

# Cooperative interactions between the central spindle and the contractile ring during *Drosophila* cytokinesis

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We analyzed male meiosis in mutants of the *chickadee* (*chic*) locus, a *Drosophila melanogaster* gene that encodes profilin, a low molecular weight actin-binding protein that modulates F-actin polymerization. These mutants are severely defective in meiotic cytokinesis. During ana-telophase of both meiotic divisions, they exhibit a central spindle less dense than wild type; certain *chic* allelic combinations cause almost complete disappearance of the central spindle. Moreover, *chic* mutant spermatocytes fail to form an actomyosin contractile ring. To further investigate the relationships between the central spindle and the contractile ring, we examined meiosis in the cytokinesis-defective mutants *KLP3A* and *diaphanous* and in testes treated with cytochalasin B. In all cases, we found that the central spindle and the contractile ring in meiotic ana-telophases were simultaneously absent. Together, these results suggest a cooperative interaction between elements of the actin-based contractile ring and the central spindle microtubules: When one of these structures is disrupted, the proper assembly of the other is also affected. In addition to effects on the central spindle and the cytokinetic apparatus, we observed another consequence of *chic* mutations: A large fraction of *chic* spermatocytes exhibit abnormal positioning and delayed migration of asters to the cell poles. A similar phenotype was seen in testes treated with cytochalasin B and has been noted previously in mutants at the *twinstar* locus, a gene that encodes a *Drosophila* member of the cofilin/ADF family of actin-severing proteins. These observations all indicate that proper actin assembly is necessary for centrosome separation and migration.

[Key Words: *chickadee* mutations; cytokinesis; central spindle; contractile ring; profilin; *Drosophila*]

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In animal cells, cytokinesis is accomplished by the contractile ring, a transient structure containing actin and myosin II filaments that is anchored to the equatorial cortex. Interactions between these filaments lead to the constriction of the ring, which pinches the dividing cell in the middle like a purse string until cleavage is completed (for review, see Satterwhite and Pollard 1992; Fishkind and Wang 1995; Goldberg et al. 1997).

Many experiments have clearly shown that the spindle controls the occurrence and positioning of the cytokinetic event (e.g., Hiramoto 1956, 1971; Hamaguchi 1975; Rappaport 1986, 1991). In large echinoderm eggs that contain prominent asters and a small central spindle/spindle midzone (two names for the bundle of interdigitated microtubules between the separating chromosome complements during anaphase and telophase), the stimu-

lus for assembly of the contractile ring is provided primarily by the asters. This was elegantly shown by Rappaport (1961), who manipulated sand dollar eggs containing two spindles into a horseshoe shape. In these eggs, cytokinesis occurred not only in the canonical positions equidistant between the two asters of each spindle, but also between the asters of different spindles. Further evidence for a key role of asters in signaling cytokinesis was obtained by selective removal of different parts of the spindle from sea urchin eggs (Hiramoto 1971). Other micromanipulation experiments on echinoderm eggs, however, have shown that the central spindle can also play an important role in promoting cleavage furrow formation (Rappaport and Rappaport 1974, 1983; Rappaport 1985).

In animal cells that contain small asters and a relatively large central spindle, the stimulus for cytokinesis appears to be provided primarily by the central spindle. Experiments on grasshopper neuroblasts showed that when the spindle is dislocated from its normal position

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by micromanipulation, the midzone of the repositioned spindle has the ability to stimulate cleavage (Kawamura 1977). This relationship may also extend to vertebrate eggs: Miller et al. (1993) noticed that when cytokinesis of *Xenopus* eggs was perturbed by calcium buffer injection, the location of the reforming furrow was dependent on the position of the central spindle rather than the poles. A strong correlation between the spindle midzone and the cleavage plane has also been shown by examination of multipolar mitotic figures in living rat kidney cells (Wheatley and Wang 1996) and in cultured human cells (Eckley et al. 1997). Moreover, modifications of the spindle midzone microtubules by either nocodazole or taxol result in specific and opposite effects on the organization of actin filaments in the cleavage furrow (Fishkind et al. 1996). Finally, Cao and Wang (1996) have recently obtained direct evidence that the central spindle of rat kidney cells provides the signals that stimulate cytokinesis. By puncturing these cells with a blunted needle, they created perforation barriers between different regions of the spindle and the cortex. Only barriers between the spindle midzone and the cortex suppressed cleavage in the equatorial region facing the perforation. Interestingly, whereas the interzonal microtubules were properly organized in the cleaving region of these cells, they were completely disorganized in the noncleaving part of the cells, suggesting an interaction between central spindle microtubules and the equatorial cortex (Cao and Wang 1996).

Our approach to elucidate the mechanisms underlying cytokinesis in animal cells is the identification and molecular characterization of genes that control this process in *Drosophila melanogaster*. *Drosophila* not only offers well-known advantages for genetic analysis, but also provides unique opportunities for cytological examination of mutant phenotypes in different cell types (for review, see Giansanti et al. 1996). A *Drosophila* cell system that is highly suitable for phenotypic characterization of mutants defective in cytokinesis is male meiosis (Cenci et al. 1994; Gunsalus et al. 1995; Williams et al. 1995; Giansanti et al. 1996). The existence of cytokinesis problems in mutant testes is readily apparent through effects on the easily recognized products of meiosis, the spermatids (see below). Moreover, cytokinesis defects can be analyzed with exquisite cytological resolution because of the large size of the spermatocytes. The meiotic spindles of *Drosophila* males are correspondingly large and exhibit a prominent central spindle that is pinched in the middle during cytokinesis (Cenci et al. 1994); these cells also exhibit an actin-based contractile ring around the spindle midzone where pinching occurs (Gunsalus et al. 1995).

