 μM (g); see *Materials and Methods* and *Results* for details on derivation of stoichiometry and affinity. (E) The F-actin decrease at steady state (2.5 μM total actin concentration) is plotted as a function of tetraThymosin β concentration using actin filaments with gelsolin-capped (Δ) or elongation competent free barbed ends (\blacktriangle). The calculated curves (in gray) show the hypothetical decreases for the latter condition if only sequestering would occur for complexes of tetraThymosin β -actin with stoichiometries 1:1 (top) to 1:3 (bottom); see text. The dashed black horizontal line is the reference value for F-actin concentration in the absence of tetraThymosin β . (F) Cosedimentation of tetraThymosin β WT and mutant (T β R $_3$) with F-actin in function of increasing total actin concentration. The amounts were derived from densitometry of pellet fractions analyzed on Coomassie-stained SDS mini-slab gels. The results shown are corrected for the small amount of spontaneously sedimenting tetraThymosin β WT or mutant in a control sample without actin (0.7 and 0.8% of added WT and mutant, respectively). TetraThymosin β WT (white bars) and mutant tT β R $_3$ (having only repeat 3 intact; gray bars) were preincubated at the indicated concentration with prepolymerized actin filaments before spinning.

Figure 8. The four repeats of tetraThymosin β display differences in G- and F-actin interaction and cooperate in sequestering and inhibition of salt-induced polymerization. (A) Synthetic peptides r1 to r4 (see Figure 1) were incubated with 10 μ M prepolymerized actin before EDC-cross-linking and subsequently spun. SDS-PAGE analysis of equal amounts of supernatant (S, containing G-actin, G-actin-peptide cross-link, and free peptide) and of pellets (P, containing F-actin and peptide that cross-links to F-actin subunits) are shown for each peptide concentration; FA is a control sample with only F-actin; marker proteins (M) are 43 and 67 kDa. The actin-peptide cross-link migrates at slightly higher molecular weight than actin (45 vs. 42 kDa). (B) Change in pyrene fluorescence corresponding to the amount of gelsolin capped F-actin at steady state (actin concentration 2.5 μ M) is plotted as a function of tetraThymosin β concentration (WT or mutants tT β R₁, tT β R₂, tT β R₃, and tT β R₄ having only the indicated repeat intact as indicated above the graph; see also Table 1). If these mutants are inactive, the starting fluorescence will not change (dotted gray line). (C) Salt-induced actin polymerization is followed by recording pyrene fluorescence in time for actin (10 μ M, control), actin (10 μ M) incubated with 2 μ M tetraThymosin β WT, or incubated with 25 μ M mutant tT β R₁, tT β R₂, tT β R₃, or tT β R₄. (D) Concentration-dependent effect of the strongest sequestering mutant tT β R₄ on salt-induced polymerization (as in Figure 8C); 10 μ M actin control (gray), actin in the presence of 2, 10, 20, and 25 μ M tT β R₄ (black curves). (E) Salt-induced polymerization of 10 μ M actin in absence (control) or presence of tetraThymosin β WT or mutants. The experiment is performed as in Figure 8C but the mutants T β R_{2,3,4} (a), T β R_{1,3,4} (b), T β R_{1,2,4} (c), and T β R_{1,2,3} (d) having only one repeat mutated (as indicated, see also Table 1) are used at 2 μ M as is WT tetraThymosin β .



to actin protomers for the tetraThymosin β -actin ratio's used here. This low ratio may point at a relatively weak interaction or alternatively suggests that tetraThymosin β targets only ATP-protomers at the barbed end of the actin filaments in line with its effect on polymerization at this end (see also *Discussion*). The significance of the F-actin interaction is also shown using individual tetraThymosin β repeats and mutant tetraThymosin β proteins (see below).

