Twinfilin is required for actin-dependent developmental processes in Drosophila

Gudrun Wahlström,¹ Maria Vartiainen,² Lumi Yamamoto,¹ Pieta K. Mattila,² Pekka Lappalainen,² and Tapio I. Heino¹

¹Developmental Biology Program and ²Cellular Biotechnology Program, Institute of Biotechnology, Viikki Biocenter, FIN-00014 University of Helsinki, Finland

The actin cytoskeleton is essential for cellular remodeling and many developmental and morphological processes. Twinfilin is a ubiquitous actin monomer–binding protein whose biological function has remained unclear. We discovered and cloned the Drosophila twinfilin homologue, and show that this protein is ubiquitously expressed in different tissues and developmental stages. A mutation in the twf gene leads to a number of developmental defects, including aberrant bristle morphology. This results from uncontrolled polymerization of actin filaments and misorientation of actin bundles in developing bristles. In wild-type bristles, twinfilin localizes diffusively to cytoplasm and to the ends of actin bundles, and may therefore be involved in localization of actin monomers in cells. We also show that twinfilin and the ADF/cofilin encoding gene twinstar interact genetically in bristle morphogenesis. These results demonstrate that the accurate regulation of size and dynamics of the actin monomer pool by twinfilin is essential for a number of actin-dependent developmental processes in multicellular eukaryotes.

Introduction

The actin cytoskeleton is fundamental in processes such as cell division, polarized growth, migration, and endocytosis, and has a central role in the development of multicellular organisms. In nonmuscle cells, actin filaments are highly dynamic, and the structure and dynamics of the actin cytoskeleton is spatially and temporally regulated by a large number of actin-binding proteins. These proteins regulate different aspects of actin filament turnover; the Arp2/3 complex regulates actin filament nucleation, whereas heterodimeric capping proteins regulate filament capping, and ADF/cofilins regulate the depolymerization of filaments (for review see Pollard et al., 2000).

Actin monomer–binding proteins also have a central role in regulating cytoskeletal dynamics. For example, profilin is a small actin monomer–binding protein found in all eukaryotes that promotes the assembly of actin filaments and sequesters actin monomers in the absence of free filament ends (Vinson et al., 1998). Profilin also catalyzes the exchange of the nucleotide bound to an actin monomer, and at least in yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, this activity is important in the in vivo actin filament turnover (Wolven et al., 2000; Lu and Pollard, 2001). In Drosophila, profilin is ubiquitously expressed throughout development, and mutations in the profilin-encoding chickadee gene lead to defects in actin-dependent processes such as cytoplasmic transport during oogenesis (Cooley et al., 1992), bristle formation (Verheyen and Cooley, 1994), and motor axon outgrowth (Wills et al., 1999).

Small actin monomer–binding proteins of the thymosin family also regulate actin dynamics in various organisms. Thymosin β4 is an actin monomer–sequestering protein in vertebrates, and is involved in maintaining the cellular ATP–actin monomer pool (Yu et al., 1994). Ciboulot is a Drosophila brain-specific protein with three thymosin β–like repeats. Unlike vertebrate thymosin β4, ciboulot promotes the assembly of actin monomers at the barbed ends of filaments in a manner similar to profilin (Boquet et al., 2000).

ADF/cofilins regulate actin dynamics by depolymerizing actin filaments at their pointed ends and bind actin monomers with high affinities (Carlier et al., 1997). Mutations in the Drosophila ADF/cofilin homologue, twinstar, lead to defects in centrosome migration, cytokinesis, and border cell migration (Edwards et al., 1994; Gunsalus et al., 1995; Chen et al., 2001).

Twinfilin is an ~40-kD actin monomer–binding protein found in eukaryotes ranging from yeast to mammals, and is
composed of two ADF-H domains that are homologous to ADF/cofilins (Lappalainen et al., 1998); however, twofilin binds actin monomers with a 1:1 molar ratio and prevents their assembly into filaments and does not bind or depolymerize actin filaments like ADF/cofilins. In yeast and cultured mammalian cells, twofilin is diffusely distributed in the cytoplasm, and concentrated at the cortical actin cytoskeleton. Twinfilin is involved in the in vivo regulation of actin dynamics, because overexpression of twofilin in yeast and mammalian cells results in the formation of abnormal actin structures (Goode et al., 1998; Vartiainen et al., 2000). However, the lack of a clear phenotype in twofilin deletion strains of budding yeast has hampered the elucidation of this ubiquitous actin-binding protein’s biological role (Goode et al., 1998).

