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Profilin mutations disrupt multiple actin-dependent processes during *Drosophila* development

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SUMMARY

The *chickadee* gene of *Drosophila* encodes profilin, a small actin binding protein. We present the first analysis of the effects of profilin deletion in a multicellular organism. Genomic deletions of the *chickadee* locus result in a late embryonic lethal phenotype indicating that profilin is essential in flies. In addition, viable alleles of *chickadee* with defects in oogenesis, spermatogenesis and bristle formation provide insight into profilin function in a variety of cell types. Defects in oogenesis include the previously described failure to assemble nurse cell actin filament bundles in addition to abnormal regulation of mitosis, binucleate cells and stalled cell migration. Malformed bristles are a result of aberrant actin assembly. Monoclonal antibodies against *Drosophila* profilin were generated to study profilin's cellular and subcellular localization.

Key words: *Drosophila*, profilin, actin cytoskeleton, oogenesis, bristles

INTRODUCTION

The analysis of cytoskeletal components has been facilitated by the use of mutational and genetic analyses. This approach has been a successful way to reconcile and support in vitro data with in vivo observations. One difficulty often encountered in such studies is that mutagenizing cytoskeletal proteins generally leads to death. We have used oogenesis in *Drosophila* as a model system in which to dissect genetically the components of the actin-dependent process of cytoplasm transport. This strategy has identified mutations that affect the dynamics of the actin cytoskeleton, without affecting the viability of the fly (Cooley et al., 1992; Xue and Cooley, 1993; Cant et al., 1994). We have previously reported that the *chickadee* (*chic*) gene encodes *Drosophila* profilin and have described female sterile *chic* mutations that block polymerization of extensive networks of cytoplasmic actin filaments in the nurse cells of the egg chamber (Cooley et al., 1992).

Extensive biochemical analysis of profilin has led to several models of the physiological functions of the protein. Profilin is a 12-15×10^3 M_r ubiquitous cytoplasmic protein that binds actin monomers in a 1:1 complex and regulates filament polymerization (Stossel et al., 1985; Pollard and Cooper, 1986). Profilin binding to actin monomers prevents spontaneous nucleation of actin filaments (Pollard and Cooper, 1984). This prevention of nucleation by profilin does not affect elongation of existing filaments (Tilney et al., 1983; Pollard and Cooper, 1984). Profilin may also affect actin polymerization through the stimulation of nucleotide and divalent cation exchange on actin monomers (Mockrin and Korn, 1980; Nishida, 1985; Goldschmidt-Clermont et al., 1991). Since ATP-actin monomers add to growing filaments at a faster rate than ADP-actin monomers and have a lower critical concentration (Pollard, 1986), it has been proposed that in the presence of excess ATP, the stimulation of nucleotide exchange by profilin may accelerate actin polymerization (Goldschmidt-Clermont et al., 1991).

In addition to regulating actin assembly, both in vitro and in vivo evidence suggest a role for profilin in signal transduction. Profilin binds a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), with high affinity and can regulate its hydrolysis by unphosphorylated phospholipase C-γ1 (Lassing and Lindberg, 1985; Goldschmidt-Clermont et al., 1990). The phosphorylated form of phospholipase C-γ1 can hydrolyze PtdIns(4,5)P_2 bound to profilin, presumably releasing profilin into the cytoplasm (Goldschmidt-Clermont and Janmey, 1991). In addition, evidence from *Saccaromyces cerevisiae* suggests a link between profilin's regulation of PtdIns(4,5)P_2 hydrolysis and the RAS signaling pathway (Vojtek et al., 1991). Activation of the adenylyl cyclase complex by RAS requires an associated protein called CAP. Disruption of the carboxy terminus of CAP results in morphological and nutritional defects. Such mutations in CAP can be suppressed by overexpression of profilin (Vojtek et al., 1991).

Analysis of profilin null mutations in yeast have provided the first genetic evidence for an important in vivo role for profilin (Magdolen et al., 1988; Haarer et al., 1990). Although profilin is non-essential in yeast under permissive growing conditions, mutant cells display a number of aberrant phenotypes. Cells lacking profilin grow more slowly, become larger than wild-type cells and are often multinucleate. In profilin mutant cells, actin cables are no longer detected and actin spots appear

delocalized. Anti-actin antibodies were able to detect thick actin bars, which may have formed as a result of improper regulation of actin assembly (Haarer et al., 1990).

The *chickadee* gene in *Drosophila* contains two promoters that produce two transcripts sharing the same open reading frame encoding profilin (Cooley et al., 1992). *chickadee* female sterile mutations result from the disruption of the ovary-specific profilin transcript. The second transcript, that is constitutively expressed, is unaffected in homozygous mutant females. Here we report the analysis of new alleles that reduce or eliminate the constitutive expression of profilin. These new *chic* alleles can be organized into an allelic series of increasing severity ranging from effects on germ cell proliferation and cytokinesis, bristle and eye development to embryonic lethality in null alleles. In addition to phenotypic characterization of *chickadee* mutations, we raised monoclonal antibodies to *Drosophila* profilin to study the protein distribution and subcellular localization in various tissues.

MATERIALS AND METHODS

Drosophila stocks

All fly stocks were maintained under standard culturing conditions. Wild-type genomic DNA for Southern blot analysis was obtained from *cn*;*ry*^{506}. Female sterility was assessed by placing homozygous females in vials with an excess of males and scoring for any progeny after 10 days. Homozygous males were tested with five virgin females and the presence of progeny was scored 10 days later. Males and females were classified as weakly fertile if they produced less than 25% of the wild-type number of progeny.

Antibody production

Hybridoma cell lines were derived from a mouse immunized with a fusion protein containing the profilin open reading frame cloned into the expression vector pGEX-3X (Smith and Johnson, 1988). After induction of the fusion protein in *E. coli*, the protein was purified from crude lysates by affinity chromatography on immobilized glutathione (Smith and Johnson, 1988). 50 μg purified fusion protein was used to immunize mice by intraperitoneal injection. Antisera were screened by western blotting to the fusion protein and immunostaining to ovaries. Monoclonal cell lines were generated following standard procedures (Harlow and Lane, 1988).

Western blot analysis

Drosophila ovaries or whole animals were ground in SDS sample buffer and protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories). 15% polyacrylamide gels were loaded with 40 μg of protein per lane and transferred to Immobilon-P membrane (Millipore). Blocking, antibody incubation and detection were carried out according to Xue and Cooley (1993). Monoclonal anti-profilin cell culture supernatant from line 6F was used at a 1:10 dilution.

Immunofluorescence staining

The procedure for antibody staining of ovaries was described previously (Xue and Cooley, 1993). Undiluted anti-profilin monoclonal supernatant from cell line 4H was used. The secondary FITC-conjugated goat anti-mouse IgG (Pierce) was used at a concentration of 1:200.

Embryos were fixed and stained according to the method of Patel et al. (1989) with the following exception. After incubation with an FITC-conjugated goat anti-mouse secondary antibody diluted 1:200, embryos were washed and mounted in 50% glycerol. Anti-profilin monoclonal supernatant from cell line 6F was used undiluted.

Egg chamber staining procedures

Ovaries were dissected in *Drosophila* saline solution and stained for β-galactosidase activity according to Cooley et al. (1992). For actin staining, ovaries were dissected into individual egg chambers, fixed in devitellinizing buffer and heptane (see Cooley et al., 1992), and rinsed thoroughly in saline. Rhodamine-conjugated phalloidin (10 μl) (Molecular Probes no. R-415) was vacuum dried and resuspended in 100 μl of saline. Egg chambers were incubated for 20 minutes in the dark with 50-100 μl rhodamine-phalloidin and then rinsed with saline. For DAPI staining, fixed egg chambers were incubated for 5 minutes in 1 μg/ml DAPI in saline followed by several washes in saline. Egg chambers were mounted in 50% glycerol in PBS.

To count the number of nurse cells in mutant egg chambers, either rhodamine-conjugated phalloidin was used to stain cell membranes or antibodies to the kelch protein (Xue and Cooley, 1993) were used to stain ring canals. We found that in every mutant egg chamber examined the number of ring canals corresponded to the number of nurse cells.