All Four TetraThymosin β Repeats Can Interact with Actin

We set out to determine the contribution of each repeat of the full-length tetraThymosin β protein in the multiple effects on actin described above and hence to understand why this protein has a repeated structure. Initially, we determined which repeats function in G- or F-actin binding. The derived stoichiometry of the tetraThymosin β -G-actin complex already indicates that multiple tetraThymosin β repeats contribute to the formation of a large tetraThymosin β -actin complex. It does however not allow evaluating the contribution of each of the different tetraThymosin β repeats. To gain more insight in this, we initially studied four peptides (r1–r4, Figure 1) expected to mimic each repeat in actin binding based on the known binding determinants of thymosin β 4 (Van Troys *et al.*, 1996b). All four peptides can be EDC-cross-linked to G-actin in a concentration-dependent manner, albeit to different extents, with r3 showing lowest efficiency (unpublished data). This is informative about relative affinities of the repeats although differences in cross-linking efficiency cannot be ruled out. As the intact protein

has affinity for actin filaments, we also analyzed F-actin interaction under physiological salt conditions using cross-linking of the individual peptides to prepolymerized actin followed by sedimentation. Two of the four tetraThymosin β peptides, namely r2 and r3, interact with protomers in the actin filament (Figure 8A), already at the lowest peptide concentrations tested. In contrast for the repeat 1 and 4 peptides, we do not observe significant cross-linking to F-actin, proving the specificity of the method. Note that, under these polymer conditions, peptide r4-G-actin cross-linking was again observed, combined with an increase in G-actin (Figure 8A, supernatant), which suggests a strong sequestering activity for peptide r4. The latter additionally suggests that the actin interaction for repeat r3 is most likely not the effect of dynamic incorporation of G-actin-peptide cross-linked complexes into the filament but rather bona fide F-actin binding by this repeat. Under these conditions, repeat 2 cross-links both to F-actin and in a lower extent to G-actin (see supernatant fraction, Figure 8A).

The Repeats Cooperate during Actin Sequestering and Inhibition of Polymerization

Given that two of four repeats display F-actin-binding capacity, we investigated the contribution of each repeat in the sequestering activity of tetraThymosin β . Therefore we performed the sequestering assay with capped filament ends (Figure 8B) using four tetraThymosin β full-length mutants in which each time three of four repeats were inactivated by mutation of their motif sequences (Table 1: tT β R₁–tT β R₄, the

repeat indicated in the name is unchanged). These mutant proteins display similar cross-linking characteristics as their peptide counterparts r1–r4 (unpublished data). Figure 8B shows that these mutants display significant differences in sequestering capacities. $tT\beta R_4$ is the strongest sequester of the four mutants, although it is less efficient than WT. Next to $tT\beta R_4$ only $tT\beta R_1$ has sequestering activity; hence, the WT sequestering affinity is likely due to the combined activities of these two repeats. In contrast, mutants $tT\beta R_3$ and $tT\beta R_2$ have completely lost their sequestering capacity and intriguingly increase fluorescence as a function of concentration. For $tT\beta R_3$, this is consistent with the r3-F-actin interaction (see above) and possibly with F-actin stabilization by this repeat at steady state. Figure 7F shows that this mutant $tT\beta R_3$ also cosediments with actin filaments (gray bars, corresponding to 1.4, 3.1, 4.9, and 16.4% of total $tT\beta R_3$ in the presence of 2.5, 5.0, 7.5, and 12 μM actin, respectively; 0.8% of total $tT\beta R_3$ sediments in the absence of actin). We note that, performing the same sequestration experiment as in Figure 8B with the peptide mimetics r1–r4 yielded identical results except for the second repeat (unpublished results), because peptide r2 still has weak sequestering activity. Hence, repeat 2 apparently has a mixed G- and F-actin-binding phenotype (see also Figure 8A). The result obtained for $tT\beta R_2$ (Figure 8B), may thus be the effect of (weak) F-actin-binding capacity of repeat 2 enhanced by residual F-actin-binding present in repeat 3 via residues outside the mutated motif.