In this paper we report that mutations in the *chicka-dee* (*chic*) locus disrupt cytokinesis during male meiosis, preventing the formation of both the central spindle and the contractile ring. The *chic* gene encodes a *Drosophila* profilin, a low molecular weight actin-binding protein that regulates actin polymerization (Cooley et al. 1992). In addition, we observed a simultaneous disruption of both the central spindle and the contractile ring in mu-

nants at the *diaphanous* (*dia*) (Castrillon and Wasserman 1994) and *KLP3A* (Williams et al. 1995) loci, and in meiotic cells treated with cytochalasin B. Together, these results strongly suggest that during *Drosophila* male meiosis there is a cooperative interaction between the central spindle microtubules and elements of the actomyosin contractile ring, so that impairment of either of these structures prevents the formation of the other.

## Results

### Mutations at the *chic* locus

To isolate mutations affecting meiotic cell division in *Drosophila* males, we used P-element mutagenesis to generate a collection of autosomal male sterile mutants, and screened these stocks for defects in onion stage spermatids (for the mutagenesis scheme, see Gatti and Goldberg 1991). The morphology of these cells, which consist of a nucleus associated with a mitochondrial derivative called the Nebenkern, is diagnostic of errors in chromosome segregation and/or cytokinesis that occurred during the previous meiotic divisions (Gonzalez et al. 1989; Fuller 1993). We isolated four mutations associated with aberrations in onion-stage spermatids that were suggestive of defects in meiotic cytokinesis (see below). All four mutations were mapped by in situ hybridization to the 26A interval in polytene chromosomes (data not shown). Transposase-generated precise excisions of the marked P elements in these strains reverted the male sterility and the defective spermatid phenotype, indicating that the P element was indeed the cause of the aberrant phenotypes. All four mutations failed to complement *Df(2L)GpdhA* (which removes bands 25D7-E1 to 26A8-9) for both male sterility and the aberrant meiotic phenotype, but complemented *Df(2L)clot7* (which removes bands 25D5-6 to 26A7). These findings prompted us to perform complementation tests between these mutations and previously described mutations in the *chic* locus a hot spot for P-element mutagenesis mapping to the same chromosomal interval (Cooley et al. 1992). All four mutations were allelic to each other and behaved as alleles of *chic* (Table 1); we refer to these subsequently as *chic*<sup>R1</sup>, *chic*<sup>8A</sup>, *chic*<sup>13E</sup>, and *chic*<sup>35A</sup>. This conclusion was verified by examining DNA adjacent to the site of P-element insertion in each of these four mutant strains (see Materials and Methods for details). The four P-element insertions were found within the *chic* gene (Fig. 1).

The male sterile *chic* alleles we recovered are not null mutations. Verheyen and Cooley (1994) have shown that *chic* is an essential gene; deletions of the coding region cause recessive embryonic lethality, whereas less severe alleles affect fertility in males and females and also cause bending and kinking of adult bristles. The gene produces two major transcripts, both of which can encode the *chic* gene product: a constitutively synthesized 1.2-kb RNA and a 1.0-kb RNA specific to ovaries in females and probably to the germ line in males (Cooley et al. 1992). The four P-element insertions we obtained are clustered within 100 bp of the first intronic region for the 1.0-kb

**Table 1.** Complementation analysis among *chic* mutant alleles

<b>R 1</b>	<i>Fs MS AB</i>					
<b>8 A</b>	<i>Fs MS AB</i>	<i>Fs MS AB</i>				
<b>13 E</b>	<i>Fs MS AB</i>	<i>FF MS AB</i>	<i>Fs MS AB</i>			
<b>35 A</b>	<i>FS MS NB</i>	<i>FF MS AB</i>	<i>FS MS NB</i>	<i>FS MS NB</i>		
<b>37</b>	<i>FS MS AB</i>	<i>FS MS AB</i>	<i>FS MS AB</i>	<i>FS MS AB</i>	<i>FS MS AB</i>	
<b>1320</b>	<i>Fs MS AB</i>	<i>FF MS AB</i>	<i>FS MS AB</i>	<i>FS MS NB</i>	<i>FS MS AB</i>	<i>FS MS AB</i>
<b>DfGpdhA</b>	<i>FS MS AB</i>	<i>FS MS AB</i>	<i>FS MS AB</i>	<i>FS MS AB</i>	<i>SL AB</i>	<i>FS MS AB</i>
	<b>R 1</b>	<b>8 A</b>	<b>13 E</b>	<b>35 A</b>	<b>37</b>	<b>1320</b>

(FF) Female fertile; (Fs) female semisterile, 2%–5% wild type fertility; (FS) female sterile; (MS) male sterile; (SL) semilethal; (NB) normal bristles; (AB) abnormal bristles. Note that escapers from semilethal mutant combinations are sterile in both sexes.

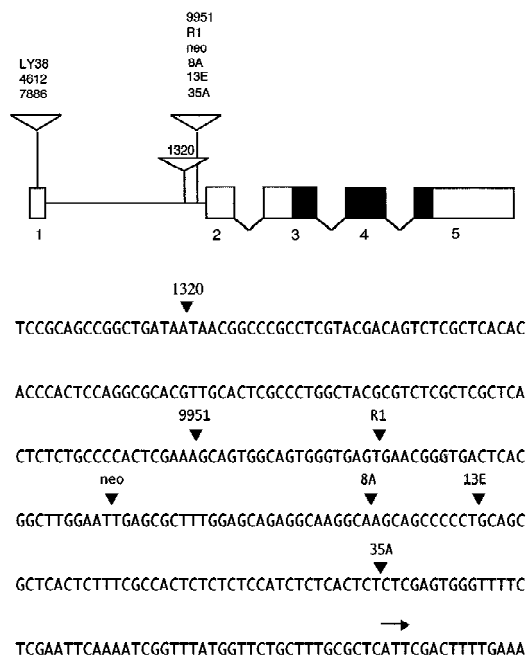
transcript (Fig. 1). Cooley et al. (1992) have shown that other sterile mutations caused by P-element insertions within or very close to the same region (*chic<sup>neo</sup>*, *chic<sup>1320</sup>*, and *chic<sup>9951</sup>*) abolish the 1.0-kb transcript, but still produce at least some of the 1.2-kb *chic* mRNA from the constitutive promoter (see Fig. 1). Though we have no direct evidence, we anticipate that the four mutations we isolated affect expression of the *chic* locus in a similar manner. The phenotypic effects of all of these mutations on viability, male fertility, female fertility, and bristle morphology as homozygotes or as hemizygotes [over the deletion *Df(2L)GpdhA*] are similar, as reported in Table 1. Relatively modest variations between the phenotypes associated with these alleles (Table 1) are likely to result from small differences in the amount of *chic* protein expressed in specific germ-line cells or in bristle-producing trichogen cells at specific times during development.