Tissue dissection

White prepupae were collected and the nota and heads were dissected at 38-42 hours of pupal development according to Schweisguth and Posakony (1992). After fixation, the tissue was stained with rhodamine-conjugated phalloidin as described above. The tissue was washed extensively in PBS and mounted in 50% glycerol. Bristle actin was examined on a confocal microscope where optical sections were superimposed.

Testes were dissected out of adult males in PEM (0.1 M Pipes, 1 mM $MgCl_2$, 1 mM EGTA pH 6.9). They were fixed in 4% paraformaldehyde in PEX (PEM + 0.1% Triton X-100) (Gönczy et al., 1992). After fixation, the testes were mounted in 50% glycerol.

Microscopy

Microscopy was carried out on a Zeiss Axiophot microscope equipped with differential interference contrast and epiflourescence optics. Confocal images were collected using the MRC 600 system (Bio Rad). Optical sections were combined using the program 'Project'. For the scanning electron microscope, flies were ethanol dehydrated, mounted on stubs and air dried. Flies were viewed on an ISI-SS40 electron microscope.

P element excision strategy

To remobilize the P element in *chic*^{7886}, a Δ2-3 jumpstarter strain (Laski et al., 1984; Robertson et al., 1988) was crossed into the *chic*^{7886} background to supply a source of transposase. Flies in which excision events had occurred were detected by scoring progeny for loss of the *rosy*$^{+}$ eye color marker carried by the P element. The phenotype of the *rosy*$^{-}$ revertants was determined by making the mutant chromosomes homozygous. Lines with phenotypes more severe than the original *chic*^{7886} phenotype were further characterized by genetic complementation testing and Southern blotting.

Nucleic acid blotting

Genomic DNA for Southern blot analysis was isolated from adults using standard procedures (protocol 48 in Ashburner, 1989). DNA gels were blotted by capillary transfer to Hybond-N (Amersham) and ultraviolet cross-linked (Stratalinker, Stratagene). ^{32}P-labeled DNA probes were prepared using random hexamer priming according to Feinberg and Vogelstein (1983). Hybridization was in aqueous solution at 65°C with agitation (Church and Gilbert, 1984).

RESULTS

Expression of profilin throughout development

The expression of profilin during various stages of *Drosophila*

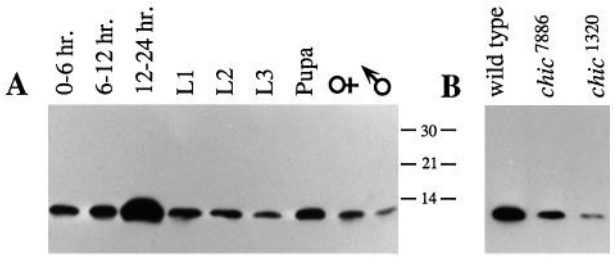

Fig. 1. Western blot analysis of profilin expression during development and in the ovary. (A) Western blot analysis in which 40 μg of total protein were loaded per lane from wild-type animals in specific stages of development. The blot was probed with monoclonal antibody 6F against *Drosophila* profilin which detects a single 12×10^3 M_r protein. Protein molecular mass markers are indicated ($\times10^{-3}$) between A and B. The first three lanes correspond to hours of embryonic development. L1, L2 and L3 correspond to the three larval instars. The pupal lane contains protein from a mixed collection of pupal stages. The male and female protein was obtained from whole adult flies. (B) Western blot of ovary protein extracts from wild type, weakly fertile *chic*^{7886} and the female sterile *chic*^{1320} alleles of *chickadee*. Equal amounts of protein (40 μg) were loaded per lane.

development was examined by western immunoblotting (Fig. 1A). Profilin was present throughout development with a strong surge in expression in 12- to 24-hour embryos and a second less dramatic increase during pupal stages. The adult extracts showed the lowest expression levels. The reduced expression may reflect the reduced mitotic activity of adult tissue.

Because the original *chic* alleles were female sterile, we examined profilin in ovary extracts from wild type, *chic*^{7886} (a weakly fertile allele) and *chic*^{1320} (a fully sterile allele; Fig. 1B). The amount of profilin was reduced in the mutant ovaries, with the level of reduction corresponding to the severity of the phenotype. Since female sterile alleles do not express the ovary-specific transcript (Cooley et al., 1992), the remaining protein detected in the *chic*^{1320} lane corresponds to expression from the constitutive promoter.

The subcellular localization of profilin in egg chambers was examined, in part to determine the distribution of the residual protein in *chickadee* mutant ovaries. The oocyte develops in a structure called the egg chamber that is formed at the anterior of the ovary in the germarium (for oogenesis reviews, see King, 1970 and Spradling, 1994). Incomplete cytokinesis during the four mitotic divisions of a germline stem cell daughter results in sixteen germline cells interconnected by fifteen cytoplasmic bridges, or ring canals. One becomes the oocyte and the other 15 differentiate into nurse cells. After the mitotic divisions, the cluster of cells is enclosed by somatic follicle cells. The egg chambers develop in ovariole tubes, with more mature egg chambers moving posteriorly in the ovary. As the egg chamber matures, the 15 nurse cells synthesize and transport into the oocyte components that are required for subsequent embryonic development. Late in oogenesis, during stage 10B, extensive networks of actin filaments polymerize in the cytoplasm of each nurse cell. In stage 11, the nurse cells contract and their remaining cytoplasmic contents are rapidly transported into the oocyte. This results in regression of the nurse cell cluster, and doubling of the oocyte volume.

In wild-type ovaries, profilin was highly expressed in the cytoplasm of the somatic follicle cells as these cells migrate and enclose germline clusters to form egg chambers in the germarium (Fig. 2A). In these somatically derived cells, low levels of profilin were found associated with the plasma membrane (arrow in 2A). The high cytoplasmic expression in the follicle cells decreased after stage 2 (arrowhead in Fig. 2B), but the protein remained expressed in these cells throughout oogenesis (Fig. 2B). In the follicle cells covering the oocyte at stage 10 we also detected low levels of membrane associated profilin in addition to cytoplasmic profilin (not shown). Profilin was very abundantly expressed in a specialized set of follicle cells, called border cells, that migrate through the nurse cell cluster from the anterior of the egg chamber to the anterior edge of the developing oocyte (arrow in Fig. 2B). This cluster of cells was often observed extending cell surface projections during the migration. In the germarium, profilin was also expressed in the cytoplasm of the germline cells, however at a much lower level than in the somatic tissue. Profilin was detected in the nurse cell cytoplasm throughout oogenesis (Fig. 2B), with the highest level of expression at stage 10 (Fig. 2C).

Examination of ovaries from *chic*^{1320}, a strong female sterile allele, revealed that only a subset of the profilin expression pattern was affected. The expression in somatic follicle cells throughout oogenesis appeared unaffected, while expression in the nurse cell cytoplasm was dramatically reduced (Fig. 2D). This reduction was most apparent at stage 10, where the low level of profilin in the nurse cell cytoplasm correlated with the failure to polymerize extensive actin filament networks (Cooley et al., 1992). Thus, the reduction in total protein in the ovary detected by western blot (Fig. 1B) is likely to result from an absence of high levels of the protein in the nurse cell cytoplasm. These results indicate that the ovary-specific transcript that is disrupted in female sterile alleles of *chickadee* (Cooley et al., 1992) is in fact nurse cell-specific. It does appear that the constitutive transcript provides a basal level of profilin expression in the germline cells, which is still present in *chic*^{1320}.

Generation of more severe *chickadee* alleles

To examine further the function of profilin in *Drosophila* we determined the phenotype of a profilin null mutation by inducing deletions at the locus using P element excision. A source of transposase was genetically introduced into an insertion allele of *chickadee* (*chic*^{7886}) to catalyze the mobilization of the P element. The orientation of the P element places the *rosy*$^+$ gene adjacent to the rest of the *chickadee* gene. This proved to be important for obtaining deletions extending into *chickadee* since we scored for excisions by selecting for loss of the *rosy*$^+$ eye color marker. 333 independent *rosy*$^-$ excision lines were isolated after mobilizing the P element in *chic*^{7886} and their phenotypes were analyzed by making the mutant chromosomes homozygous. 261 of the lines displayed homozygous phenotypes roughly the same as *chic*^{7886}, and corresponded to internal P element excisions. The second most common event was precise excision of the P element, restoring fertility in 57 homozygous *rosy*$^-$ lines. Eleven lines displayed phenotypes more severe than *chic*^{7886}. Only one of these lines was homozygous viable (*chic*^{37}), with a severe female sterile phenotype, and the addition of male sterility and bristle defects. The other ten *rosy*$^-$ lines were homozygous lethal.