Analyzing the effect of this mutant series on salt-induced actin polymerization (Figure 8C) confirms the functional differences between the repeats. WT tetraThymosin β at 2 μM completely inhibits polymerization of 10 μM actin during the time span of the experiment, although polymerization reaches its plateau value after overnight incubation. This inhibitory activity is weaker or lost for every mutant tetraThymosin β protein tested that contains only one intact repeat because only higher concentrations of the mutants generate an effect (shown in a concentration-dependent manner for $tT\beta R_4$, Figure 8D). Consistent with its stronger sequestering activity, $tT\beta R_4$ is the most efficient of this mutant series. A same concentration of the mutants $tT\beta R_1$ or $tT\beta R_2$ also inhibit salt-induced actin polymerization but always to a lesser extent than WT tetraThymosin β . $tT\beta R_3$ at 25 μM appears to have no inhibitory effect on polymerization indicating this mutant has lost the sequestering capacity, consistent with its observed F-actin interaction.

Using the same assay, we tested a second series of mutants in which only one of four repeats was inactivated ($tT\beta R_{2,3,4}$, $tT\beta R_{1,3,4}$, $tT\beta R_{1,2,4}$ to $tT\beta R_{1,2,3}$, Table 1, the indicated repeats are unchanged). Figure 8E shows that none of these mutants, tested like WT at 2 μM , displays WT activity. This again indicates that all repeats participate in actin binding and that none of the repeats is dispensable to obtain the WT inhibitory effect on actin polymerization.

We cannot exclude that the decreased effects of the mutants on actin are in part induced by altered folding of intact repeats when adjacent repeats are mutated. However the observations that each of the repeats also functions under its individual form of peptide mimetic is a strong argument against a determining role for folding differences in the mutant activities.

Taken together, the data obtained with peptide mimetics, with mutants with only one repeat intact or with those that have one repeat inactivated, demonstrate that in none of the cases a single repeat displays WT activity.

Full Effect of tetraThymosine β on the G-/F-actin Balance at Steady State Using Uncapped Filaments Requires the Combined Activity of All Repeats: Repeat 3 Is Essential but Not Sufficient for This Activity

TetraThymosin β , like ciboulot and actobindin, behaves differently with respect to actin filaments with free or capped barbed ends (see above). This difference has been proposed to result from a positive effect on barbed end elongation and to act via a same mechanism as profilin, i.e., by forming a 1:1 complex with actin (Hertzog *et al.*, 2002). Our results above however show the functionality of multiple actin-binding repeats in the tetraThymosin β -actin interaction. To gain further insight in the underlying mechanism, we performed for WT tetraThymosin β and selected mutants the *in vitro* assay presented by Pantaloni and Carlier (1993) in which a possible barbed end elongation promoting effect of the protein of interest is derived from the nonadditive shift in critical monomer concentration (CMC) of actin polymerization observed in the additional presence of the sequestering agent thymosin $\beta 4$. For WT tetraThymosin β (Figure 9A) this non-additive CMC-shift is indeed clearly observed: The CMC-shift induced by tetraThymosin β in the presence of thymosin $\beta 4$ is much smaller than the shift for thymosin $\beta 4$ (Figure 9A, detailed view in Figure 9B). In Figure 9C, the effect of tetraThymosin β mutant $tT\beta R_{1,2,4}$ on the CMC shift is shown using the same assay. This shows that the effect of thymosin $\beta 4$ and of this mutant, that lacks the filament binding repeat 3, on the CMC appears additive: the WT effect is lost. We also tested tetraThymosin β variants, $tT\beta R_{2,3,4}$, $tT\beta R_{1,3,4}$, and $tT\beta R_{1,2,3}$, in which only repeat 1, 2, and 4, respectively, are mutated (Table 2: these data for tetraThymosin β WT, and all four mutants were determined independently of the data presented in Figure 9). Note that for these three mutants, the nonadditive effect on CMC shift in the presence of thymosin $\beta 4$ is still observed but less pronounced. Thus, our data indicate that repeat 3 is essential and repeats 1, 2, and 4 all three contribute (to more or less similar extent) in generating the WT effect in this assay.

