Twinfilin is required for actin-dependent developmental processes in *Drosophila*

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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 misori-

entation 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 β4–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, twinfilin 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, twinfilin 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 twinfilin 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 twinfilin 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 twinfilin 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 twinfilin revealed a single gene coding for a putative 343 amino acid protein. This protein is 26% identical to yeast and 49% identical to murine twinfilin (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
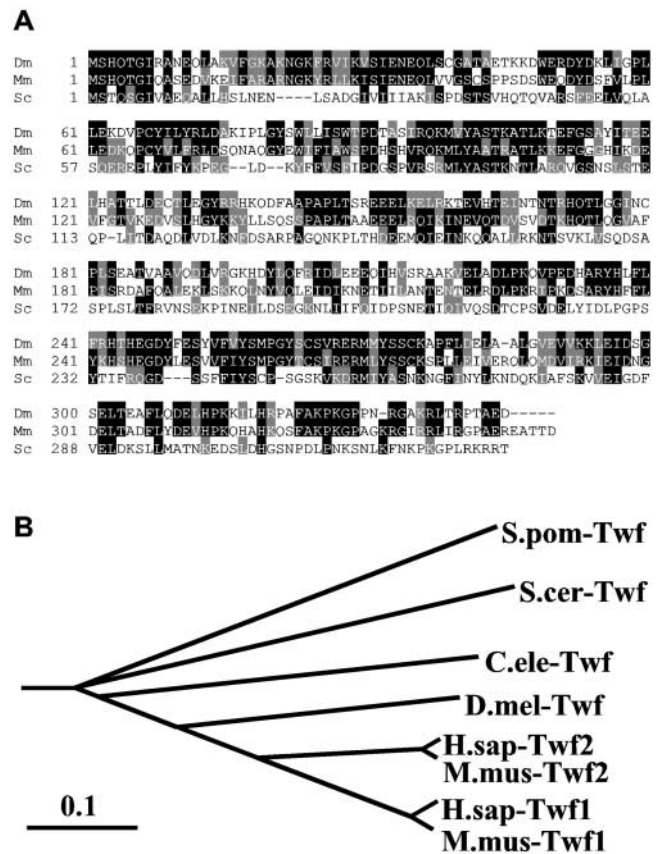
Figure 1. ***Drosophila twf* gene encodes a homologue of yeast and mammalian twinfilins.** (A) Sequence alignment of *Drosophila* (Dm), murine (Mm), and budding yeast (Sc) twinfilins. (B) Phylogenetic tree of all known twinfilins produced by Clustal-X software with a bar showing 10% divergence. Database and accession nos. for twinfilin sequences are *S. pombe* (GenBank/EMBL/DDBJ accession no. AL034490), *S. cerevisiae* (accession no. Z72865), *Caenorhabditis elegans* (accession no. U46668), *Drosophila melanogaster* (accession no. AE003703), *Homo sapiens* twinfilin-2 (accession no. Y17169), *Mus musculus* twinfilin-2 (accession no. Y17808), *H. sapiens* twinfilin-1 (PIR A55922), and *M. musculus* twinfilin-1 (accession no. U82324).

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*^3701^. 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*^3701^ strain, whereas *twinfilin* mRNA expression is significantly reduced (Fig. 3 B). This shows that the P element insertion line, *twf*^3701^, represents a specific and strong hypomorphic *twinfilin* mutant.