#### Mutations in *chic* block cytokinesis during male meiosis

As mentioned above, our interest in *chic* was originally sparked by aberrations in spermatid morphology in mutant testes that suggested difficulties in meiotic cytokinesis. These abnormalities are immediately obvious because of the characteristic appearance of spermatids directly after their formation at the so-called onion stage. Wild-type onion stage spermatids contain a single phase-light nucleus and a similarly sized phase-dark Nebenkern (Fig. 2A). The uniformity of nuclear and Nebenkern sizes in these spermatids is assured by cell behavior during cytokinesis. During the two meiotic divisions, mito-

chondria are arrayed along the central spindle and are equally partitioned between the two daughter cells when the central spindle is pinched in the middle during cytokinesis (Fuller 1993; Cenci et al. 1994). Thus, failures in cytokinesis result in abnormally large Nebenkern associated with multiple normal-sized nuclei (Fuller 1993; Castrillon and Wasserman 1994; Gunsalus et al. 1995; Williams et al. 1995).

In testes of males either homozygous for a variety of *chic* alleles or heterozygous for various combinations of alleles or deletions of the locus, a large fraction (>90% in the strongest alleles) of onion-stage spermatids contain a single Nebenkern of larger than normal size, associated with two or more normal-sized nuclei (Fig. 2B; Table 2). A substantial proportion have two nuclei with an intermediate-size Nebekern, but most frequently, these aberrant spermatids contain four nuclei and a very large Nebenkern (Table 2). These phenotypes reflect failures of cytokinesis at either one or both meiotic divisions, respectively, which would prevent proper subdivision of mitochondria and nuclei into daughter spermatids. A smaller number of mutant spermatids have three nuclei; as explained by Gunsalus et al. (1995), these could result from secondary consequences of a failure in cytokinesis I. Yet, smaller percentages of abnormal spermatids have more than four nuclei, indicating cytokinesis failures in both premeiotic gonial divisions and meiotic divisions. The occurrence of some failures in gonial cytokinesis is further suggested by the finding that in *chic<sup>35A</sup>* ~3% of primary spermatocytes are polyploid (data not shown). Rarely, and especially in the strongest allele, *chic<sup>35A</sup>*, we observed spermatids with irregularly sized nuclei (Table 2), indicative of occasional errors in meiotic chromosome segregation.



**Figure 1.** Map and partial sequence of the *chic* locus. (Top) Map of *chic* indicating the exon/intron organization and the positions of P-element insertions associated with *chic* mutations. This map is redrawn from Cooley et al. (1992) and Verheyen et al. (1994). The *chic* transcription unit produces two mRNAs: one of 1.0 kb that includes exons 1, 3, 4, and 5; and another of 1.2 kb comprised of exons 2, 3, 4, and 5. (Open boxes) Exons that do not encode parts of the *chic* protein; (solid boxes) protein-coding exons. The first intron is in scale with respect to the exons, whereas the other introns are not in scale. (Large, open inverted triangles) Locations of P-element insertions. (Bottom) Partial DNA sequence of the first intron showing the position of the P-element insertions (▼) associated with *chic* mutations. The locations of the insertions associated with 1320, 9951, and *neo* have been determined by the laboratory of Dr. Lynn Cooley (pers. comm.). The locations of the *R1*, *8A*, *13E*, and *35A* insertions have been determined in the present study (see Materials and Methods for details). The horizontal arrow indicates the start site of exon 2.

#### Mutations in *chic* disrupt contractile ring assembly and central spindle formation

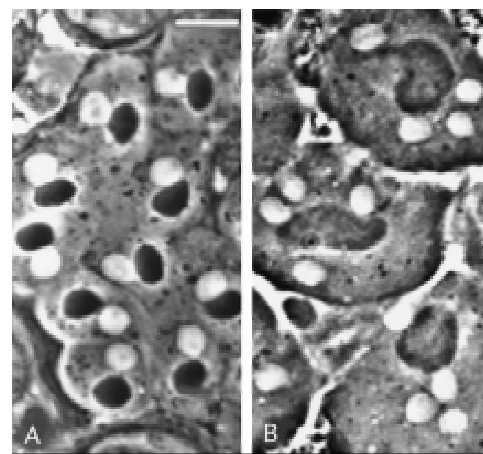
To identify the primary defect that impairs meiotic cytokinesis in *chic* males, we analyzed mutant testes stained with anti-tubulin antibodies, rhodamine-conjugated phalloidin, and Hoechst 33258. This procedure allows simultaneous detection of microtubules, F-actin, and chromatin. In wild-type testes, phalloidin staining reveals an F-actin-enriched contractile ring that encircles the spindle midzone from late anaphase through telophase of both meiotic divisions (Fig. 3A; Gunsalus et al. 1995). In contrast, in ~60% of *chic*<sup>13E</sup> ana-telophases I and II, and in >95% of the ana-telophases I and II of the strongest *chic* mutants (*chic*<sup>R1</sup>, *chic*<sup>8A</sup>, *chic*<sup>35A</sup>), no actin staining was visible at the cell equator (Fig. 3C,D). Moreover, in most mutant ana-telophases with equatorial ac-

tin staining, only irregular patches of F-actin were observed (Fig. 3B).