DISCUSSION

The *in vivo* data on tetraThymosin β presented in this work suggest an active role for this *C. elegans* protein in actin-based motile processes in cells. Our data show that tetraThymosin β is strikingly present in embryonic neuronal tissue, i.e., in the developing nerve ring. Expression of tetraThymosin β is however absent in the adult nerve ring, indeed suggesting that its presence in these neuronal cells is mainly required during formation of the neuronal bundle, a process driven by actin polymerization. Similarly, ciboulot is important in fly brain metamorphosis (Boquet *et al.*, 2000). *tth-1(gk43)*-animals, having no detectable levels of tetraThymosin β , cannot survive. Some of the phenotypes of the knock out animals are consistent with a crucial role of tetraThymosin β in specific actin-related processes during the worm life cycle. Most notably, formation of the cortical actin in oocytes during their maturation appears dependent on tetraThymosin β . Deletion of tetraThymosin β causes defects in this actin structure and prevents the normal ovulation process and consequently reproduction. It is intriguing that both tetraThymosin β mRNA and protein can be detected at all developmental stages, whereas the observable phenotypes detected in knockouts are only limited: a dumpy phenotype and fragile oocytes. The maternal-effect lethality of the *tth-1* mutation suggests that the maternal supply of the tetraThymosin β protein from the heterozygous parents is