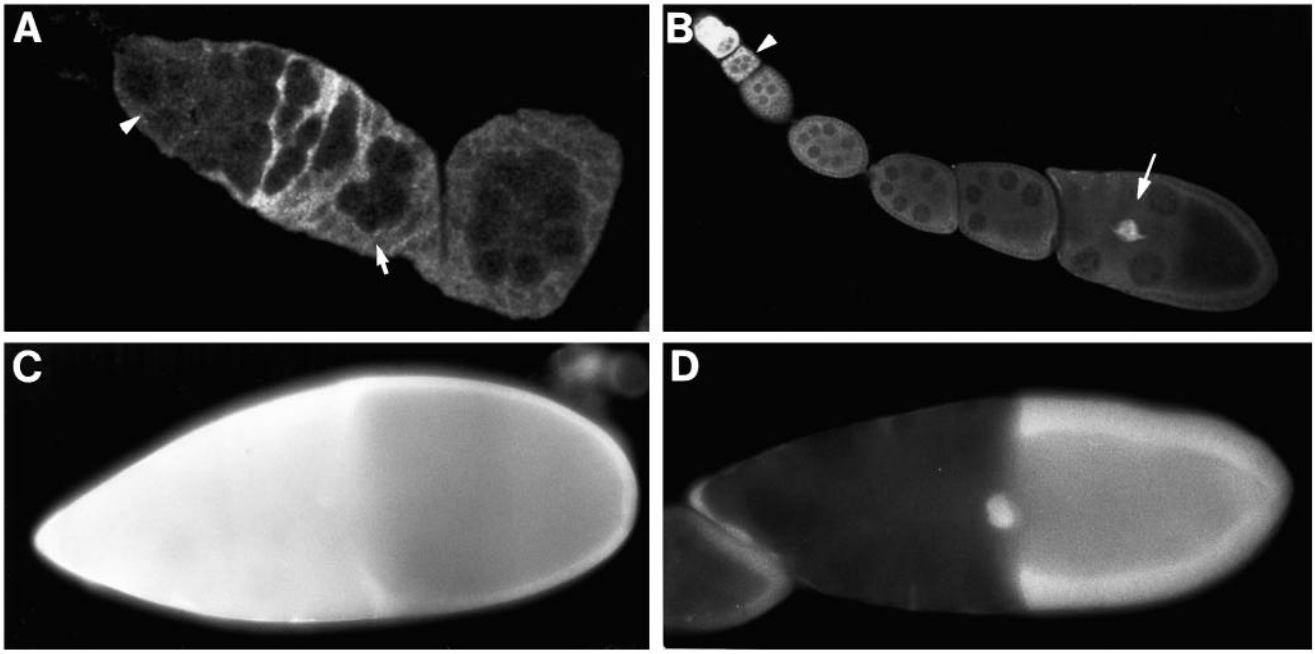

Fig. 2. The subcellular localization of profilin in egg chambers. In the germarium, profilin is present in the cytoplasm of germline cells (arrowhead in A). It is highly abundant in the cytoplasm and at the plasma membrane (arrow in A) in the somatic follicle cells as they migrate to enclose the egg chambers. After stage 2 (arrowhead in B) profilin is expressed in the cytoplasm of both germline and somatic tissue (B). During stage 9, the migrating border cells express very high levels of profilin (arrow in B). At stage 10, there is strong expression in both the germline and somatic tissue (C). In ovaries from a *chic*^{1320} female, the expression in the somatic follicle cells is unaffected, however there is a dramatic reduction of profilin in the nurse cell cytoplasm (D). We showed standard micrographs in C and D (instead of confocal sections as in A and B) to emphasize this reduction.

Previously we described a divergently transcribed gene in close proximity to the *chickadee* locus (Cooley et al., 1992). This gene is now known to encode the *Drosophila* homolog of the eukaryotic translation initiation factor 4A (eIF4A; L. C. and E. M. V., data not shown; Dorn et al., 1993). While characterizing this genetic interval, we determined that mutations in the *eIF4A* gene are lethal and allelic to *l(2)gpdh-4* (Knipple and MacIntyre, 1984). Recently, Dorn et al. (1993) described lethal mutations of *eIF4A* and designated the gene *l(2L) 162*^{neo} after a P element insertion mutation at the locus. We will refer to the gene encoding the eIF4A protein as *eIF4A* in this paper.

Profilin is an essential protein

To establish whether the lethality of the excision lines was caused by deletion of *chickadee*, *eIF4A* or both, genetic and molecular approaches were undertaken. Complementation testing showed that the ten lethal lines fell into three groups (Fig. 3A). The group I lethals (106, 112 and 129 in Fig. 3A) failed to complement both *chickadee* and *eIF4A* mutations and are therefore deletions affecting proper functioning of both genes. These three lines were lethal in combinations with all other deletion lines obtained. The group II lethals (38, 103, 139) failed to complement the group I lethals, but partially complemented the group III lethals (*chic*^{221}, *chic*^{281}, 211, and 321). Complementation was scored as incomplete because *trans*-allelic flies were female sterile, male sterile and had bristle defects. These phenotypes were also produced by the one viable deletion line (*chic*^{37}) obtained from the excision screen. These data suggested that the deletions carried in the partially complementing group II and III lethals overlapped only in the region that was also deleted in *chic*^{37} (Fig. 3A). The group III lethal lines failed to complement *chickadee*, but fully complemented the eIF4A mutant, *l(2)gdh-4*.

After genetically determining that the group III lethals map to *chickadee*, we mapped the deletion breakpoints molecularly for three of the lines using Southern blotting (Fig. 3C). Genomic DNA was prepared from *chic*^{37}, *chic*^{221}/CyO and *chic*^{281}/CyO. Breakpoints were mapped by probing for restriction fragment length polymorphisms induced by the deletion. The breakpoints for the two lethal lines, *chic*^{221} and *chic*^{281}, both mapped within the fragment detected by probe I (Fig. 3C, left panel). This probe detects only *chickadee* transcripts on a northern blot (L. C., data not shown). The breakpoint for the viable line *chic*^{37} was found using probe II, which is a small *Eco*RI-*Xba*I fragment located between the first and second exons of *chickadee* (Fig. 3C, center panel). The genetic complementation data with *l(2)gdh-4* suggested that the *eIF4A* gene was probably not affected by these deletions. Probe III, which detects the DNA directly flanking the *chic*^{7886} P element insertion site, was used to confirm that the *eIF4A* gene was intact (Fig. 3C, right panel). Thus the two lethal lines *chic*^{221} and *chic*^{281} are deletions that affect only the *chickadee* gene, and are obligate null alleles.

Genetic analysis revealed that *chickadee* alleles can be arranged in a phenotypic series which correlates increasingly severe phenotypes with reduced levels of profilin expression (Table 1). The original female sterile phenotype was previously described in detail (Cooley et al., 1992) and represents loss of function for one aspect of profilin function. In subse-

quent sections we describe the pleiotropic consequences of reducing somatic and germline profilin concentrations using various combinations of our new *chickadee* alleles.

Profilin disruption causes aberrations in germline nuclear and cell number

In the female sterile allele *chic*^{1320}, only the nurse cell-specific transcript was absent (Cooley et al., 1992). When this allele was made hemizygous with the null mutation *chic*^{221}, or a larger deficiency for the region, *Df(2)GdhA*, more severe egg chamber defects were observed. These were presumably due to halving the expression of the constitutive transcript in both the somatic and germline tissue. The defects were first observed by staining nuclear material with the dye DAPI. We observed variations from the normal number of fifteen nurse cell nuclei (Fig. 4D,E; Fig. 5). Often the nuclei appeared to be in pairs, so we investigated whether all the nurse cells were binucleate. Egg chambers were double stained with DAPI and rhodamine-conjugated phalloidin. Staining of the actin allowed us to map the plasma membranes of nurse cells and deduce the number of nurse cells in an egg chamber (Fig. 4A,B,C). We found that the majority of egg chambers had too few nurse cells and these were mostly binucleate, as determined by counting the number of nuclei within the egg chamber (Fig. 4D,E). The variation in cell number was great, with egg chambers containing between 2 and over 30 nurse cells (Fig. 5). Egg chambers with more than eight nurse cells had too many nuclei to count accurately. However, we were able to determine that nuclei were always in excess of nurse cell number.