In addition to the absence of the contractile ring, the ana-telophases of all the *chic* mutants exhibit another obvious abnormality: a defect in central spindle morphology. In wild-type ana-telophases of both meiotic divisions, the two daughter nuclei are connected by a prominent bundle of interdigitating microtubules (Figs. 3A and 4A,B; Cenci et al. 1994). In *chic* mutants, this central spindle structure is considerably less dense than in wild type, and microtubules show very little or no interdigitation (Figs. 3C,D and 4C,D). In wild type, the central spindle is already evident by mid-anaphase, before the actin ring constriction has caused substantial equatorial pinching (Fig. 4A,B; Cenci et al. 1994). In the strongest *chic* mutants, we never observed these early stages of central spindle formation (Fig. 4), indicating that the *chic* phenotype reflects a failure of central spindle assembly rather than a degradation of this structure in the absence of the actin ring. Interestingly, we found a strong positive correlation between the presence of the contractile ring and the density of central spindle microtubules. In all the cases in which there was some actin staining at the cell equator, the density of central spindle microtubules was higher than that observed in cells completely devoid of an actin ring (Fig. 3B). Together, these observations indicate that *chic* mutations disrupt two major cytokinetic structures: the microtubule-based central spindle and the actomyosin contractile ring.

#### Profilin accumulates at the equator of meiotic ana-telophases

The fact that *chic* mutations have such strong effects on both of the major structures present at the equator of meiotic ana-telophases I and II, suggested to us the possibility that *Drosophila* profilin, the product of this gene,



**Figure 2.** Abnormal spermatids in *chic* mutants. Live testes were examined by phase-contrast microscopy to determine spermatid defects in onion-stage cysts. (A) Wild-type spermatids showing nuclei and Nebenkern of similar sizes; (B) spermatids of *chic*<sup>R1</sup> males with large Nebenkern associated with four nuclei of regular size. Bar, 10  $\mu$ m.



**Table 2.** Abnormal spermatids in *chic* mutants

Genotype <sup>a</sup>	Number of spermatids	Percent spermatids <sup>b</sup>										Percent abnormal
		1:1	2:1	3:1	4:1	5:1	6:1	7:1	8:1	>8:1	irr	
Oregon-R	500	100	0	0	0	0	0	0	0	0	0	0
<i>R1/R1</i>	622	17.6	25.3	5	49.2	0.6	1.1	0.1	0.8	0.3	0	82.4
<i>8A/8A</i>	688	8.8	15.5	5.6	61.4	1.2	2.9	0.3	1.2	1.2	1.9	91.2
<i>13E/13E</i>	448	58.7	23.2	4.7	10.5	0.4	0.2	0	0.6	0.4	1.3	41.3
<i>35A/35A</i>	714	6.6	10.5	7.5	57.4	1.8	3.9	2.0	4.9	1.2	4.2	93.4
<i>37/37</i>	142	2.3	9.7	4.4	65.4	0.7	5.1	2.2	10.2	0	0	97.7
<i>37/R1</i>	69	1.4	18.0	2.9	67.6	2.9	2.9	0	1.4	2.9	0	98.6
<i>1320/R1</i>	168	0.6	28.0	0.6	64.3	0	3.5	0	1.2	0.6	1.2	99.4
<i>1320/8A</i>	273	4.4	16.8	5.5	65.0	0	1.8	0	5.8	0.7	0	95.6

<sup>a</sup>The Oregon-R stock was used as wild-type control; the *chic*<sup>37</sup>/*chic*<sup>37</sup>, *chic*<sup>37</sup>/*chic*<sup>R1</sup>, *chic*<sup>1320</sup>/*chic*<sup>R1</sup>, *chic*<sup>1320</sup>/*chic*<sup>8A</sup> males are severely defective in germ-line cell proliferation.

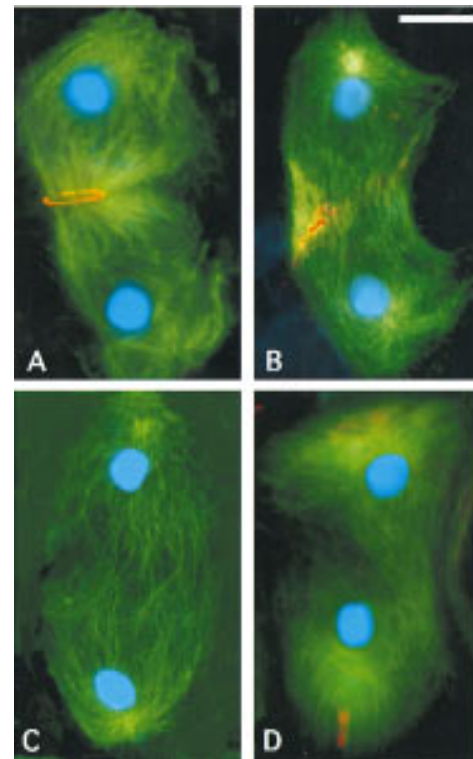
<sup>b</sup>Regular spermatids contain one nucleus and one Nebenkern (1:1) of similar size. The other ratios refer to aberrant spermatids containing one abnormally large Nebenkern associated with multiple nuclei. The irregular class (irr) refers to spermatids with multiple nuclei containing at least one nucleus of irregular size.

might, in fact, be concentrated in the same region of dividing spermatocytes. Staining of fixed testes with an antibody directed against the *chic* protein (Verheyen and Cooley 1994) shows this supposition to be true. Profilin is distributed in a fairly diffuse pattern, but clear concentrations are observed near the cell cortex, particularly in the equatorial zone (Fig. 5). In the strongest *chic* mutants (*chic*<sup>R1</sup>, *chic*<sup>8A</sup>, *chic*<sup>35A</sup>), profilin immunostaining is considerably weaker than in wild type, and profilin does not accumulate at the cell equator (Fig. 5).