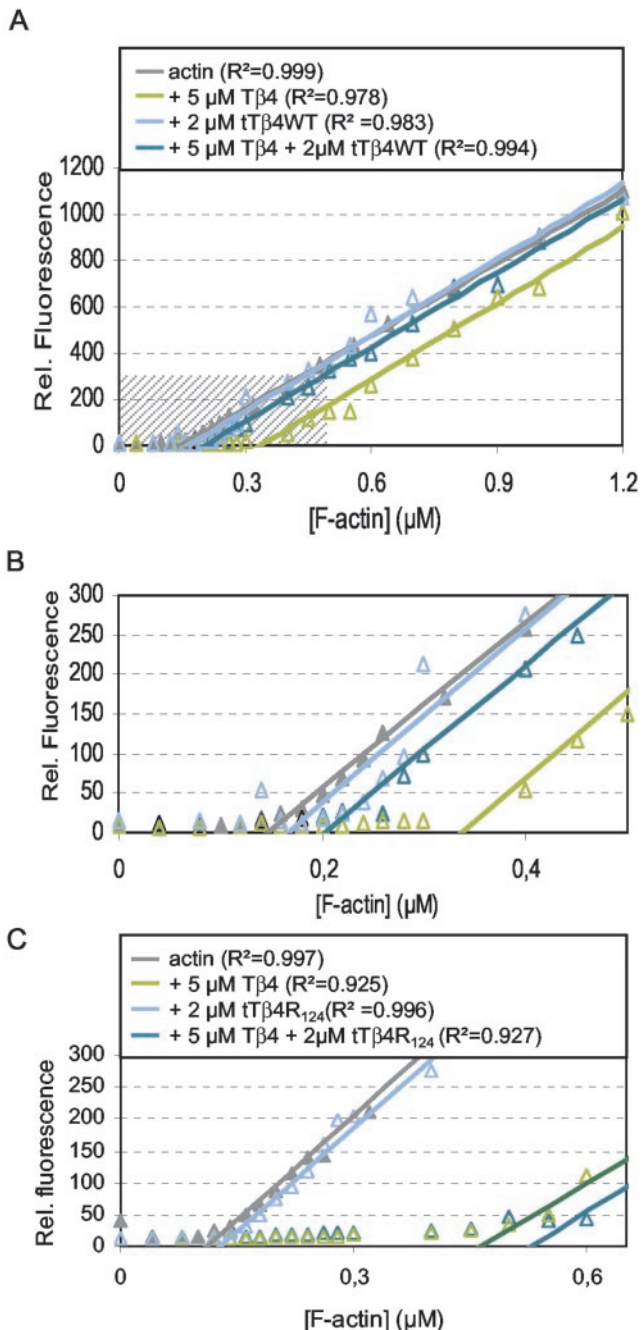


Figure 9. All four tetraThymosin β repeats combine to produce the WT effect on G-/F-actin balance at steady state in the presence of actin filaments with free barbed ends. (A and B) Determination of shifts in critical monomer concentration (Δ CMC) vs. pure actin: actin (gray, closed gray triangle), actin with 2 μ M WT tetraThymosin β (light blue), actin with 5 μ M thymosin β 4 (green), and in presence of both tetraThymosin β (2 μ M) and thymosin β 4 (5 μ M) (dark blue). In B the part of graph A indicated by the gray box is enlarged to better show the differences in induced CMC-shift. For each curve, the CMC, corresponding to the actin concentration at which the fluorescence starts increasing, is determined from the intercept of linear fitting through all data points with higher than basal G-actin fluorescence (correlation coefficients shown). The shift in critical monomer concentration induced by both tetraThymosin β WT and thymosin β 4 is already smaller than the one induced by thymosin β 4 alone demonstrating a nonadditive effect on the CMC for tetraThymosin β and thymosin β 4 (see text). (C) Same experiment as in A and B, but using tetraThymosin β mutant tT β R $_{1,2,4}$ that is

Table 2. Effect of WT and mutant tetraThymosin β on the induced CMC shift in the presence or absence of thymosin β 4

	activity (%) ^a
WT tetraThymosin β	100
tT β R $_{2,3,4}$	50
tT β R $_{1,3,4}$	60
tT β R $_{1,2,4}$	0
tT β R $_{1,2,3}$	40

^a The ratio of CMC shifts $\Delta 2/\Delta 1$ for WT is set to 100%. $\Delta 1$: CMC-shift induced by tetraThymosin β alone, $\Delta 2$: CMC-shift induced by tetraThymosin β in the presence of thymosin β 4 corrected for the shift induced by thymosin β 4 alone (Δ CMC $_{tT\beta+T\beta4}$ - Δ CMC $_{T\beta4}$).

sufficient for many morphogenetic processes in the homozygous mutants. It is also possible that the tetraThymosin β activity can in a number of processes be compensated for by the activity of other, simultaneously expressed actin-binding proteins. Prime candidates herein may be the three profilin isoforms present in *C. elegans* (Polet *et al.*, unpublished results). In *D. melanogaster*, overexpression of *chicadee*, the fly profilin, indeed rescues the brain defects caused by lack of ciboulot (Boquet *et al.*, 2000). One of the *C. elegans* profilins PFN-1 is expressed in early embryos and required for cytokinesis (Severson *et al.*, 2002), and the other two profilins are expressed in many other cell types (Polet *et al.*, unpublished results).

TetraThymosin β expression patterns in WT worms and the defects observed in *tth*^{-/-} homozygotes reveal its crucial role in actin polymerization based processes. This is underscored by biochemical data presented here that show tetraThymosin β to be a true member of a subgroup within a family of actin-binding proteins, the multirepeat β -thymosins, that we previously defined (Van Troys *et al.*, 1999). Like the other members ciboulot and actobindin (Boquet *et al.*, 2000; Hertzog *et al.*, 2002), tetraThymosin β is characterized by the ability to sequester actin monomers only when actin filament barbed ends are capped (Figure 7E) and to induce a nonadditive CMC-shift in the presence of a pure sequestering agent (thymosin β 4) when filaments are uncapped (Figure 9A). Taken together, this suggests that, next to a sequestration function in cells, tetraThymosin β -actin complexes may under given conditions participate in filament formation.