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*Abbreviations used in this paper: dAbi, *Drosophila* Abelson interacting protein; SEM, scanning electron microscopy.
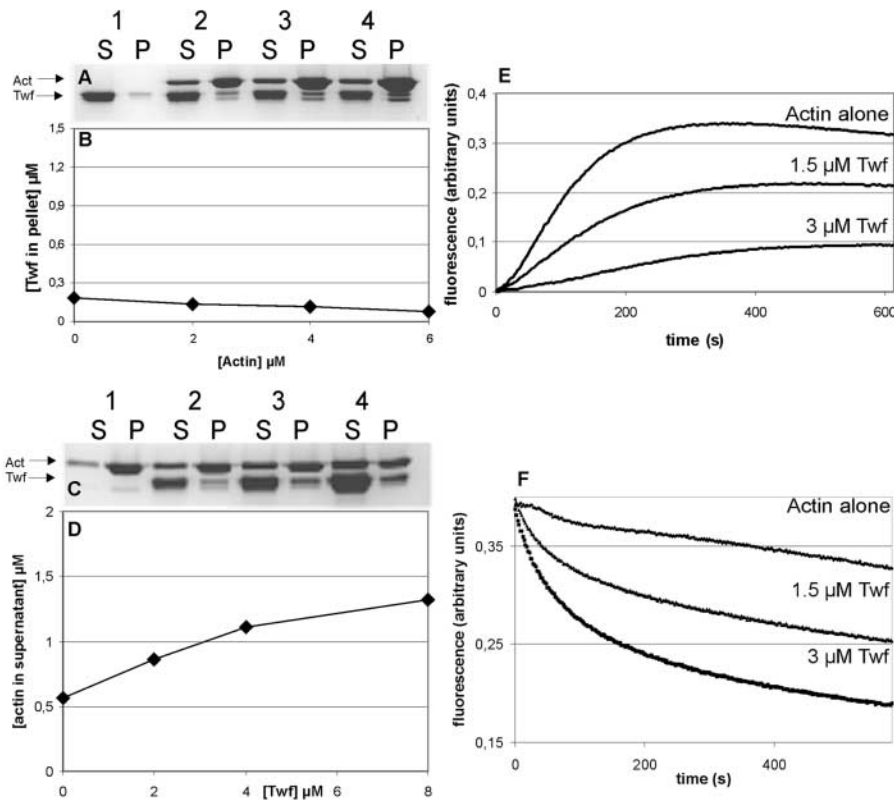
Figure 2. ***Drosophila* twinfilin is an actin monomer–sequestering protein.** (A) *Drosophila* twinfilin does not cosediment with actin filaments. Twinfilin (1.5 μM) was mixed with 0- (lane 1), 2- (lane 2), 4- (lane 3), and 6- (lane 4) μM actin filaments, and the filaments were sedimented by centrifugation. (B) The amount of twinfilin in the pellet and supernatant fractions was quantified from three independent experiments. (C) *Drosophila* twinfilin increases the amount of monomeric actin. Actin filaments (3 μM) were incubated with 0 (lane 1), 2 (lane 2), 4 (lane 3), and 8 (lane 4) μM twinfilin for 30 min and the filaments were sedimented by centrifugation. (D) The amount of actin in the supernatant and pellet fractions was quantified from three independent experiments. (E) Twinfilin prevents the assembly of actin filaments. Polymerization of 3 μM actin (1:6 pyrene actin:human platelet actin) in the presence of 0, 1.5, or 3 μM twinfilin was initiated by the addition of 0.1 M KCl, 2 mM MgCl$_2$, and 0.5 mM ATP. Polymerization of filaments was followed by the increase in pyrene fluorescence. (F) Disassembly of prepolymerized actin filaments (3.3 μM, 1:6 pyrene actin:human platelet actin) was induced by mixing 72-μl actin filaments with 8 μl of 0, 1.5, or 3 μM twinfilin. Depolymerization of filaments was followed by the decrease in pyrene fluorescence.