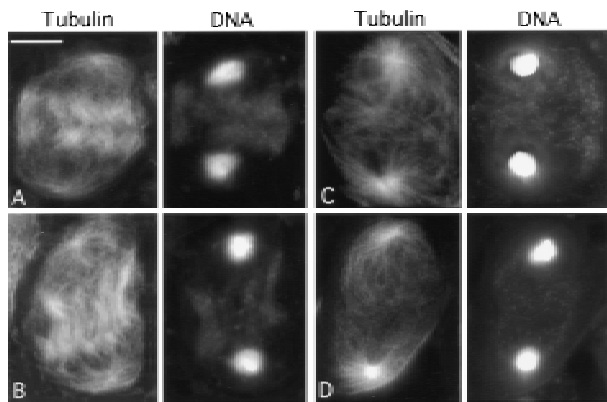
#### Mutations in *chic* produce abnormal accumulations of F-actin

In addition to preventing contractile ring formation, *chic* mutations disrupt at least one more aspect of actin cytoskeleton dynamics, as they elicit the formation of F-actin aggregates that are not seen in wild-type testis cells. Abnormal aggregations of F-actin in *chic* mutants are observed primarily in premeiotic mature spermatocytes at the S5 stage (Fig. 6C; for designations of the stages of *Drosophila* spermatogenesis, see Cenci et al. 1994). In the strongest *chic* mutants and in hemizygous individuals (mutant/Df), these aggregates persist until prometaphase I (stages M1 and M2) and then disintegrate.

We believe that these actin clusters represent remnants of the fusome, a structure that traverses the ring canals of male and female germ-line cells, connecting all the cells present in any given cyst (Lin et al. 1994; Gunsalus et al. 1995; Hime et al. 1996; Robinson and Cooley 1996). Although the male and female fusomes appear to have similar properties, they differ in their molecular composition. They both contain  $\alpha$ -spectrin, but the male fusome, in contrast to its female counterpart, is highly enriched in F-actin (Gunsalus et al. 1995; Hime et al. 1996). In wild-type males, the fusome connects the 16 primary spermatocytes during the early stages of their growth, but regresses in mature spermatocytes (stage S5), which only exhibit small fusome fragments (Gunsalus et



**Figure 3.** Defects in central spindle formation and contractile ring assembly during the first meiotic division in *chic* mutant males. Telophases from wild-type controls (A), *chic*<sup>13E</sup> mutants (B), *chic*<sup>35A</sup> mutants (C), and *chic*<sup>R1</sup> mutants (D). Cells were sequentially stained with anti-tubulin antibodies and rhodamine-phalloidin to detect tubulin (green) and F-actin (red); they were then stained with Hoechst 33258 to visualize chromatin (blue). The fluorescent signals were recorded separately by a CCD camera and merged in pseudocolors. Note that the *chic*<sup>35A</sup> and *chic*<sup>R1</sup> telophases are severely defective in the central spindle and lack the actin-based contractile ring. In the *chic*<sup>13E</sup> cell, the central spindle is irregular and less dense than in wild type; it exhibits only a patch of F-actin at the midzone (see text for further explanation). Bar, 10  $\mu$ m.



**Figure 4.** Failure of central spindle formation in *chic* mutants. (A,B) Wild-type mid-anaphases I showing microtubule interdigitation in the spindle midzone. (C,D) *chic*<sup>R1</sup> (C) and *chic*<sup>35A</sup> (D) anaphases with a low density of noninterdigitating central spindle microtubules. Bar, 10  $\mu$ m.

al. 1995). Although young *chic* spermatocytes have a normal fusome (Fig. 6A), the prominent F-actin-enriched aggregates in mature *chic* spermatocytes (stage S5) have the appearance of aberrantly long-lived fusome remnants (Fig. 6C,D). To test whether these actin structures were indeed fusome derivatives, *chic* testes immunostained with either an anti- $\alpha$ -spectrin antibody or an anti-anillin antibody were sequentially stained with rhodamine-phalloidin. Anillin has been shown previously to be a stable component of the male ring canals through which the fusome extends (Field and Alberts 1995; Hime et al. 1996). This analysis showed that the F-actin aggregates seen in *chic* S5 spermatocytes are enriched in  $\alpha$ -spectrin (Fig. 6D–F) and are almost invariably associated with ring canals (Fig. 6G–I). Together, these findings suggest that these actin aggregates are in fact relatively undegraded remnants of the male fusome.

#### Mutations in *chic* affect aster migration

In wild-type primary spermatocytes during the prophase–prometaphase transition of the first meiotic division (stage M1), centrosomes migrate from a position just under the plasma membrane to the nuclear envelope, where they nucleate prominent asters (Tates 1971; Cenci et al. 1994). The two asters then separate and move around the periphery of the nuclear membrane, so as to establish a bipolar spindle (Fig. 7A; Cenci et al. 1994). Similarly, during late telophase of the first meiotic division and the short interphase between meiosis I and meiosis II (stages M5–M6b), asters separate and migrate to the opposite poles of secondary spermatocytes (Fig. 7C; Cenci et al. 1994). In contrast, at the corresponding meiotic stages in *chic* mutants, the two asters often fail to separate from each other (Fig. 7D,F; Table 3). Furthermore, the asters in *chic* spermatocytes often fail to associate with the nucleus, remaining in ectopic po-

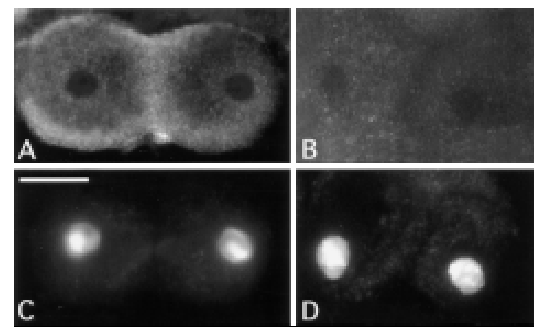
sitions in the middle of the cytoplasm or near the plasma membrane (Fig. 7D,F).