We show that the *Drosophila* *twf* gene encodes a homologue of yeast and mammalian twinfilins. A mutation in the *twf* gene leads to several developmental defects, including aberrant bristle morphology, that results from misorientation of actin filaments in developing bristles. Our findings demonstrate that twofilin is essential for actin-dependent morphological processes in multicellular organisms.

**Results**

**Cloning and biochemical activities of *Drosophila* twinfilin**

A TBLASTN search of the complete *Drosophila* genome with yeast twofilin revealed a single gene coding for a putative 343 amino acid protein. This protein is 26% identical to yeast and 49% identical to murine twofilin (Fig. 1 A). Twinfilins form an evolutionarily conserved family of proteins, and phylogenetic analysis showed that *Drosophila* twinfilin is currently the most similar invertebrate twinfilin to mammalian twinfilins (Fig. 1 B).

To examine if the *Drosophila* twinfilin protein has similar biochemical activities as yeast and mammalian twinfilins, we cloned the full-length ORF of *Drosophila* twinfilin from an embryonic cDNA library and expressed the protein in *Escherichia coli*. In actin filament cosedimentation assays, there was no significant increase in the amount of twinfilin in the pellet fraction with actin concentrations ranging from 0 to 6 μM, suggesting that *Drosophila* twinfilin does not bind actin filaments with a detectable affinity (Fig. 2, A and B). On the other hand, *Drosophila* twinfilin was able to prevent actin filament assembly in a manner similar to yeast and mouse twinfilins (Goode et al., 1998; Vartiainen et al., 2000). In this assay, actin filaments were incubated with 0–8 μM of *Drosophila* twinfilin and were then sedimented by centrifugation. A small but reproducible increase in the amount of actin in the supernatant fraction was seen in the presence of twinfilin (Fig. 2, C and D). Purified *Drosophila* twinfilin also prevents actin filament assembly and promotes filament disassembly (Fig. 2, E and F), showing that the *Drosophila* twinfilin is an actin monomer–sequestering protein and a biochemical homologue of yeast and mammalian twinfilins.

**Isolation of a twinfilin mutant**

The Berkeley *Drosophila* Genome Project (Berkeley, CA) has identified a P element insertion (Fig. 3 A) that mapped to twinfilin’s first intron. Southern blotting experiments showed that the strain has a single P element (unpublished data). In this strain, EP(3)3701, very few homozygotes were observed. This semilethality was not due to the P element insertion, because a normal number of transheterozygotes appeared in crosses of the EP(3)3701 strain with several strains carrying large deletions uncovering the twinfilin gene. This putative second-site mutation was outcrossed from the EP(3)3701 strain, resulting in a viable homozygous stock. We designated this outcrossed strain as *twf*<sup>3701</sup>. In addition to twinfilin, the gene encoding the *Drosophila* Abelson interacting protein (dAbi)* (Juang and Hoffmann, 1999) lies close to the P element insertion site (Fig. 3 A). Northern blot analyses revealed that the level of dAbi transcription is not affected in the *twf*<sup>3701</sup> strain, whereas twinfilin mRNA expression is significantly reduced (Fig. 3 B). This shows that the P element insertion line, *twf*<sup>3701</sup>, represents a specific and strong hypomorphic twinfilin mutant.

*Abbreviations used in this paper: dAbi, *Drosophila* Abelson interacting protein; SEM, scanning electron microscopy.
Twinfilin is ubiquitously expressed

We raised rabbit antiserum against purified recombinant Drosophila twinfilin in order to study its expression during development. In extracts of larvae, the twinfilin antiserum identified a single, ~40-kD band, suggesting that the antiserum is specific and that Drosophila twinfilin does not have any major posttranslational modifications. Furthermore, very little twinfilin was detected in the twf<sup>3701</sup> mutant larval extracts (Fig. 3 C). A Northern blot of total RNA from different developmental stages hybridized with a twinfilin probe showed that twinfilin is ubiquitously expressed (Fig. 3 D).