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*[3701] 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 immunostainings of wild-type (Fig. 4 A) and *twf*[3701] 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*[3701] 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*[3701] 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*[3701] mutant flies displayed reduced or completely lost flight ability. The hatching frequency was also slightly reduced in *twf*[3701] mutants. The *twf*[3701] 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*[3701] 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*[3701] 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).
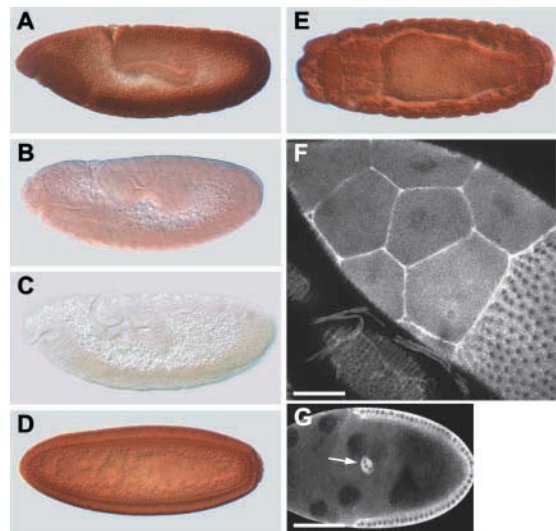
Twinfilin mutants have defective bristle morphology

The most obvious external phenotype of the *twf*[3701] 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
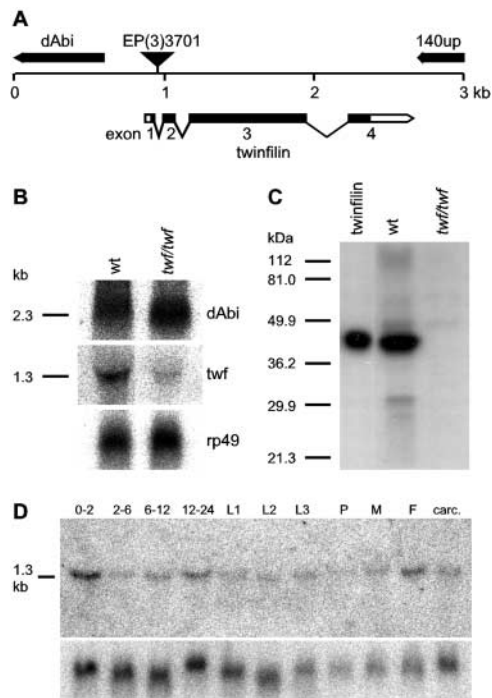
Figure 3. **Identification of a strong hypomorphic mutation in the twinfilin locus.** (A) Genomic organization of *twf* and its surrounding loci. The arrows represent the direction of transcription. The four exons of *twinfilin* are shown; the empty boxes represent 5′ and 3′ UTRs, and the black box represents the coding region. The inverted triangle shows the location of the insertion site of EP(3)3701 (*twf*[3701]). (B) Northern blot containing 40 μg of total RNA isolated from wild-type and *twinfilin* mutant pupae hybridized with *dAbi*, *twf*, and *rp49* (loading control) probes. The level of *twf* transcript is reduced in the *twf*[3701] mutant. (C) Western blot containing 60 ng of bacterially expressed twinfilin and equal amounts of extracts (100 μg) from wild-type and *twf*[3701] 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 4. **Twinfilin is ubiquitously expressed in embryos and ovaries.** Wild-type (A and C–E) and *twf*[3701] 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*[3701] 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.

al., 2000a). In the *twf*[3701] mutant, all bristles were affected (Fig. 5, A–I), macrochaetae (Fig. 5, E–G) more severely than microchaetae (Fig. 5, C and D). The hairs are also slightly shorter than normal (Fig. 5, C and D). The macrochaetae in *twf*[3701] mutant flies are shorter than in the wild-type flies and have a rough surface. SEM revealed that the ridges and grooves on the *twf*[3701] mutant's macrochaetae are highly irregular (Fig. 5, F and G) as compared with the completely straight ridges and grooves seen in wild-type bristles (Fig. 5 E). The bristles often had a thicker portion somewhere along the shaft (Fig. 5 F), and in the thickenings the ridges were oriented perpendicular to the long axis. The tip of the bristle was also not as thin as in the wild-type flies, and had a smooth surface with only short, randomly oriented ridges (Fig. 5, H and I). The macrochaetae, but not microchaetae, were often bent, split or branched (Fig. 5 B). The described bristle phenotypes are equally severe in *twf*[3701]/