Although the asters in *chic* mutants are improperly positioned by the start of prometaphase, relatively normal-looking bipolar spindles eventually form by late metaphase (Fig. 7E). It is remarkable that the function of these spindles, with respect to chromosome segregation, appears to be largely unimpaired, because we find only a very low frequency of onion-stage spermatids containing irregularly sized nuclei such as micronuclei (Table 2).

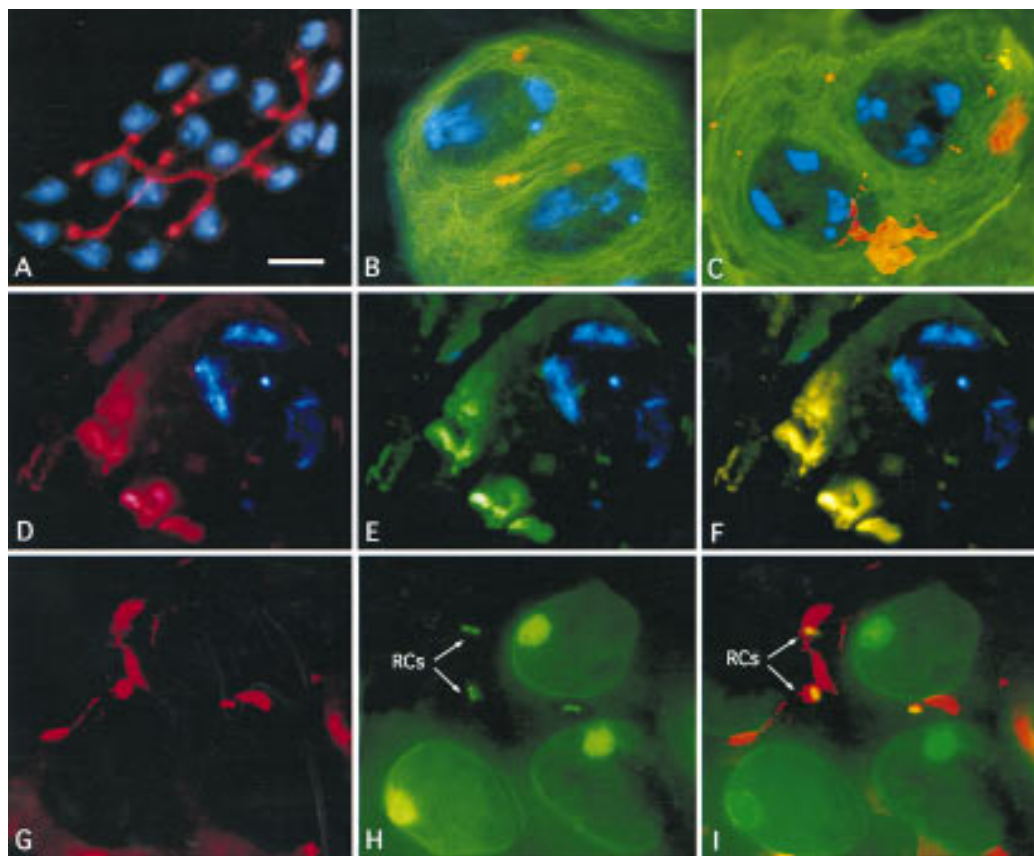
#### *chic* is epistatic to *twinstar*

Our previous investigations (Gunsalus et al. 1995) revealed that lesions in *twinstar* (*tsr*), a gene encoding a *Drosophila* cofilin (an actin severing and depolymerizing protein), cause a syndrome of phenotypic effects that have both similarities and differences to those described above for *chic* mutations. In *tsr*, as in *chic* spermatocytes, centrosome separation and migration are defective, abnormal accumulations of F-actin are apparent, and cytokinesis often fails after one or both meiotic divisions. *tsr* and *chic* mutant phenotypes, however, can be easily distinguished on the basis of several criteria. In *tsr* mutants, the central spindle is normal, and the contractile ring still forms (though in misshapen and enlarged form), in contrast to the situation for *chic* described above. Furthermore, as explained in the Discussion, the types of F-actin aggregates formed in *tsr* spermatocytes are much different from those in *chic*.

To further characterize the relationship between *chic* and *tsr*, we constructed flies simultaneously mutant for both *tsr*<sup>1</sup> and *chic*<sup>R1</sup>. These double mutants die at the larval/pupal transition just as *tsr*<sup>1</sup> single mutants. Testes from larvae simultaneously homozygous for *chic*<sup>R1</sup> and *tsr*<sup>1</sup>, however, have a phenotype almost identical to that observed in *chic*<sup>R1</sup> single mutants, indicating that *chic* is epistatic to *tsr* (Fig. 8). *chic*<sup>R1</sup> *tsr*<sup>1</sup> individuals show frequent defects in aster positioning and separation (>95% of the cells are affected), fail to form either a normal central spindle or an actomyosin contractile ring (in con-



**Figure 5.** Localization of profilin during ana-telophase I of *Drosophila* male meiosis. (A,B) Immunostaining with anti-profilin antibody; (C,D) chromatin staining with Hoechst 33258. (A,C) Wild-type telophase I; (B,D) *chic*<sup>35A</sup> telophase I. Note that in the mutant, the overall intensity of profilin immunostaining is lower than in wild type; in addition, in the *chic*<sup>35A</sup> cell there is no profilin accumulation at the cell equator. Bar, 10  $\mu$ m.