An additional defect seen in about 10% of the egg chambers from *chic*^{1320}/*chic*^{221} females was a failure of proper border cell migration. As described above, profilin is highly expressed in the somatically derived border cells and allelic combinations that reduced the somatic expression of profilin delayed the migration of these cells through the nurse cell cluster (data not shown).

Profilin is required for germline proliferation

The one homozygous viable mutant obtained in the excision screen, *chic*^{37}, contained a genomic deletion between the first and second exons of *chickadee*. Examination of gonads from *chic*^{37} homozygous animals revealed dramatic defects in the proliferation of both the male and female germline. Mutant

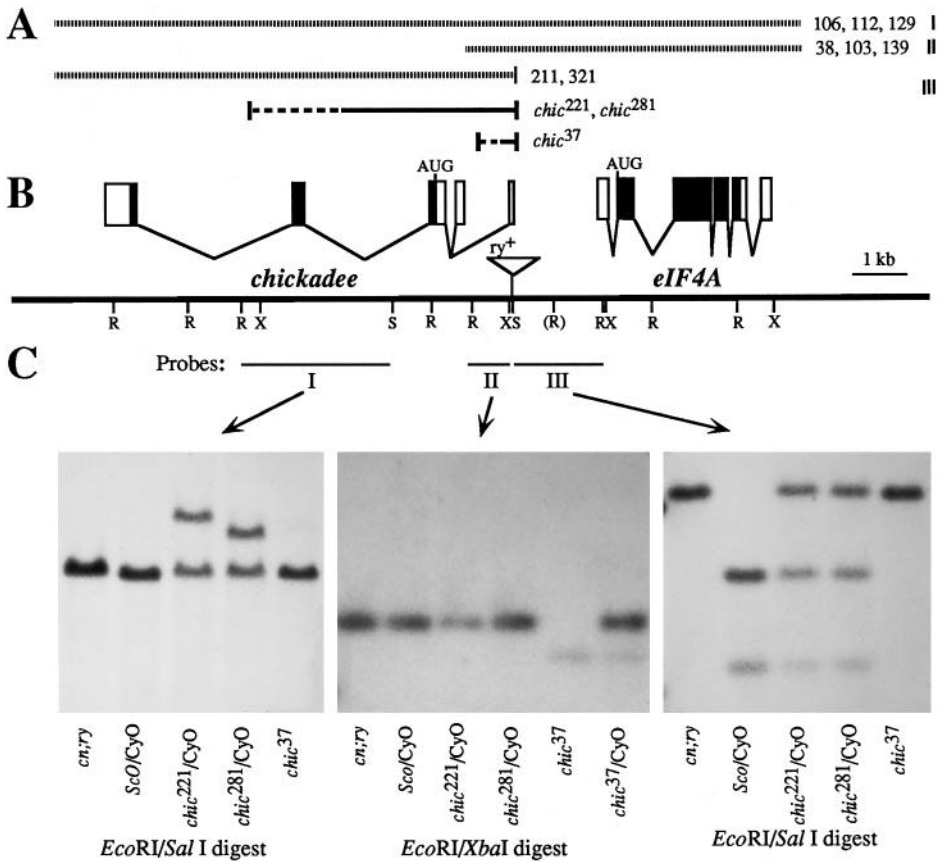

Fig. 3. Mapping deletions at the *chickadee* locus. Excision lines with phenotypes more severe than the original *chic*^{7886} phenotype were characterized. One viable stock (*chic*^{37}) was generated in addition to ten homozygous lethal stocks (*chic*^{221}, *chic*^{281}, 211, 321, 38, 103, 139, 106, 112, 129). The extent of the P element excision-induced deletions was determined both genetically and through Southern blot analysis. (A) Genetic mapping of induced deletions. Complementation testing between the lethal lines allowed them to be placed into several groups, corresponding to the extent of their deletions. The striped lines represent the extent of deletions as determined genetically. Group I (106, 112 and 129) and group II (38, 103 and 139) lethals have deletions that affect the *eIF4A* transcription unit, in addition to affecting the *chickadee* locus. *chic*^{37} and group III lethals (*chic*^{221}, *chic*^{281}, 211 and 321) fully complemented the eIF4A mutant, *l(2) gdh-4*. (B) Genomic map and gene organization of the divergently transcribed *chickadee* and *eIF4A* genes. The *chic*^{7886} P element insertion site in the first exon of *chickadee* is indicated by a triangle. Transcription of *chickadee* beginning at exon 1 is nurse cell specific while transcription from exon 2 is constitutive. (C) Southern blot analysis of putative *chickadee* deletions. Genomic DNA from three of the deletion mutants was isolated, restriction digested with the enzymes indicated below, blotted and hybridized with probes I, II or III. *cn;ry* DNA was used as wild-type, and *Sco*/CyO is DNA from the balancer chromosome stock used to maintain the homozygous lethal lines. Deletion breakpoint-induced restriction fragment length polymorphisms were found using probe I (left blot) for the two lethal lines (221, 281). The band in the mutant lanes, which is the same size as wild type, corresponds to balancer chromosome DNA. The band of altered size represents the deletion chromosome. Probe II was used to map the deletion breakpoint-induced polymorphism for line 37 (center blot). The extent of the molecularly mapped deletion is indicated by the solid lines. The dashes at the ends of these lines indicate that the breakpoints map to within the *Eco*RI-*Sal* I or *Eco*RI-*Xba*I fragments, but the precise location has not been mapped. Probe III detects the genomic DNA directly flanking the original P element insertion site. This probe was used to establish whether the three deletions extended toward *eIF4A*. All three lines are intact to the *eIF4A* side of the P element (right blot). Restriction enzymes used: RI, *Eco*RI; S, *Sal*I; X, *Xba*I. (R) indicates a polymorphic *Eco*RI restriction site present in the balancer stock but absent in wild-type DNA, as detected by probe III.

Table 1. Phenotypes of *chickadee* alleles

	Females		Males		Bristles†	
Allele*	Homozygous	Hemizygous‡	Homozygous	Hemizygous‡	Homozygous	Hemizygous‡
7886	Weak fertile	Sterile	Fertile	Sterile	+	+
1320	Sterile	Sterile	Sterile	Sterile	Extra	Bent
37	Severe sterile¶	Semi-lethal**	Severe sterile¶	Semi-lethal**	Strong bent	Extreme bent
221, 281	Lethal	Lethal	Lethal	Lethal	–	–

*This is not a complete list of *chic* alleles, however each of these represent a phenotypic class. †Adult bristle phenotypes: extra, extra macrochaete bristles; bent, some bristles bent and forked; strong, many bristles affected, thickened, bent and forked; extreme, almost every bristle very bent and kinked. ‡Hemizygous over the deficiency *Df(2)Gdha* (Knipple and MacIntyre, 1984). ¶Severe sterile, germline proliferation defect, germlineless gonads. **Semi-lethal, agametic gonads, mild rough eye defects and extremely reduced viability.

ovaries dissected from pupae or newly eclosed adult females contained only 10-20 egg chambers, corresponding to one or two egg chambers per ovariole (Fig. 6B). The germarium appeared devoid of germline material, resulting in 'empty' follicle cell stacks (small arrow in Fig. 6B). The few egg chambers that did develop (long arrow in Fig. 6B) resembled the most severe mutant egg chambers seen in the allelic combination *chic*^{1320}/*chic*^{221} described above. These egg chambers must be resorbed into the abdomen since in older females no germline tissue was visible, and the follicle cell stacks were elongated (Fig. 6C). It thus appeared that the general organization of the follicle cells was unaffected and they formed empty pseudo-ovarioles devoid of germline cells.

Testes from *chic*^{37} males had a similar defect to that seen in females. Testes from newly eclosed males contained a few spermatid bundles (data not shown). Testes from older males were markedly smaller than wild type and appeared agametic (Fig. 6E). As indicated in Table 1, some less severe *chic* alleles were also male sterile. These alleles had a slightly milder defect, in which mutant testes still contained some spermatid bundles, but mature sperm were non-motile (data not shown; Castrillon et al., 1993).