Df(3R)SuHw[7] transheterozygotes, which carry a large deletion uncovering the *twf* gene (unpublished data). This suggests that the *twf*[3701] 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* (*tsr*) 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*[k05633], which has a lethal insertion in *twinstar*, with the *twf*[3701] 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*[k05633]/ +; *twf*[3701]/+ flies, 66% of the bristles were split, branched, or had a rough surface, whereas only 2% of the bristles on *tsr*[k05633]/+; +/+ flies and none of the bristles on +/+; *twf*[3701]/+ flies had this phenotype. The presence of a single *tsr*[k05633] allele in the *twf*[3701] 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*[3701] mu-
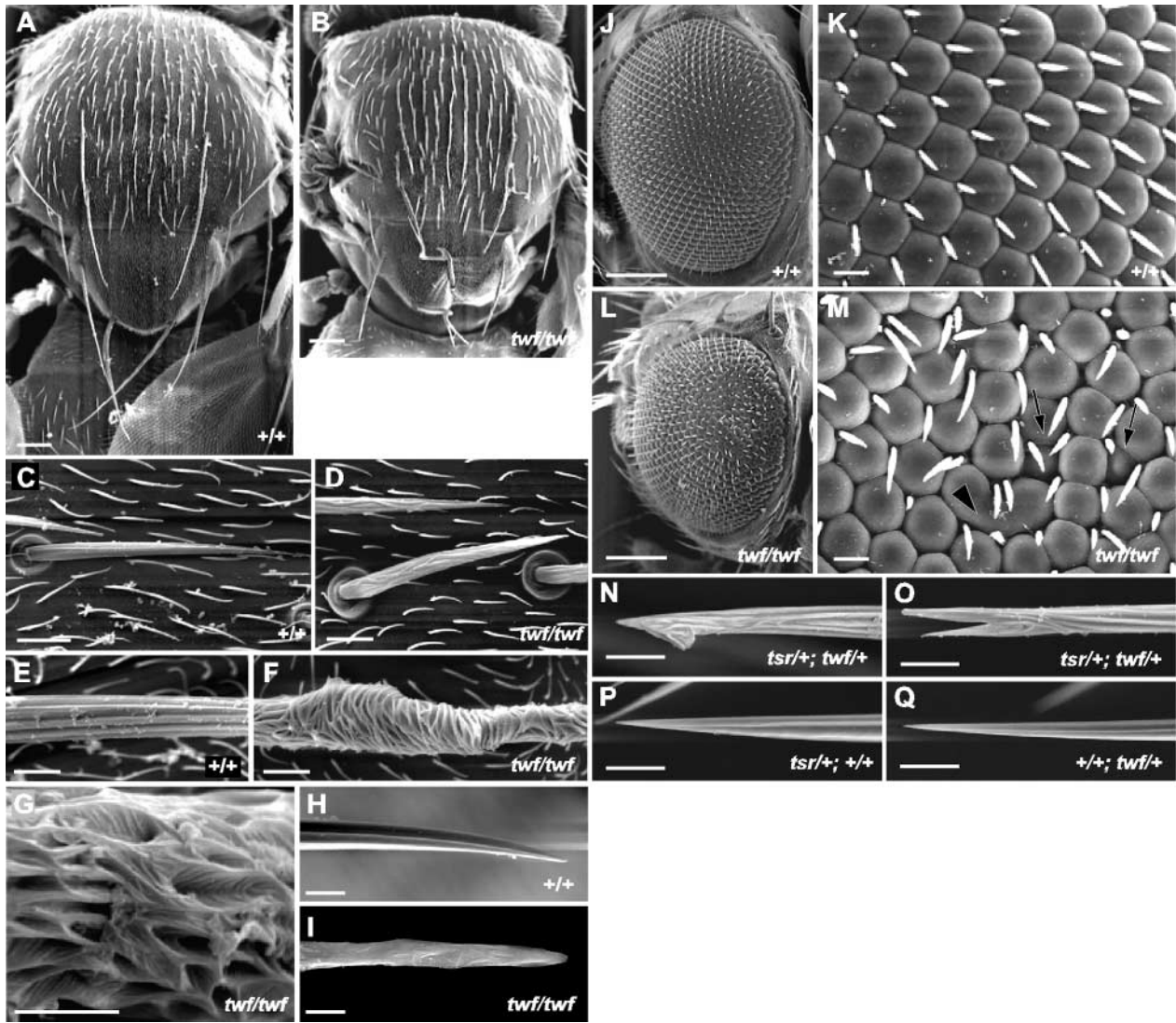
Figure 5.   ***twf* mutants have defects in bristle morphology and in eyes and interact genetically with *twinstar (tsr)* mutants.** Scanning electron micrographs of bristles (A–I and N–Q) and eyes (J–M). (A) The whole thorax from wild-type and (B) *twf*[3701] mutant adult. Note the bent and split bristles. (C) Microchaete from wild-type and (D) from the *twf*[3701] mutant. Note the irregularly arranged ridges on the surface and shorter hairs than in the wild-type. (E) Surface of a wild-type macrochaete showing straight longitudinal ridges. (F) Thickening on a mutant *twf*[3701] macrochaete with ridges arranged perpendicularly to the long axis of the bristle. (G) High magnification image of a *twf*[3701] macrochaete with highly irregular ridges. (H) Tip of a wild-type bristle and (I) tip of a *twf*[3701] mutant bristle. Note the smooth surface compared with the wild-type bristle. (J) wild-type eye and (L) *twf*[3701] mutant eye. (K) High magnification of wild-type ommatidia and interommatidial bristles. (M) In *twf*[3701] 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/+; twf/+* double heterozygotes showing a hooked (N) or a split tip (O). (P and Q) Normal tip of a *tsr/+; +/+* (P) and a *+/+; twf/+* (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.