**Figure 6.** Actin behavior in *chic* mutants. To visualize DNA and actin, larval testes were stained with Hoechst 33258 and rhodamine-phalloidin. Tubulin,  $\alpha$ -spectrin and anillin were detected by immunostaining with specific antibodies. The fluorescent signals were recorded separately by a CCD camera and merged in pseudocolors. (A) Complete cyst from a *chic<sup>R1</sup>* mutant, including 16 young spermatocytes and two cyst cells (DNA in blue), that exhibits a normal actin-enriched fusome (red). Wild type (B) and *chic<sup>R1</sup>* (C) premeiotic, mature primary spermatocytes at the S5 stage stained for chromatin (blue), tubulin (green), and F-actin (red); note the prominent F-actin aggregates present in the mutant. *chic<sup>R1</sup>* primary spermatocyte at the S5 stage stained for chromatin (blue) plus F-actin (red) in D, and for chromatin (blue) and  $\alpha$ -spectrin (green) in E; F is a merge of D and E showing colocalization of F-actin and  $\alpha$ -spectrin (overlap in yellow). *chic<sup>R1</sup>* primary spermatocytes at the S5 stage stained for actin (red, G) and anillin (green, H); (I) merge of G and H. Note that the ring canals (RCs, arrows) are traversed by the F-actin-enriched fusome remnants. Bar, 10  $\mu$ m.

trast with *tsr*), and exhibit many abnormal spermatids (>95%) that contain a large Nebenkern associated with two or more nuclei. The actin aggregates present in mature *chic<sup>R1</sup> tsr<sup>1</sup>* spermatocytes are similar in nature to those in *chic<sup>R1</sup>* alone, but in the double mutant these actin masses are slightly larger and persist longer, until anaphase I (Fig. 8).

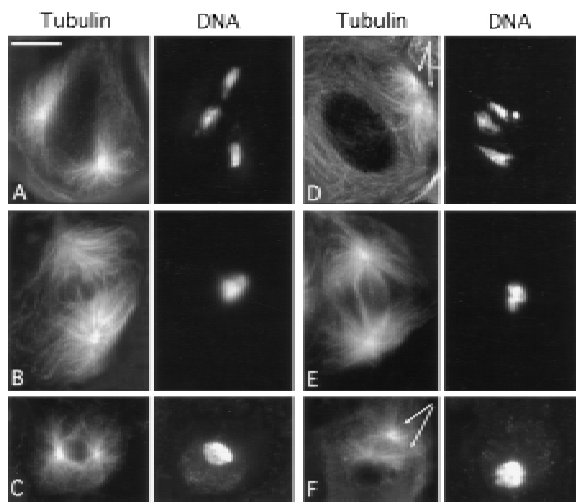
#### Relationship between the central spindle and the contractile ring

There is accumulating evidence that signals from the central spindle are required for proper organization of the actomyosin contractile ring (see introductory section). In this paper, we have shown that mutations in a gene encoding a *Drosophila* profilin disrupt not only contractile ring assembly, but also the organization of the ana-telophase central spindle. One possible explanation for these

findings is that signaling between these structures could work in both directions: Microtubules of the central spindle and the F-actin-based contractile ring may interact cooperatively at the cell equator. To test this hypothesis, we examined the effects on male meiosis of additional mutations or drug treatments that primarily disrupt either the microtubule or the actin cytoskeleton.

We have shown previously that the *KLP3A* gene encodes a kinesin-like protein that accumulates in the central spindle midzone during anaphase and telophase of both meiotic divisions. Accordingly, mutations in this gene disrupt central spindle formation and cause frequent failures in meiotic cytokinesis (Williams et al. 1995). To check whether the defect in central spindle integrity observed in *KLP3A* mutants also affects actin ring assembly, we stained *KLP3A<sup>e4</sup>* mutant testes with rhodamine-labeled phalloidin. The results of this experiment clearly showed that most mutant ana-telophases





**Figure 7.** Abnormal aster positioning and migration in *chic* mutants. (A) Wild-type prometaphase at the M1 stage of the first meiotic division; (B) wild-type metaphase I at the M3 stage; (C) wild-type interphase secondary spermatocyte at the M6b stage; (D) *chic<sup>R1</sup>* prometaphase at the M1 stage with ectopically positioned asters (arrows) that have failed to migrate to the cell poles. (E) *chic<sup>R1</sup>* metaphase I at the M3 stage; note that despite the delay in migration, the asters eventually attain a bipolar arrangement. (F) *chic<sup>R1</sup>* secondary spermatocyte at the M6b stage with ectopic, unseparated asters (arrows). Bar, 10  $\mu$ m.

(90%) were completely devoid of actin rings (Fig. 9B). The rare ana-telophases that exhibited thin and incomplete actin rings also contained central spindles more dense than those of cells completely lacking contractile rings. Despite the absence of the contractile ring, *KLP3A* mutants did not exhibit aberrant actin accumulations or problems in aster migration like those described above for *chic* and *tsr* mutants.

The *dia* locus is required for cytokinesis during both neuroblast mitosis and male meiosis. The *dia* gene encodes a protein homologous to the products of the *limb deformity* gene of mouse and the *BN11* gene of *Saccharomyces cerevisiae* (Castrillon and Wasserman 1994); *dia* also has a homolog in *Schizosaccharomyces pombe*

(*cdc12*) needed for actin ring formation and cytokinesis. The Cdc12 protein colocalizes with the actin ring and interacts with profilin through its proline-rich domain (Chang et al. 1996, 1997). We thus tested whether *dia* is similarly involved in actin ring assembly during *Drosophila* male meiosis. All the ana-telophases present in testes homozygous for either the lethal allele *dia<sup>3</sup>* or the male sterile allele *dia<sup>1</sup>* were completely devoid of actin rings (Fig. 9C). It is of interest that these figures also showed severe defects in the central spindle, similar to those observed in *chic* and *KLP3A*: Central spindle microtubules were much less dense than in wild type and failed to interdigitate at the midzone (Fig. 9C). In addition, ~50% of the meiosis I prometaphases in *dia* mutants exhibited ectopic positioning and delayed migration of asters (not shown).