Profilin is required for proper bristle morphology

In *chic*^{37} homozygous flies, in addition to defects in oogenesis and spermatogenesis, the formation of the peripheral nervous system is disrupted. Both macrochaete and microchaete bristles on the head, thorax, legs and wings are affected to varying degrees (Fig. 7). The bristle shaft is formed as a cytoplasmic extension of the trichogen cell. Its structure is provided by a core of microtubules surrounded by fiber bundles (Overton, 1967). Staining of pupae with rhodamine-phalloidin established that the fibrillar structures were actin filament bundles (Fig. 8; Appel et al., 1993). The ridges seen on the bristle cuticle are formed by cytoplasm of the trichogen cell protrud-

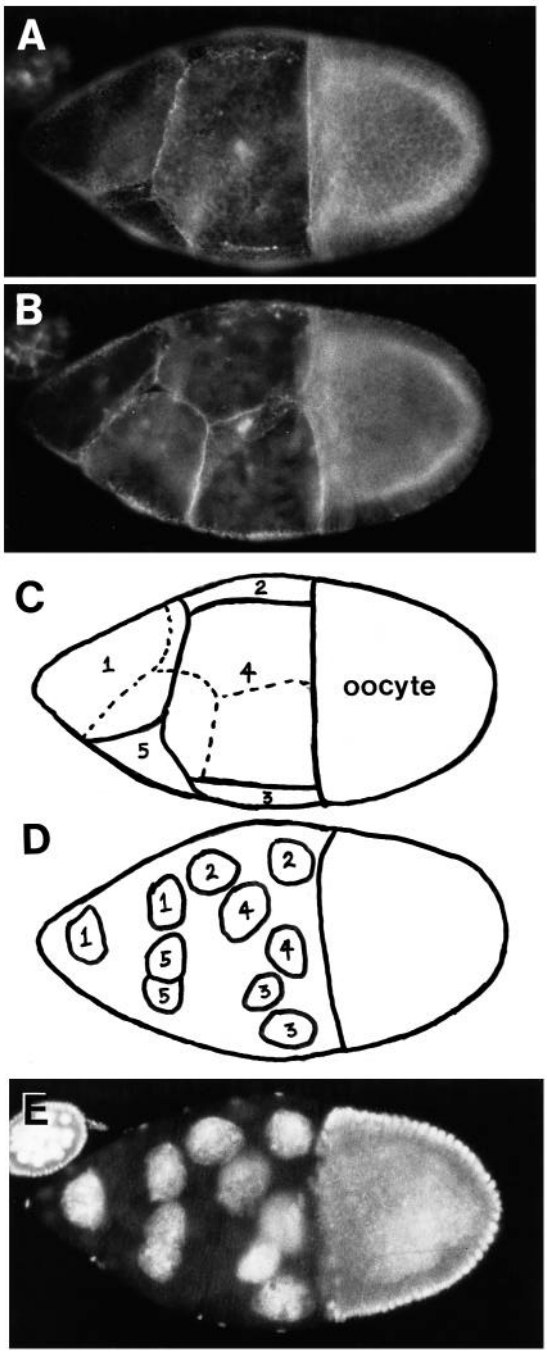

Fig. 4. Egg chambers from hemizygous females are multinucleate. A, B and E are photographs of the same egg chamber from a *chic*^{1320}/*Df(2)GdhA* female taken in two focal planes. Staining with rhodamine-conjugated phalloidin labels the subcortical actin filaments of each cell (A, B), and allows the nurse cell number to be determined. C is a cartoon of the staining in A and B showing that there are only five nurse cells. The nurse cells have been numbered 1-5. DAPI staining reveals the presence of ten nurse cell nuclei (E). In D, the nuclei have been numbered according to which nurse cell they reside in.

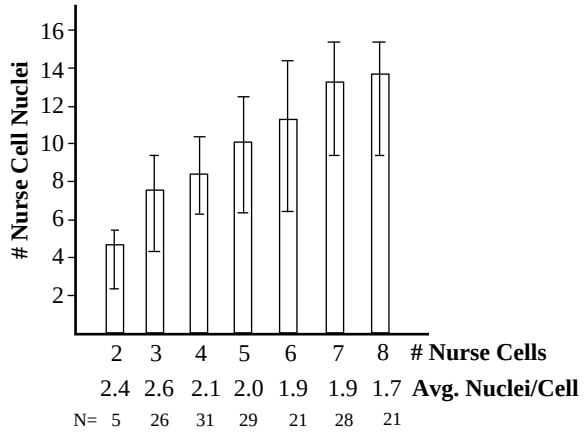

Fig. 5. Evidence for aberrant germline mitoses. The number of nurse cells and their nuclei were determined by counting ring canals and DAPI staining, respectively. The bars indicate the average number of nuclei in an egg chamber, with the range indicated. N= the number of egg chambers analyzed.

ing between the actin filament bundles, and the ridges are therefore representative of the number of bundles (Fig. 7D). Normally, wild-type bristles are long and thin, and taper towards the tip (Fig. 7A, D). We compared wild-type and mutant bristles from the same region of the head bordering the eye socket. *chic*^{37} mutant bristles were thicker and shorter, with sharp bends, kinks and forked ends (Fig. 7B,C,E,F,G). The ridges on mutant bristles were often thinner, more numerous and disorganized. Not every bristle on a *chic*^{37} fly was affected, but almost every class of bristle had some defective members. Although there was great variety in the morphology of mutant bristles, the phenotype seen in Fig. 7C was extremely common for the ocellar bristles. Mutant bristles were also observed in hemizygous strong female sterile *chickadee* alleles (Table 1). In addition, large mosaic cuticle clones generated with a null allele had the same bristle morphology as *chic*^{37} (data not shown).

We examined the distribution of actin filaments in bristles by staining 38- to 43-hour old pupae with rhodamine-phalloidin. In the wild-type bristles, actin was seen in 8-12 discrete bundles (Fig. 8A; Overton, 1967). Mutant bristles appeared to contain abundant actin filament bundles, but they were more numerous and somewhat thinner than wild type (Fig. 8B,C,D). Often the ends of individual bundles appeared to have separated from the core of the bristle and became bent. Overall, the actin filament bundles were more disorganized in the *chic*^{37} mutant. Thus the aberrant external ridge morphology correlated with the condition of the underlying actin filament bundles.

Embryonic expression of profilin

As described above, we generated deletion mutations (*chic*^{221}, *chic*^{281}) that are profilin null alleles. These mutations cause a very late embryonic lethal phenotype in which the homozygous null mutant embryos develop to an advanced stage. The timing of the lethal phase is quite broad, beginning during embryonic stages 16 and 17 and ending during the first larval instar period. The few larvae that hatch from the egg cases are smaller than wild-type and display uncoordinated and greatly reduced movement (Fig. 9E). These larvae die soon after hatching. Cuticle preparations of dead embryos failed to reveal any obvious structural defects (data not shown).

To gain some insight into the role of profilin during embryogenesis, we determined the protein localization in wild-type embryos. Profilin was expressed abundantly and ubiquitously throughout embryogenesis, with specific regions of enrichment during development. During cellularization, profilin appeared to be enriched at the inner, leading edge of the cells, where actin and myosin also localize (Fig. 9A; Warn and Robert-Nicoud, 1990; Young et al., 1991). During gastrulation, the protein was more highly expressed in cells undergoing dramatic movements, such as those in the ventral and cephalic furrows (Fig. 9B). Staining of a stage 16 embryo revealed high levels of profilin in the ventral nerve cord (Fig. 9C).

DISCUSSION

Profilin has been found in every non-muscle cell type examined. Numerous biochemical analyses implicate profilin in the regulation of assembly of actin into filaments. Such experiments, however, have been unable to indicate whether profilin is an essential protein. We demonstrate that deletion of the *chickadee* gene results in a recessive embryonic lethal phenotype indicating that in *Drosophila* profilin is essential for proper development. The lateness of the lethal phase may be explained by a perdurance of maternally supplied profilin that is eventually degraded, inducing systemic failure in the zygotically null embryos. The fact that profilin is essential in *Drosophila* contrasts with yeast. Although profilin null mutations have severe effects on yeast cell growth and morphology, they are not lethal (Magdolen et al., 1988; Haarer et al., 1990). This suggests that multicellular eukaryotes have a more stringent requirement for profilin than unicellular ones.