tant alone (unpublished data). These results show that *twinfilin* interacts genetically with the ADF/cofilin encoding gene *twinstar* during bristle and eye morphogenesis.

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*[3701]/*twf*[3701] and *twf*[3701]/*Df(3R)SuHw*[7] 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 bris-

tle 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*[3701] 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

Figure 6. Lack of twinfilin results in disorganized actin filament bundles in developing bristles. Projections of confocal sections through wild-type (A and F), *twf*^3701^/*twf*^3701^ (B–D) and *twf*^3701^/*Df(3R)SuHw*^7^ (E and G–I) pupal bristles. The bristles were dissected from 48-h-old pupae, except for C, which is from a 41-h-old pupa. (A) Middle portion of a wild-type bristle showing straight actin bundles. (B) Mutant bristle with F-actin containing spots between the bundles. Note the variation in bundle thickness. (C) Mutant bristle with thin bundles perpendicular to the long axis of the bristle. (D) Bundles are misoriented perpendicular to the long axis and form a knob on the bristle. (E) Weakly fluorescent bundles twist around the bristle, while straight strongly fluorescent bundles appear to be located internally. (F) Tip of a wild-type bristle. (G) Numerous tiny bundles are oriented perpendicular to the main bundles in a mutant bristle tip. (H) Surface view of a mutant bristle tip where some thicker bundles are visible among the tiny bundles. (I) A mutant bristle with two bends. The main bundles in the basal part of the bristle end at the bending point. Bars, 10 μm.