We also used the antiserum in whole mount immuno- staining of wild-type (Fig. 4 A) and twf<sup>3701</sup> embryos (Fig. 4 B). The preimmune serum did not stain the wholemounts (Fig. 4 C) and the twinfilin amount in mutant embryos is strongly reduced. In the wild-type embryos, the twinfilin protein is uniformly distributed throughout embryogenesis (Fig. 4, A, D, and E) and is also maternally provided, because the protein is already present in early embryos before the onset of zygotic transcription (Fig. 4 D).

Twinfilin is present in the cytoplasm and at the plasma membrane of all ovarian cells (Fig. 4 F), and is abundant in the border cells (Fig. 4 G). The twinstar gene, which encodes Drosophila’s ADF/cofilin, is crucial for ovarian border cell migration (Chen et al., 2001). This led us to examine whether this process is also affected in twf<sup>3701</sup> mutant egg chambers. However, we did not detect any defects in border cell migration (unpublished data), suggesting that in contrast to ADF/cofilin, the border cells do not depend on high levels of twinfilin for migration.

Morphological and developmental defects in twinfilin mutant flies

The twf<sup>3701</sup> mutant flies can be maintained as a homozygous stock. However, the mutant flies are slightly smaller and appear to be less active than wild-type flies. In flight tests, older twf<sup>3701</sup> mutant flies displayed reduced or completely lost flight ability. The hatching frequency was also slightly reduced in twf<sup>3701</sup> mutants. The twf<sup>3701</sup> mutants also had significantly prolonged larval periods compared with wild-type flies, but the duration of the pupal periods were the same (unpublished data).

The twf<sup>3702</sup> mutant has a rough eye phenotype, and closer examination by scanning electron microscopy (SEM) (Fig. 5, J–M) showed that the interommatidial bristles were often tufted (Fig. 5 M), whereas in wild-type eyes the bristles are arranged in regular arrays between the ommatidia (Fig. 5 K). The ommatidia in the twf<sup>3701</sup> mutant were sometimes pitted and occasionally fused. Interestingly, similar phenotypes have been previously reported in Drosophila bifocal mutant eyes, which also result from alterations in the actin cytoskeleton (Bahri et al., 1997).

Twinfilin mutants have defective bristle morphology

The most obvious external phenotype of the twf<sup>3701</sup> flies was defects in the bristles’ morphology. Adult bristles of Drosophila are chitinous structures with ridges and grooves that run along the bristles, which taper toward the tip. The bristle shaft is formed from a single cell, and actin filament bundles dispersed at the plasma membrane along the length of the developing bristle determine the external shape of the adult bristle (Overton, 1967; Appel et al., 1993; Tilney et
Identification of a strong hypomorphic mutation in the twinfilin locus. (A) Genomic organization of twinfilin and its surrounding loci. The arrows represent the direction of transcription. The four exons of twinfilin are shown; the empty boxes represent 5' and 3' UTRs, and the black box represents the coding region. The inverted triangle shows the location of the insertion site of EP(3)3701 (twf<sup>3701</sup>). (B) Northern blot containing 40 µg of total RNA isolated from wild-type and twinfilin mutant pupae hybridized with dAbi, twinfilin, and rp49 (loading control) probes. The level of twinfilin transcript is reduced in the twf<sup>3701</sup> mutant. (C) Western blot containing 60 ng of bacterially expressed twinfilin and equal amounts of extracts (100 µg) from wild-type and twf<sup>3701</sup> mutant larvae probed with anti-twinfilin antiserum. No twinfilin protein is detected in the mutant larval extract. Small amounts could be detected after overexposure of the blot (unpublished data). (D) Northern blot containing total RNA from all developmental stages probed with twinfilin: embryos of the indicated age in hours (lanes 1–4), the three larval instars (lanes 5–7), pupae (lane 8), adult males (lane 9), adult females (lane 10), and females without ovaries (lane 11). The same blot probed with rp49 as loading control is shown below.