We show that all repeats can interact with actin; two out of four are also able to interact with actin filaments. Importantly, mutants with only one intact repeat score positive in at least one actin-binding assay: repeat 3 in cosedimentation, repeats 1 and 4 in sequestration and inhibition of polymerization, repeat 2 also inhibits salt-induced polymerization. In addition, the activities of mutants with three of four repeats intact, never mimic full WT activity. This allows concluding that each tetraThymosin β repeat is functional. Moreover, we here show that the WT protein can bind at least three mono-

Figure 9 (cont). mutated only in repeat 3 (actin (gray, closed gray triangle), actin with 4 μ M tT β R $_{1,2,4}$ (light blue), actin with 5 μ M thymosin β 4 (green), or with both tT β R $_{1,2,4}$ (4 μ M) and thymosin β 4 (5 μ M) (dark blue). Here Δ CMC $_{tT\beta R_{1,2,4}+T\beta4}$ is larger than Δ CMC $_{T\beta4}$ indicating an additive effect. Only part of the data points is shown, and the lines shown are the results of linear regression analysis (least square method) of all data points (same number as in A) yielding R^2 values larger than 0.92 as indicated.

mers simultaneously, most probably via repeats 1, 2, and 4. Similar to thymosin β 4 (Safer *et al.*, 1997), tetraThymosin β likely adopts an extended conformation (it is largely unstructured as a free protein, our unpublished results) enabling the formation of complexes with varying stoichiometry. Analogously to our results, actobindin is shown to contain two functional actin-binding modules (Vancompernelle *et al.*, 1991) and to complex an actin dimer (Bubb *et al.*, 1994). For the homolog ciboulot, however, a 1:1 complex with G-actin was proposed (Boquet *et al.*, 2000) and during revision of our manuscript Hertzog *et al.* (2004) reported that only domain 1 of this three-repeat-containing homolog interacts with actin. However our SPR data and the NBD-actin interaction assay independently indicate that tetraThymosin β binds multiple actin molecules. This is further corroborated by cross-linking of individual repeats and by kinetic data on both sets of tetraThymosin β mutants. The discrepancy with the data on ciboulot (Hertzog *et al.*, 2004) may arise from evolutionary differences including the number of domains and variations in amino acid sequence (see also below). Indeed the fourth domain of tetraThymosin β , that has a clear sequestering activity (Figure 8B), is absent in ciboulot. In addition, the observed lack of actin binding by individual ciboulot repeats 2 and 3 (Hertzog *et al.*, 2004) may arise from differently chosen domain definitions, mainly with regard to the inclusion of the complete hydrophobic triplet as was done in the tested tetraThymosin β peptides and mutants used here. We note that N-terminal shortening of the peptide repeats of these proteins may result both in destabilizing the N-terminal actin interaction helix as well as in reducing the contact surface.

Based on the here-derived stoichiometry and the fact that at least three repeats have sequestering abilities, it can be understood that substoichiometric amounts of tetraThymosin β allow the efficient inhibition of actin polymerization (as observed in Figure 8C). Mainly the mutants affected in sequestering (i.e., lacking functional repeats 1, 2, or 4) display strongly reduced inhibitory potential. This suggests that the observed inhibition results from actin monomer sequestration by forming a large actin-tetraThymosin β complex. It can however not be excluded that the inhibition is in part mediated by the fact that tetraThymosin β repeats may associate with one or both actin protomers at the pointed end of filaments and influence growth at this end. Such capping ability—described for ciboulot and actobindin to only affect pointed end growth and not depolymerization (Hertzog *et al.*, 2002)—could contribute to the observed deceleration of salt-induced actin polymerization in the presence of tetraThymosin β .

To yield its effects on actin, the multirepeat nature of the tetraThymosin β protein is fully exploited: Multiple repeats function in monomer binding and/or sequestering and, in addition, we demonstrate that repeat 3 and, to a smaller extent, repeat 2 are F-actin-interacting modules. Intriguingly, for repeat 3, this gain in function is accompanied by a complete loss in sequestering ability. The action of repeat 2 is less outspoken: depending on the assay used its activity appears more similar to the action of either repeat 1 or 3. The structural basis of these functional differences between the highly similar repeats is not obvious from their primary structure. Thymosin β 4 can also be cross-linked to F-actin, albeit only at very high concentrations (Carlier *et al.*, 1996). Hence, F-actin-binding by this module is not unprecedented (see also Ballweber *et al.*, 2002). Note that, in the nonsequestering, F-actin-binding and thus functionally most divergent, repeat 3, the hexapeptide motif is identical to the one of thymosin β 4, whereas in repeat 4, the pure