and G). 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, whereas the remaining straight strongly fluorescent bundles appeared to be located more 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 con-

densed 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*^3701^ 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*^3701^ 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*^3701^ mutant flies, the adult macrochaetae are split, branched, or bent and have a highly irregular ridge pattern. This pheno-

| phalloidin | anti-twinfilin | merged |
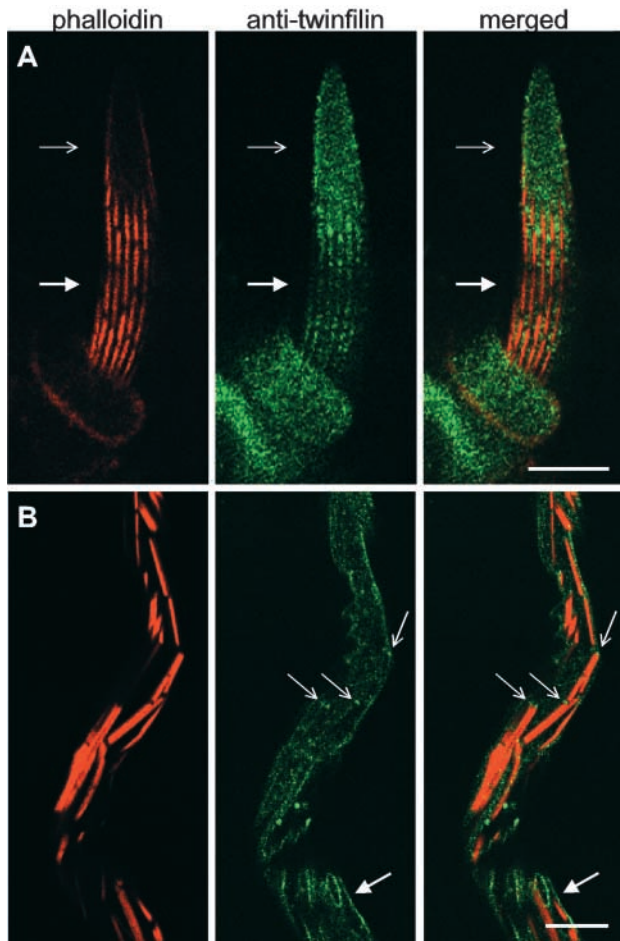
Figure 7. **Twinfilin shows cytoplasmic and cortical localization and is present at actin filament ends.** (A) Confocal section through a 32-h-old wild-type bristle stained with phalloidin and anti-twinfilin antiserum. The bristle is oriented so that the section is cut through the center of the bristle in the upper part (thin arrow) and at the surface in the lower part (thick arrow). Twinfilin is evenly distributed in the cytoplasm of the bristle's center (thin arrow) and as spots between the actin filament bundles at the surface (thick arrow). (B) A confocal section from a 48-h-old wild-type bristle. Twinfilin is localized at the plasma membrane (thick arrow) and as spots at the tip-most end of bundle modules (thin arrows). Bar, 10 μm.

(Verheyen and Cooley, 1994) or the β-subunit of capping protein (Hopmann et al., 1996), all of them proteins that are involved in regulation of actin dynamics, lead to defects in bristle morphology. Interestingly, the severely affected bristles in the capping protein mutant (Hopmann et al., 1996) are almost identical to the bristles in the *twf*[3701] mutant.

In addition to unstable main bundles, we observed ectopic actin filament-containing spots or perpendicular tiny bundles in fully elongated *twf*[3701] 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 *twf*[3701] 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 spots in *twf*[3701] mutant macrochaetae are reminiscent of 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. 7 B). 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/+; *twfl+* 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 *twfl+* mutation, lead to defects in bristle morphogenesis.