Figure 3

Twinfilin is ubiquitously expressed in embryos and ovaries. Wild-type (A and C–E) and twf<sup>3701</sup> mutant (B) embryos stained with anti-twinfilin antiserum or preimmune serum. (D) Twinfilin is highly and ubiquitously expressed in a wild-type stage 10 embryo (lateral view). (B) The staining is strongly reduced in the twf<sup>3701</sup> mutant. (C) No staining was seen with the preimmune serum. (D) An early stage 5 embryo had large amounts of maternally provided twinfilin. (E) Twinfilin is present in large amounts throughout embryogenesis as exemplified with a late stage 14 embryo (dorsal view). (F and G) Confocal sections of wild-type stage 10 (F) and stage 9 (G) egg chambers stained with anti-twinfilin antiserum showing twinfilin localization in the cytoplasm and at the cell membranes. Twinfilin is also strongly expressed in border cells (arrow in G). Bar, 50 µm.

Figure 4

Df(3R)SuHu<sup>+</sup> transheterozygotes, which carry a large deletion uncovering the tuf gene (unpublished data). This suggests that the tuf<sup>60563I</sup> allele is close to null in regards to bristle development.

Twinfilin shows genetic interaction with twinstar

In yeast, lack of twinfilin does not result in a detectable phenotype, except for slightly enlarged cortical actin patches. In combination with a temperature-sensitive cofilin allele, twinfilin causes lethality at the permissive temperature (Goode et al., 1998). In Drosophila, cofilin is encoded by the twinstar (tstar) gene (Edwards et al., 1994; Gunsalus et al., 1995). To investigate the possible genetic interaction between twinfilin and twinstar, we crossed the P element line tsr<sup>0563I</sup>, which has a lethal insertion in twinstar, with the twf<sup>3701</sup> homozygotes, and examined the resulting double heterozygotes for a bristle phenotype. Nearly all flies had at least one macrochaete with defects at bristle tip (Fig. 5, N–Q). The bilateral posterior scutellar and anterior dorsocentral bristles on the thorax were by far the most frequently affected. We scored these four bristles for abnormalities under higher magnification. In tsr<sup>60563I</sup>+/ twf<sup>3701</sup>/+ flies, 66% of the bristles were split, branched, or had a rough surface, whereas only 2% of the bristles on tsr<sup>60563I</sup>/+; +/+ flies and none of the bristles on +/+; twf<sup>3701</sup>/+ flies had this phenotype. The presence of a single tsr<sup>60563I</sup> allele in the twf<sup>3701</sup> homozygous background results in a more dramatic eye defect, whereas the severity of the bristle phenotype appears to be equal to the one in the twf<sup>3701</sup> mu-
tant alone (unpublished data). These results show that twinfilin interacts genetically with twinstar (tsr) mutants. Scanning electron micrographs of bristles (A–I and N–Q) and eyes (J–M). (A) The whole thorax from wild-type and (B) twf\textsuperscript{2701} mutant adult. Note the bent and split bristles. (C) Microchaete from wild-type and (D) from the twf\textsuperscript{2701} mutant. Note the irregularly arranged ridges on the surface and shorter hairs than in the wild-type. (E) Surface of a wild-type macrochaeta showing straight longitudinal ridges. (F) Thickening on a mutant twf\textsuperscript{2701} macrochaeta with ridges arranged perpendicularly to the long axis of the bristle. (G) High magnification image of a twf\textsuperscript{2701} macrochaetae with highly irregular ridges. (H) Tip of a wild-type bristle and (l) tip of a twf\textsuperscript{2701} mutant bristle. Note the smooth surface compared with the wild-type bristle. (J) wild-type eye and (L) twf\textsuperscript{2701} mutant eye. (K) High magnification of wild-type ommatidia and interommatidial bristles. (M) In twf\textsuperscript{2701} mutants the interommatidial bristles are often tufted and the ommatidia are occasionally fused (arrowhead) or pitted (arrows). (N and O) Posterior scutellar bristles from tsr\textsuperscript{+/+}; twf\textsuperscript{+/+} double heterozygotes showing a hooked (N) or a split tip (O). (P and Q) Normal tip of a tsr\textsuperscript{+/+}; twf\textsuperscript{+/+} (P) and a ++ (Q) bristle. Bars: (A, B, J, and L) 100 μm; (C–F, K, and M) 10 μm; and (G–I and N–Q) 5 μm.