sequestering module, the motif is more diverse. Differences in activity and preferences for G- or F-actin as binding partner for the repeats may thus originate from amino acids both inside and outside the motif. Vancompernelle *et al.* (1992) showed that elevated concentrations of a thymosin β 4 mutant with a single amino acid exchange in the central motif could induce actin polymerization. By contrast the barbed end elongation activity of ciboulot has been contributed to two specific pairs of amino acids present in domain 1 (boxed in Figure 1; Hertzog *et al.*, 2004). Introduction of similar mutations in thymosin β 4 indeed conferred an analogous barbed end elongation activity (Hertzog *et al.*, 2004). With regard to tetraThymosin β and in light of presented differences in its activities, we note that these two suggested determinants are never present in combination in any of the four repeats. Taken together these observations of differential activities for WH2-modules may have significant implications in interpreting the function of other WH2-repeat-containing proteins for which actin monomer binding is usually unequivocally assumed.

Mutant t β R $_3$, which contains only the F-actin-binding repeat, does not affect salt-induced polymerization at a concentration more than 10 fold higher than the one at which WT is completely inhibitory. This suggests that, if pointed end capping is contributing to the observed effect of WT, repeat 3 is not the capping repeat at this filament end. Alternatively, the F-actin interaction of this repeat, and hence of the intact protein, may be important in the possible effect on barbed end growth. Indeed, only a mutant defective in this repeat has completely lost the desequestering ability in the presence of thymosin β 4 that is observed for WT tetraThymosin β (Figure 9, Table 2). This strongly suggests that F-actin binding by this repeat in the intact tetraThymosin β is required for the full efficiency of this effect. This does however not rule out that other individual repeats contribute to barbed end elongation in a similar way as reported for ciboulot D1 (Hertzog *et al.*, 2004). Combining the activities of the repeats of tetraThymosin β , a putative delivery system may be imaginable in which the repeat 3-F-actin interaction may bring the actin monomers, bound to the other repeats, in proximity of the growing filament end and makes them available for incorporation. Consequently, loss of functionality of the sequestering repeats 1, 2, and 4 should reduce the WT-effect, as is shown from measuring desequestration from the thymosin-actin pool (Table 2). As yet we have no evidence that this protein would preferably target barbed ends of actin filaments. However, the fact that all homologues of tetraThymosin β bind ATP-G-actin more strongly (Hertzog *et al.*, 2002) and the notion that tetraThymosin β displays F-actin binding may agree with a model in which repeat 3 could putatively serve as a targeting domain to these ends. The combined presence of an F-actin interaction site and actin monomer recruitment sites is not unprecedented: it is also reported, e.g., for VASP and WASP (Bachmann *et al.*, 1999; Walders-Harbeck *et al.*, 2002). For these proteins, the *in vivo* relevance hereof is underscored by a novel model for actin-based motility (Dickinson and Purich, 2002; Dickinson *et al.*, 2002). The clamped filament elongation or “load-lock and fire” model basically works on two main requirements: a direct interaction (clamp or lock) between the growing filament and the motile machinery and the possibility of direct transfer of monomers to the growing end from multiple actin monomer recruitment sites. In this light and based on its F-actin- and multiple G-actin-binding sites, tetraThymosin β putatively contains “load and fire” machinery. Insight in local subcellular tetraThymosin β concentrations or in its putative re-

cruitment to the polymerization zone in cells will be needed to support this.

In conclusion, in contrast to earlier reports on tetraThymosin β homologues, our experimental findings demonstrate that the repeats present in tetraThymosin β together form a full functional unit that binds multiple actin molecules. Although the details of the combined action of the repeats are still elusive, we demonstrate that they all contribute to the effects of the intact protein on actin polymerization. This forms a basis for understanding the essential presence of these multirepeat proteins in diverse evolutionary species as well as the conservation within the repeats. Exploiting its repeated structure renders this protein of crucial importance in generating specific cellular actin structures *in vivo*.

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