type is similar to the one seen in *singed* and *forked* mutants, which arise from mutations in actin filament–bundling proteins (Cant et al., 1994; Petersen et al., 1994; Tilney et al., 1995, 1998). The *twf*[3701] bristle phenotype is explained by our observations that in developing mutant bristles, the actin bundles are twisted and misoriented (Fig. 6). However, unlike fascin (*singed* gene product) and forked, twinfilin is an actin monomer–sequestering protein with no detectable affinity for actin filaments (Fig. 2 A). Thus, the presence of a severe bristle phenotype in the *twf*[3701] mutant demonstrates that the accurate regulation of the size and dynamics of the actin monomer pool is essential for actin filament assembly and subsequent bundle formation. This conclusion is also supported by previous experiments in which developing bristles were cultured in vitro together with drugs that affect filament elongation (Tilney et al., 2000b). Furthermore, mutations in *Drosophila* cofilin (Chen et al., 2001), profilin

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 λZap cDNA library using primers 5′-CGCGCCATGGCTCACCAAACG-GGTATCCGAGCCAAC and 5′-GCGCGAATTCTTAGTCCTCTGCGGTGG-GACGCGTGAGGCG. The products were 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′-CGTCGTTTTTCT-TGAGCACGTAC and 5′-GATGACTACCACCACCACAGGC.

Proteins and antibodies
Drosophila twinfilin was purified as a glutathione *S*-transferase fusion similar to murine twinfilin (Vartiainen 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
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 $CaCl_2$) and polymerized for 30 min by the addition of 5 μl of 10× initiation mix (20 mM $MgCl_2$, 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 centrifugating the samples for 30 min in a Beckman Optima MAX Ultracentrifuge at 217,000 g using a TLA100 rotor. 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 stain and scanned with a FluorS™-imager (Bio-Rad Laboratories). The intensities of actin and twinfilin bands were quantified with the QuantityOne program (Bio-Rad Laboratories).

Actin filament assembly assays
Kinetics of actin filament assembly and disassembly were monitored by pyrene fluorescence with excitation at 365 nm and emission at 407 nm using BioLogic MOS-250 fluorescence spectrophotometer. For assembly assays, 64 μl of G buffer (5 mM Tris, pH 7.5, 0.2 mM ATP, 0.2 mM DTT, 0.2 mM $CaCl_2$) containing 3.75 μM actin (1:6 pyrene actin:human platelet actin) was mixed with 8 μl of G-buffer or 15/30 μM twinfilin. Polymerization was induced by addition of 8 μl of 10× initiation mix. For disassembly assays, 3.3 μM actin (1:6 pyrene actin:human platelet actin) in G buffer was polymerized by the addition of 1/10 vol of 10× initiation mix for 1 h. Depolymerization of filaments was induced by mixing 72 μl of filaments with 8 μl of G buffer or 15/30 μM twinfilin.

Western blotting
Wild-type and mutant larvae were washed with PBS containing a dissolved protease inhibitor cocktail tablet (Roche). Larvae were homogenized with a Dounce homogenizer and the resulting cells were lysed with 1% Triton X-100. The proteins in the cell lysates were separated on a 12% SDS-polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and immunoblotted with a 1:2,000 dilution of anti-twinfilin antiserum.

SEM
Whole adult flies were anaesthetized with CO_2, 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 *Df(3R)SuHw7/TM6Tb* and *tsr^{k05633}/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 *tsr^{k05633}/CyO* males and *twf^{3701}* homozygous females. Bristles of the progeny with normal wings were examined by light microscopy and SEM along with *tsr^{k05633}/+* and *twf^{3701}/+* 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 U/ml. The samples were mounted in Vectashield (Vector Laboratories), and examined with a Biorad MRC 1024 confocal microscope. Optical sections were combined using the Confocal Assistant 4.02 program.

Immunostainings
Wild-type and *twf^{3701}* embryos were collected, fixed, and stained according to standard protocols. The anti-twinfilin antiserum 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 antiserum 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 antiserum 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 ($\epsilon = 34\,990$ $M^{-1}cm^{-1}$) and for actin at 290 nm ($\epsilon = 26\,600$ $M^{-1}cm^{-1}$). Total RNA was isolated using the TRIZOL reagent (GIBCO BRL; Life Technologies) according to the manufacturers' instructions and blotted and hybridized according to standard protocols.

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