Figure 5. twf mutants have defects in bristle morphology and in eyes and interact genetically with twinstar (tsr) mutants. Scanning electron micrographs of bristles (A–I and N–Q) and eyes (J–M). (A) The whole thorax from wild-type and (B) twf\textsuperscript{2701} mutant adult. Note the bent and split bristles. (C) Microchaete from wild-type and (D) from the twf\textsuperscript{2701} mutant. Note the irregularly arranged ridges on the surface and shorter hairs than in the wild-type. (E) Surface of a wild-type macrochaeta showing straight longitudinal ridges. (F) Thickening on a mutant twf\textsuperscript{2701} macrochaeta with ridges arranged perpendicularly to the long axis of the bristle. (G) High magnification image of a twf\textsuperscript{2701} macrochaetae with highly irregular ridges. (H) Tip of a wild-type bristle and (l) tip of a twf\textsuperscript{2701} mutant bristle. Note the smooth surface compared with the wild-type bristle. (J) wild-type eye and (L) twf\textsuperscript{2701} mutant eye. (K) High magnification of wild-type ommatidia and interommatidial bristles. (M) In twf\textsuperscript{2701} mutants the interommatidial bristles are often tufted and the ommatidia are occasionally fused (arrowhead) or pitted (arrows). (N and O) Posterior scutellar bristles from tsr\textsuperscript{+/+}; twf\textsuperscript{+/+} double heterozygotes showing a hooked (N) or a split tip (O). (P and Q) Normal tip of a tsr\textsuperscript{+/+}; twf\textsuperscript{+/+} (P) and a ++ (Q) bristle. Bars: (A, B, J, and L) 100 μm; (C–F, K, and M) 10 μm; and (G–I and N–Q) 5 μm.

Actin bundles are severely misoriented in developing twinfilin mutant bristles

To visualize the actin bundles in developing mutant macrochaetae, we stained thoracic epithelia from twf\textsuperscript{2701}/twf\textsuperscript{2701} and twf\textsuperscript{2701}/Df(3R)SuHu\textsuperscript{W} pupae with Texas red–conjugated phalloidin and examined them using confocal microscopy. In wild-type bristles, the actin bundles are located at the periphery of the developing bristle (Fig. 6 A) and during the elongation phase, new actin filaments are formed in the bristle tip. These newly formed filaments are visible as phalloidin-positive spots in the tip, as well as between the actin bundles. The filaments are gathered into tiny actin bundles that are added to the preexisting thicker bundles. In fully elongated 48-h-old bristles, actin-containing spots are no longer visible and only the actin bundles can be seen (Tilney et al., 1996).

The most striking observation was the presence of a large number of small F-actin containing spots or tiny actin bundles in 48-h-old twf\textsuperscript{2701} mutant macrochaetae. The spots were localized at the bristle surface between the main bundles (Fig. 6 B) and the tiny bundles appeared to be perpendicularly oriented to the long axis of the bristle (Fig. 6, C
The flat surface with few randomly oriented ridges seen at the bristle tip (Fig. 5 I) can be correlated with the presence of numerous short, thin bundles intermingled with a few thicker bundles (Fig. 6, G and H). In addition, the main bundles in the macrochaetae were often severely disorganized. We saw weakly fluorescent bundles that deviated toward the periphery of and then appeared to twist around the bristle, while straight strongly fluorescent bundles appear to be located internally (Fig. 6, D and E). The thickenings on some of the adult bristles seen by SEM (Fig. 5 F) corresponded to loosened bundles that perpendicularly protrude from the surface (Fig. 6 D).