Analysis of hypomorphic *chickadee* alleles reveals a phenotypic series of increasing severity, presumably corresponding to reduced transcription. Using deficiencies for the locus to halve the amount of profilin expression we find that for almost every allele, the phenotype became more severe in hemizygotes (Table 1). Our collection of *chickadee* alleles also reveals which steps of fly development are most sensitive to lowered profilin expression. Fertility in males and females is compromised in weaker alleles, while disruptions of bristle development and more extreme defects in the germline are found in stronger alleles. In allelic combinations that are only semiviable, defects in eye development (E. M. V., unpublished data) are added to fertility and bristle abnormalities. Finally, complete deletions for the locus are lethal. These data show that while profilin is absolutely required for viability, certain tissues are sensitive to even mild reductions in profilin expression. Similarly, in mammalian cultured cells actin filament populations have differing levels of sensitivity to overexpression of profilin (Cao et al., 1992). Taken together, these results suggest that certain populations of actin filaments are more sensitive than others to either increased or decreased profilin concentrations.

The regulation of profilin expression in *Drosophila* is accomplished by two transcriptional promoters in the *chickadee* gene (Cooley et al., 1992) rather than the presence of multiple gene copies as is the case in slime molds and *Acanthamoeba* (Binette et al., 1990; Pollard and Rimm, 1991). We

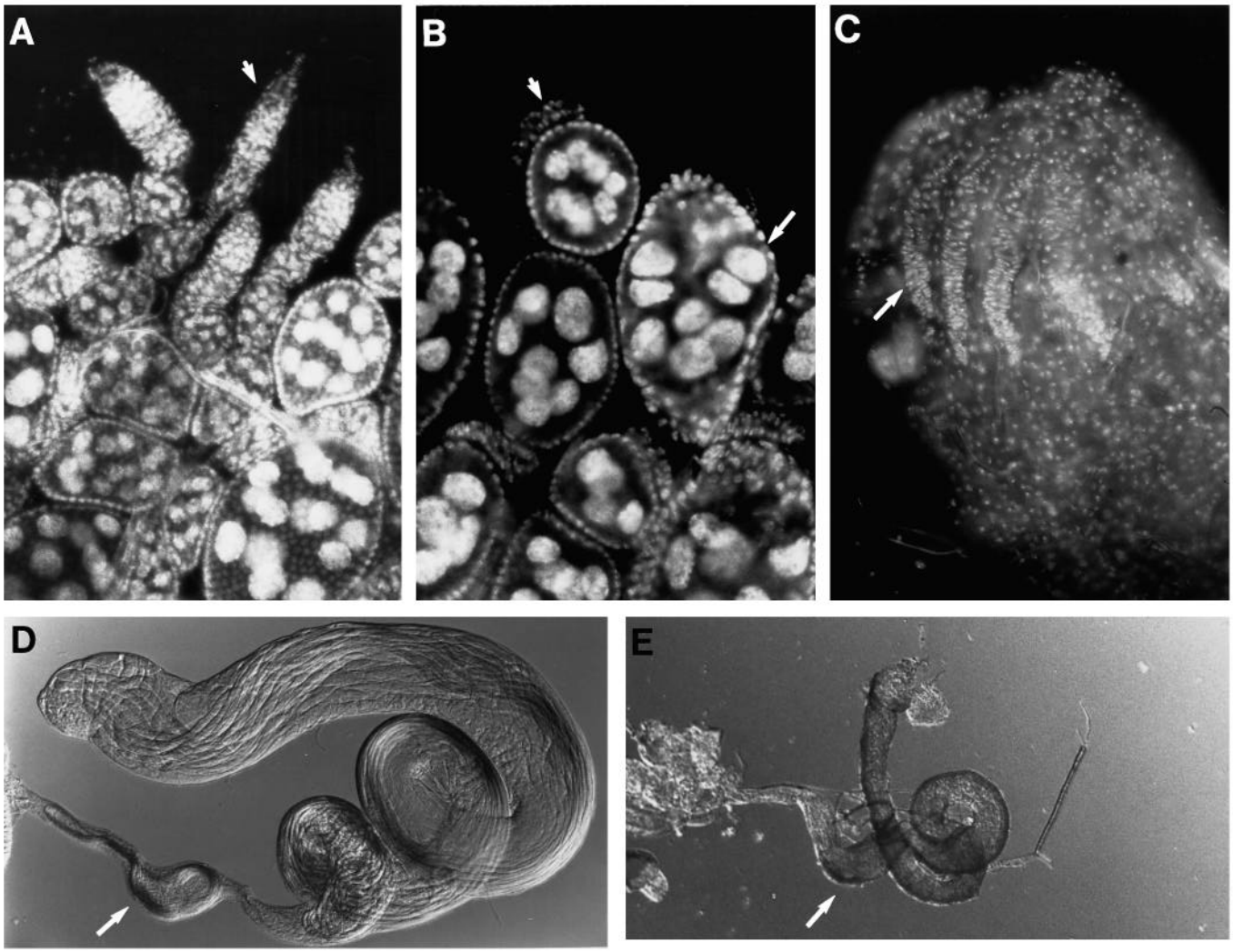

Fig. 6. Severe profilin mutation disrupts germline proliferation. Wild-type (A) and *chic*^{37} (B,C) ovaries were stained with DAPI to visualize nuclei and the general organization of the egg chambers. The large nuclei are in nurse cells, the small nuclei are in the follicle cells. In newly eclosed *chic*^{37} females, the ovaries contain 10-20 abnormal and degenerating egg chambers (B). The germaria appear to contain no new egg chambers (small arrow in B) as compared to wild-type germaria (arrow in A). Many nurse cell nuclei appear in pairs (long arrow in B). Ovaries from older *chic*^{37} females are devoid of egg chambers, and long follicle cell stacks remain (arrow in C). Testes from *chic*^{37} males appear as empty sacks of somatic tissue (E). Spermatid bundles are clearly visible in wild-type testes (D). Note that the size of the somatic seminal vesicle is unaffected in mutants (arrows in D and E).

have been unable to identify another profilin gene by low stringency blotting (unpublished data). Single copy profilin genes are also present in mice, humans and yeast (Kwiatkowski and Bruns, 1988; Magdolen et al., 1988; Sri-Widada et al., 1989). We previously determined that one of the two *chickadee* promoters is expressed exclusively in the ovary while the second is expressed ubiquitously (Cooley et al., 1992). Using antibody localization of profilin in egg chambers from wild-type and female sterile *chickadee* mutants, we now find that the ovary-specific promoter is most likely to be nurse cell-specific since nurse cell, but not follicle cell, expression is reduced in the mutant egg chambers (Fig. 2). The phenotype of this class of alleles is similarly restricted to the nurse cells where the usual cytoplasmic arrays of actin filaments fail to form.

In order to determine what function profilin is playing in cells, we have carried out a detailed analysis of the phenotypes associated with hypomorphic profilin mutations. The pleiotropic nature of these phenotypes suggests that there are roles for profilin in several distinct cellular processes, which share the need for proper regulation of a dynamic actin cytoskeleton.