Randomly oriented bundles can eventually become reorganized into main bundles that extend the bristle in a new direction. Fig. 6 I shows a macrochaete that was bent twice. The original correctly oriented main bundles that extend from the base seem to end at the bending point, instead another set of bundles grow in the new direction.

The localization of twinfilin in developing bristles
To examine twinfilin’s localization in developing bristles, we dissected wild-type pupae at 32 or 48 h after pupariation and stained them with twinfilin antiserum and phalloidin. Twinfilin was highly abundant in the cytoplasm of all hair- and bristle-producing cells (unpublished data). In addition, twinfilin was distributed throughout the developing bristle shaft. In surface confocal sections from recently sprouted (32 h) bristles (Fig. 7 A), twinfilin was localized in diffuse spots between the actin bundles, corresponding to the sites of new actin filament assembly (Tilney et al., 1996). Sections from the interior of the bristle gave a uniform signal. In fully elongated bristles (48 h) (Fig. 7 B), the twinfilin spots were still present, although they appeared more condensed and less numerous. Many of the spots colocalized with ends of the modules that make up the actin bundles. In regions with large gaps between successive modules, the spots tended to localize on the tipmost end of the module, which correspond to the barbed ends of filaments (Tilney et al., 1996).

Discussion
Previous studies on yeast have shown that twinfilin deletion strains are not detectably defective in cell growth or morphology, and therefore the specific role of this protein has remained unclear (Goode et al., 1998). The morphological defects in the *Drosophila* twf<sup>3701</sup> mutant strain (Fig. 5) provide the first genetic evidence for a biological role of this highly conserved actin-binding protein. The ubiquitous expression of twinfilin (Figs. 3 D and 4) and the number of developmental defects observed in the twf<sup>3701</sup> mutant strain suggest a fundamental role for this actin monomer–sequestering protein in the development of multicellular organisms.

Actin filaments can assemble into higher order structures via a bundling process that is involved in formation of complex structures with highly specific functions. Examples include the intestinal microvilli and inner ear hair cell stereocilia of mammals, and *Drosophila*’s mechanosensory bristles and ovarian nurse cell-specific actin filament cables and ring canals (Bartles, 2000; DeRosier and Tilney, 2000). Of these, the developing *Drosophila* bristle, which is formed as a cytoplasmic extension from a single cell, has been extensively studied as a model for actin bundle formation. In the twf<sup>3701</sup> mutant flies, the adult macrochaetae are split, branched, or bent and have a highly irregular ridge pattern. This pheno-
severe bristle phenotype in the affinity for actin filaments (Fig. 2). An actin monomer–sequestering protein with no detectable tin bundles are twisted and misoriented (Fig. 6). However, our observations that in developing mutant bristles, the actin filament-containing spots or perpendicular tiny bundles in fully elongated twf3701 mutant bristles (Fig. 6). In wild-type bristles, F-actin–containing spots are present only during the elongation phase of bristle development (Tilney et al., 1996). To our knowledge, such a mutant phenotype has not been described earlier. One explanation for the origin of the ectopic spots and/or bundles in the twf3701 mutant is that in the absence of actin monomer–sequestering twinfilin, spontaneous actin filament nucleation takes place. These filaments may then become cross-linked into tiny bundles that are not integrated with the main bundles. A second possibility is that in the absence of twinfilin, an uncontrolled polymerization of preexisting actin filaments in the main bundles takes place. The perpendicular ectopic bundles may then originate from main bundles that have split lengthwise and become separated into modules, whereas the spots are the result of further fragmentation. However, we favor the first explanation because the F-actin filament spots normally present in young bristles. In addition, the ectopic bundle pattern is clearly different from the fragmented actin bundles observed in elongating bristles treated with cytochalasin D (Tilney et al., 2000b).

Immunostainings showed that twinfilin is localized in the cytoplasm and to actin filament structures in bristles (Fig. 7). Similar localizations have been shown previously for yeast and murine twinfilins (Goode et al., 1998; Vartiainen et al., 2000). Interestingly, in fully elongated Drosophila bristles, twinfilin is localized along the actin filament bundles in spots, which may represent the barbed ends of actin filaments (Fig. 7B). Therefore, it is possible that in addition to sequestering actin monomers, the role of twinfilin in bristles may be to localize monomers at the sites of actin filament assembly. This is also supported by our recent studies showing that interactions with actin monomers and capping protein are essential for localization of twinfilin in Saccharomyces cerevisiae (Palmgren et al., 2001). Also in Drosophila bristles, the localization of twinfilin to actin bundles may be mediated through interactions with capping protein. 

tsr+/; tufl+/ double heterozygotes display a weak but significant bristle defect (Fig. 5, N–Q). A similar genetic interaction between cofilin and twinfilin has previously been demonstrated in yeast (Goode et al., 1998). In yeast cells, cofilin promotes actin dynamics by depolymerizing actin filaments, and a mutation in the cofilin gene that affects its actin filament depolymerization rate shows synthetic lethality with a twinfilin deletion (Lappalainen and Drubin, 1997; Goode et al., 1998). Analogously, we suggest that during bundle formation, the decreased actin filament depolymerization rate due to the tsr+/ mutation, together with uncontrolled filament assembly resulting from the tufl+ mutation, lead to defects in bristle morphogenesis.
This work demonstrates the essential role of twinfilin, an actin monomer–binding protein, in the development of a multicellular organism. Furthermore, we show that the accurate regulation of the size and dynamics of the actin monomer pool is important for assembly of complex actin filament structures in cells.

Materials and methods

**Plasmid construction**

The full-length ORF of twinfilin was PCR amplified from an embryonic λ Zap cDNA library using primers 5'-CGCGCAATTCGTTCTCCTGCGTTCG-3' and 5'-GCGCGGAGCCAACCAGGATATCCGAGCCAACAC-3'. The PCR fragment was cloned into the pGAT2 expression vector (Peränen et al., 1996) and sequenced. Part of the dAbi-coding region was PCR amplified from genomic DNA with primers 5'-GTGTGATTCTAATGTTTCT-3' and 5'-GATGACATCAACACACCCACC-3'.

**Proteins and antibodies**

*Drosophila* twinfilin was purified as a glutathione  S-transferase fusion similar to murine twinfilin (Variainen et al., 2000). Rabbit muscle actin was prepared from an acetone powder (Pardee and Spudich, 1982). Pyrene actin and human platelet actin were from Cytoskeleton. We immunized one New Zealand White rabbit with purified recombinant *Drosophila* twinfilin in Freund's adjuvant, and the serum was collected after four immunizations. Preimmune serum was collected from the same rabbit and used as a control in our assays.

**Actin filament cosedimentation assays**

Actin filament fractions were isolated from Drosophila actin cosedimentation assays. 40-μl aliquots of actin were diluted to desired concentrations in G buffer (20 mM Tris, pH 7.5, 0.2 mM ATP, 0.2 mM DTT, 0.2 mM CaCl₂) and polymerized for 30 min by the addition of 5 μl of 10× initiation mix (20 mM MgCl₂, 10 mM ATP, 1 M KCl). 5 μl of twinfilin in G buffer was added to filaments and incubated for 30 min. We sedimented actin filaments by centrifugation for 30 min at 10,000 g using a Beckman Optima MAX ultracentrifuge. All steps were performed at room temperature. Equal proportions of supernatants and pellets were loaded on 12% SDS-polyacrylamide gels, the gels were stained with Coomassie blue (Bio-Rad Laboratories) according to the manufacturer's instructions. The gels were imaged with a FluorImager (Bio-Rad Laboratories). Proteins and antibodies

**Actin filament cosedimentation assays**

Actin filament cosedimentation assays were performed with 40-μl aliquots of actin diluted to desired concentrations in G buffer (20 mM Tris, pH 7.5, 0.2 mM ATP, 0.2 mM DTT, 0.2 mM CaCl₂) and polymerized for 30 min by the addition of 5 μl of 10× initiation mix (20 mM MgCl₂, 10 mM ATP, 1 M KCl). 5 μl of twinfilin in G buffer was added to filaments and incubated for 30 min. We sedimented actin filaments by centrifugation for 30 min at 10,000 g using a Beckman Optima MAX ultracentrifuge. All steps were performed at room temperature. Equal proportions of supernatants and pellets were loaded on 12% SDS-polyacrylamide gels, the gels were stained with Coomassie blue (Bio-Rad Laboratories) according to the manufacturer's instructions. The gels were imaged with a FluorImager (Bio-Rad Laboratories).