Cytoplasmic actin filaments in nurse cells

The most obvious example of a role in regulating actin filament assembly was seen in mutant egg chambers that are missing the nurse cell-specific *chickadee* transcript (Cooley et al., 1992). Actin filament bundles that normally form in the cytoplasm fail to appear. The role of profilin in nurse cells may be to maintain the pool of actin monomers in the nurse cell cytoplasm in the ATP-bound form by stimulating nucleotide exchange. This would ensure a maximum rate of polymerization at the required time. Since filaments appear very rapidly in the cytoplasm, a mechanism must exist to trigger the

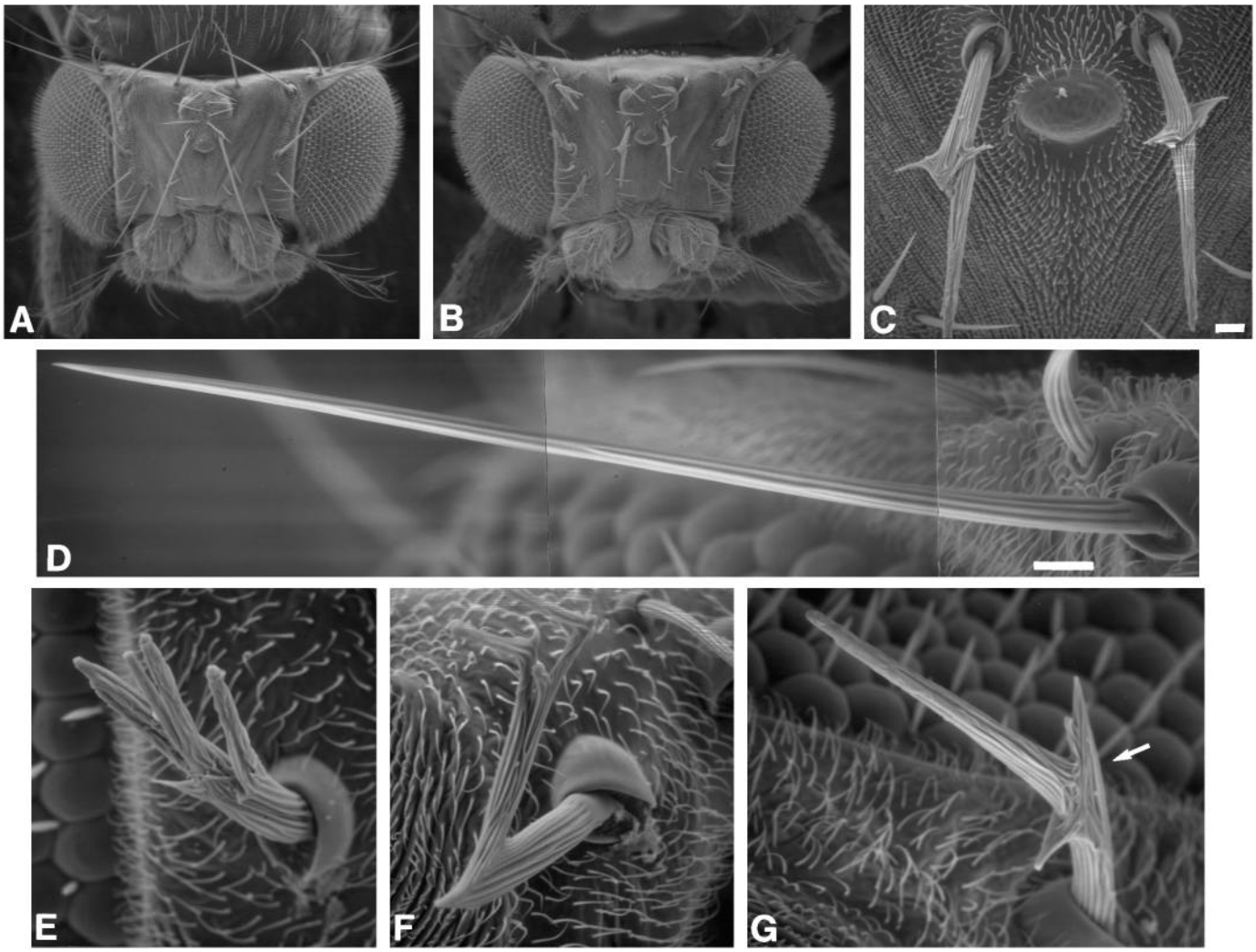

Fig. 7. Profilin mutation causes severe bristle abnormalities. Scanning electron micrographs of comparable bristles from wild-type and *chic*^{37} flies. Wild type bristles are long and thin, and taper towards the tip (A,D). *chic*^{37} mutant bristles display a wide range of abnormalities (B,C,E,F,G). An enlargement of the central ocellar bristles in B shows two bristles with a very similar defect, which is very common to bristles found at that location (C). Mutant bristles are thicker and shorter than wild-type ones (E,F,G) and have more ridges. They often bend sharply (F) split at their ends (E) or have branches off the sides (G). The bristles in D-G are at the same magnification.

dramatic actin assembly. Profilin, through its interaction with PtdIns(4,5)P_2, may be involved with transmission of an extracellular signal to trigger actin polymerization. However, the presence of PtdIns(4,5)P_2 in nurse cells has not been confirmed and we do not detect any accumulation of profilin at nurse cell plasma membranes where PtdIns(4,5)P_2 is expected to reside.

Profilin and bristle formation

The abnormal bristles in the severe allele *chic*^{37} have a very different actin phenotype than that of nurse cells. Instead of a missing population of actin filaments, actin bundles still formed in bristles, but the number of bundles was increased. The same phenotype was seen in bristles that formed within large cuticle clones of a null allele of *chickadee*. This result implies that high levels of profilin are not essential for the polymerization of filaments in bristle cells. We propose that in trichogen cells, profilin may function to sequester actin monomers to control nucleation of new filaments. This interpretation is based on the studies of the acrosomal reaction of echinoderm sperm (Tilney et al., 1983). In the sperm head, monomeric actin is complexed with profilin. Upon induction of the acrosomal reaction, the actin is rapidly assembled into a filamentous acrosomal process. As the filaments of the process are elongating, the profilin bound to actin monomers suppresses nucleation of new filaments, while allowing monomers to assemble onto the preferred end of established growing filaments at the same rate as free actin (Tilney et al., 1983; Pring et al., 1992). We suggest that a similar function is being played by profilin in the trichogen cell. When the expression of profilin is reduced in *chic*^{37}, actin nucleation becomes derepressed, leading to the formation of ectopic nuclei at the base or along the length of the bristle shaft. This results in an increased number of actin bundles.

The integrity of actin bundle organization is also compromised in *chickadee*. Frayed actin bundles are present in growing bristles (Fig. 8B-D) and extensively branched mature bristles are common (Fig. 7E-G). The branched bristles probably arise when cytoplasm containing stray actin bundles

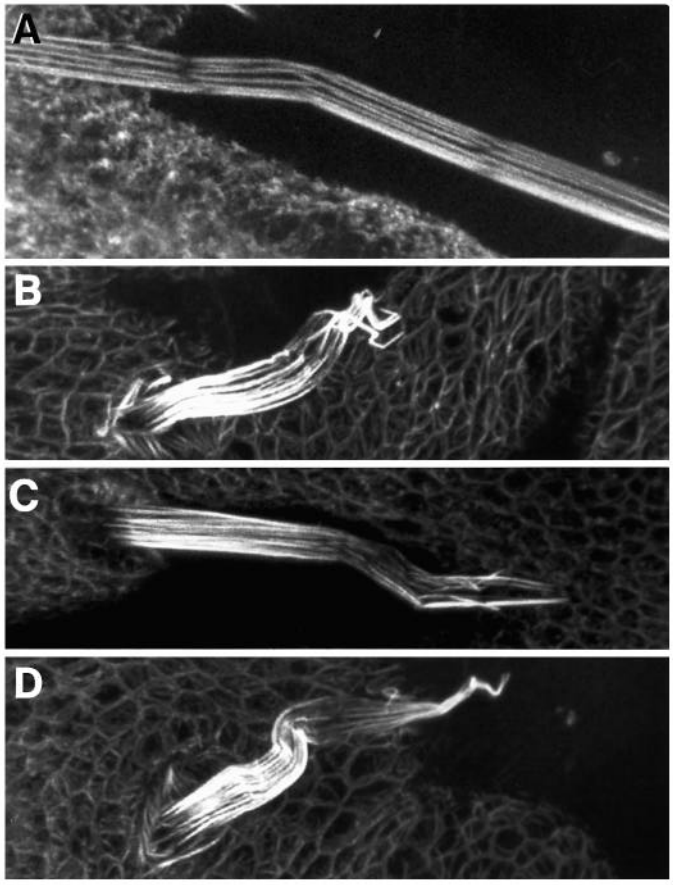

Fig. 8. Mutant bristles contain abnormal actin filament bundles. Pupae were dissected and fixed at 38- to 43-hours of pupal development and stained with rhodamine-conjugated phalloidin to visualize filamentous actin. These images are projections of serial confocal optical sections. In wild type, there are between 8-12 actin filament bundles running the length of the macrochaete bristle (A) (Appel, et al., 1993; Overton, 1967). Mutant bristles from *chic*^{37} contain abundant actin filaments, however they appear more fragile and their organization is abnormal. There are more filament bundles which are thinner than normal (B,C,D). In addition, bending (B,D) and splitting (C) of the bundles can be seen.

becomes enclosed with cuticle. The basis for actin bundle instability is not clear, although it is probably not due simply to an increase in the number of bundles. An increased number of bundles is also present in *Stubble* mutant bristles, but these do not undergo branching (Overton, 1967; Appel et al., 1993). We propose that the explanation is structural. The ridge pattern seen on the adult mutant bristles suggests that the underlying bundles are of varying thicknesses (Fig. 7E-G). Thinner or abnormally shaped bundles may be less structurally sound than wild-type bundles, and thus more susceptible to bending and splitting during the extension of the bristle shaft. Alternatively, an altered rate of polymerization of actin filaments caused by profilin reduction may perturb the normal interactions of filaments with bundling proteins leading to abnormal bundle assembly.