**SEM**

Whole adult flies were anesthetized with CO₂ and then dehydrated by 24-h incubations in a graded ethanol series. The dehydrated flies were critical point dried, mounted on SEM stubs, sputter coated with platinum, and examined with SEM.

**Fly strains and genetics**

The strains *DE3R*Sulhw/TM6B and *ts638ks1/CyO* were obtained from the Bloomington Stock Center (Bloomington, IN). The EP(3)3701 strain was identified by the Berkeley *Drosophila* Genome Project (Berkeley, CA) and obtained from the Szeged Stock Centre (Szeged, Hungary). The putative semilethal element in the original EP(3)3701 strain (Results) was outcrossed against a w strain. The outcrossed homozygous stock has the mini-w eye color marker and bristle phenotype described in the Results section. Genetic interactions between twinfilin and twinstar were examined in progeny from crosses between *ts638ks1/CyO* males and *twf3701* homozygous females. Bristles of the progeny with normal wings were examined by light microscopy and SEM along with *ts638ks1/CyO* flies as a control. Canton-S, or w strains were used as wild-type controls in all experiments. The flies were maintained on standard food at 25°C.

**Phalloidin staining of bristles**

White prepupae were collected and dissected in PBS 32–48 h after pupariation. We dissected the head and abdomen from the thorax, and removed the internal organs. The epithelial tissue was flattened after making a ventral incision, then transferred into an Eppendorf tube containing 4% paraformaldehyde in PBS and placed on ice. The specimens were further processed as described in Tilney et al. (1996). Filamentous actin was stained with Texas red-conjugated phalloidin at a concentration of 2 μM. The samples were mounted in Vectashield (Vector Laboratories), and examined with a Biorad MRC 1024 confocal microscope. Optical sections were combined using the Confoal Assistant 4.02 program.

**Immunostainings**

Wild-type and *twf3701* embryos were collected, fixed, and stained according to standard protocols. The anti-twinfilin antisem and preimmune serum were diluted to 1:20,000, and all stainings were performed under same conditions. For antibody stainings of bristles, the material was fixed and washed as above, then blocked for 1 h in 1% BSA, 0.1% Triton X-100 in PBS. The anti-twinfilin antisem was used at a 1:10,000 dilution, and FITC-conjugated secondary antibody at a 1:1,000 dilution. Ovaries were dissected, fixed, and stained with twinfilin antisem as above, except that the blocking was in 0.5% BSA, 0.1% Triton X-100 in PBS. Ovaries were mounted in Vectashield containing 0.5 μg/ml Hoechst 33258.

**Miscellaneous**

Protein concentrations were determined with a Hewlett-Packard 8452A diode array spectrophotometer using calculated extinction coefficients for *Drosophila* twinfilin at 280 nm (ε = 34 990 M⁻¹cm⁻¹) and for actin at 290 nm (ε = 26 600 M⁻¹cm⁻¹). Total RNA was isolated using the TRIZOL reagent (GIBCO BRL; Life Technologies) according to the manufacturer's instructions and blotted and hybridized according to standard protocols.

Submitted: 6 August 2001

Revised: 9 October 2001

Accepted: 12 October 2001

We thank the Szeged Stock Center (Szeged, Hungary) for providing the Drosophila twinfilin (Variainen et al., 2000). We thank the Szeged Stock Center (Szeged, Hungary) for providing the EP(3)3701 strain, and the Bloomington Stock Center (Bloomington, IN) for the other Drosophila strains. This study was supported by grants from Victoria Foundation (to G. Chen, J., D. Godt, K. Gunsalus, I. Kiss, M. Goldberg, and F.A. Laski. 2001. Cofi

**References**


Downloaded from on May 24, 2017 The Journal of Cell Biology | Volume 155, Number 5, 2001


Role of twinfoilin in Drosophila development | Wahlström et al. 795