Several mutations that result in abnormal bristle formation have been characterized in *Drosophila*. Two of these, *forked* and *singed*, produce phenotypes especially similar to *chickadee*, with wavy and gnarled bristles. *forked* encodes

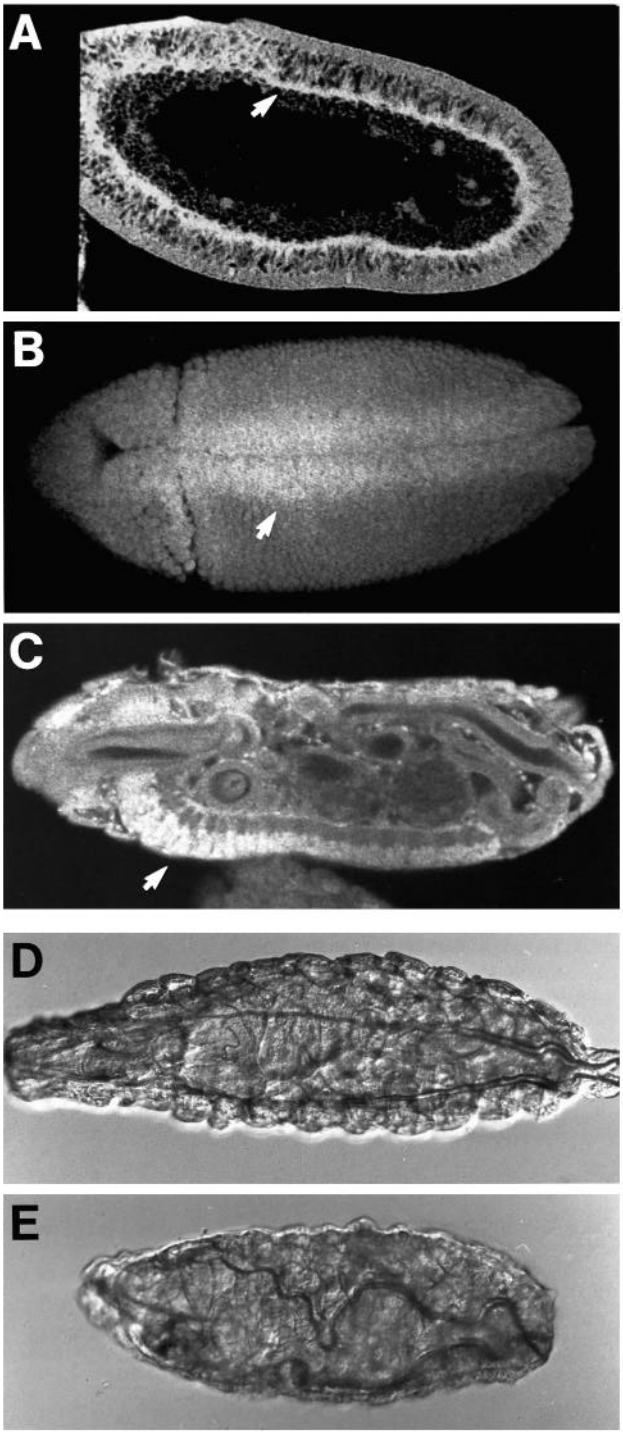

Fig. 9. Profilin is expressed abundantly throughout embryogenesis. During cellularization, profilin appears to be enriched at the inner, leading edge of the cells (arrow in A). During gastrulation, the protein is more highly expressed in cells undergoing dramatic movements, such as those in the ventral furrow (arrow in B). Staining of a stage 16 embryo reveals ubiquitous expression with high levels of profilin in the ventral nerve cord (arrow in C). Examination of dead first instar larvae from a null allele of *chickadee* (E) reveals that mutant embryos can develop to an advanced stage before dying. A wild-type first instar larvae is shown for size comparison (D).

proteins with no homology to any known actin binding protein (Hoover et al., 1993). However, the protein localizes to the actin bundles in wild-type developing bristles and actin bundles are not observed in *forked* mutant bristles (Petersen et al., 1994). This suggests that forked plays a role in actin organization. *singed* mutations also cause aberrant bristle morphology. The structure of the actin bundles is highly irregular in these mutants; the correct number of bundles is formed, but they are thinner and flattened (Overton, 1967). It is now known that *singed* encodes a protein with homology to sea urchin fascin, an actin-bundling protein (Paterson and O'Hare, 1991; Bryan et al., 1993), and that singed can bundle actin filaments in vitro (Cant et al., 1994). Thus, *chickadee*, *singed* and probably *forked* encode proteins required for proper actin polymerization or organization.

Cytoplasmic extensions and cell migration

Dynamic actin structures play a role in the migration of cells and at least one population of migrating cells is affected in *chickadee* mutants. The border cells travel between nurse cells sending out cellular extensions as they migrate (Fig. 2B). Profilin is expressed abundantly in these cells and lowered doses of profilin disrupt their migration. Cellular extensions are also vital in nervous system development and the strong expression of profilin in the ventral nerve cord suggests that profilin is involved in neuron growth and extension. Such a role in neuron growth is confirmed by the recent finding that the *stranded* mutation (Van Vactor et al., 1993), which affects motoneuron growth cone pathfinding, is a lethal allele of *chickadee* (D. Van Vactor and C. Goodman, personal communication).

Profilin and mitosis

Egg chambers from *chic*^{1320} hemizygous females contain a wide range of germline cell number and most of the cells are binucleate. These phenotypes provide a record of events that took place during mitotic divisions in the germarium. Normally, a germline stem cell daughter undergoes four highly synchronized divisions. Cytokinesis is not completed in each of these divisions so an interconnected cluster of 16 cells is formed. An early role of intercellular bridges is probably to facilitate the synchrony of the mitoses as has been postulated for human germline proliferation (Gondos, 1973). In *chickadee* mutant egg chambers, the regulation of mitoses may be disrupted such that a random number of divisions occurs. In addition, the last cytokinesis in these egg chambers appears to be wholly instead of partially incomplete resulting in predominantly binucleate cells.

These phenotypes demonstrate that profilin is required for mitosis in the germline. Its role could be to maintain a healthy actin-based cytoskeleton that can respond dynamically to signals controlling the cell cycles. When the amount of profilin falls below a certain threshold, the cells are no longer capable of supporting the normal division program. This is more starkly evident in ovaries of *chic*^{37} flies in which proliferation of the germline fails entirely. The few egg chambers that form in these ovaries are probably derived from stem cells that differentiate. The egg chambers that develop have a variable number of germline-derived cells.

Profilin may also be required in specialized structures such as cleavage furrows. Studies of *Tetrahymena* profilin found that the protein is associated with the cytokinetic cleavage furrow (Edamatsu et al., 1992). The localization of *Drosophila* profilin to the leading edge of invaginating membrane during embryonic cellularization supports such a role. The mechanism of cellularization is analogous to the formation of cleavage furrows, and is mediated by actin and myosin localized at the leading edge (Warn and Robert-Nicoud, 1990; Young et al., 1991). The failure of the last cytokinesis in *chickadee* mutant egg chambers could correlate with exhaustion of available profilin.

Conclusion

Analysis of phenotypes associated with profilin mutation demonstrated that profilin is essential to normal development. Mutations that decrease profilin expression, but do not completely abolish it, result in disruption of several processes, all of which are actin dependent. The difference in actin phenotypes in germline development versus bristle development probably reflects the different requirements for actin assembly in the two systems. Further insight into profilin's functions in vivo should be gained by genetically identifying proteins that interact with profilin.

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