The effects on the actomyosin contractile ring and the central spindle observed in *chic* and *dia* mutants could be specific consequences of lesions in the corresponding gene products. Alternatively, these effects could result from a more general disruption of the actin cytoskeleton. To discriminate between these possibilities, we treated wild-type testes with cytochalasin B prior to fixation and staining (see Materials and Methods). Cytochalasin B binds the barbed ends of actin filaments and promotes the conversion of ATP-actin monomers to ADP-actin (Cooper 1987), preventing proper assembly of the contractile ring in most cell types (Aubin et al. 1981; Inoue 1990). Remarkably, incubation with this drug produced an almost exact phenocopy of strong *chic* alleles. No F-actin staining in any contractile ring-like structures at the equator of ana-telophase cells was observed (Fig. 9D). Importantly, cytochalasin B treatment also abolished microtubule interdigitation in the central spindle in ana-telophase cells (Fig. 9D). Corresponding cytokinesis defects are indicated by a very high proportion (almost 100%) of spermatids with enlarged Nebenkern and multiple nuclei (Fig. 10C). In addition, disruption of the actin cytoskeleton with cytochalasin B produced ectopic asters in most prometaphase primary and secondary spermatocytes (stages M1 and M6b/M7, respectively), indicating effects on centrosome migration and separation (Fig. 10A,B).

**Table 3.** Aster localization and migration during meiotic divisions in *chic* mutant males

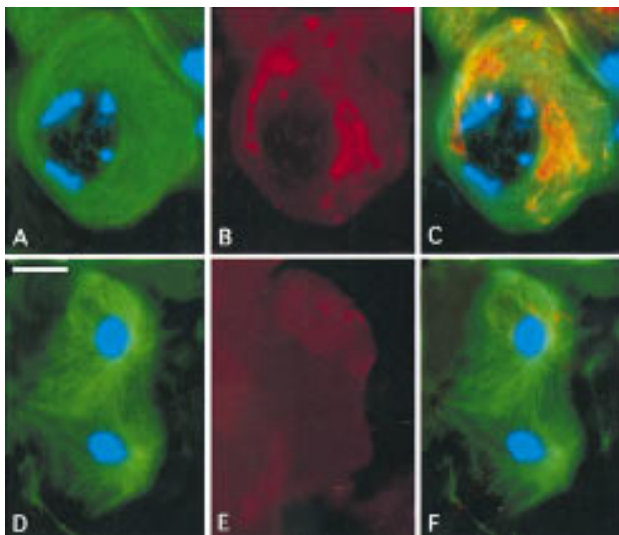
Genotype <sup>a</sup>	Prometaphase I			Prometaphase II		
	norm <sup>b</sup>	irr <sup>c</sup>	% irr	norm <sup>b</sup>	irr <sup>c</sup>	% irr
Wild type	158	10	5.9	232	10	4.1
<i>R1/R1</i>	39	69	63.9	21	10	32.2
<i>8A/8A</i>	11	36	76.6	32	21	39.6
<i>13E/13E</i>	26	12	31.5	27	6	18.2
<i>35A/35A</i>	24	36	60	53	11	17.2
<i>R1/37</i>	8	54	87	27	16	37.2
<i>R1/1320</i>	21	48	69.5	31	22	41.5

<sup>a</sup>The Oregon-R stock was used as wild-type control.

<sup>b</sup>(norm) Normal aster localization and migration.

<sup>c</sup>(irr) Irregular positioning and delayed migration of asters.





**Figure 8.** Actin and tubulin behavior in *chic<sup>R1</sup> tsr<sup>1</sup>* double mutants. A premeiotic mature spermatocyte at the S5 stage (A–C) and a telophase I (D–F) stained for tubulin (green), chromatin (blue), and actin (red). Fluorescent signals were detected separately by a CCD camera as described previously. (C) Merge of A and B; F is a merge of D and E. Bar, 10  $\mu$ m.

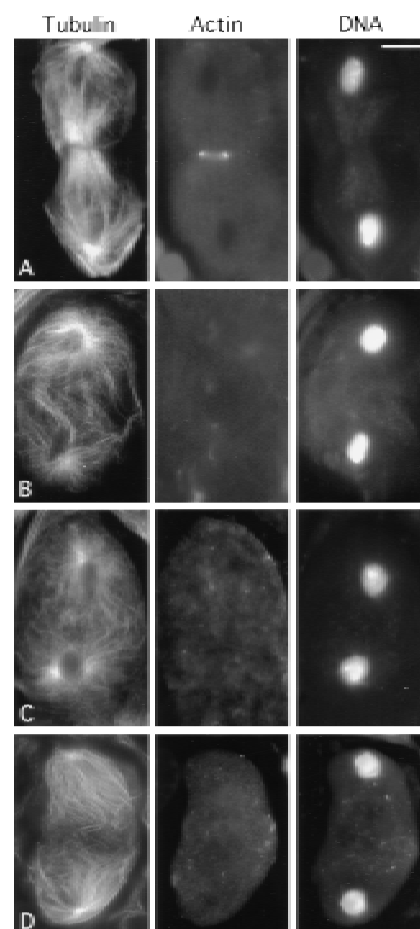
## Discussion

### Effects of *chic* mutations on F-actin-enriched structures

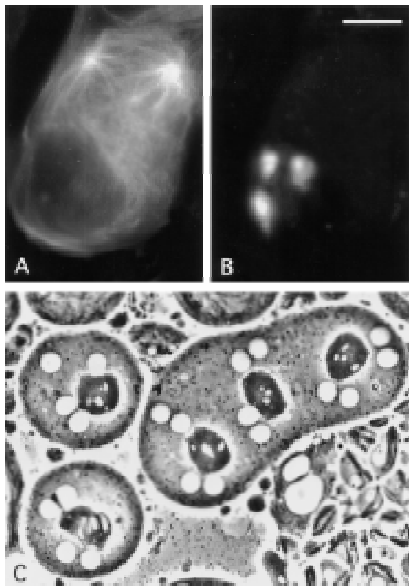
We have shown that mutations in the *chic* locus, which encodes a *Drosophila* profilin (Cooley et al. 1992), prevent the formation of the actin-based contractile ring and elicit the formation of actin aggregates that are not seen in wild type. Previously, we have observed abnormal actin accumulations in mutants at the *tsr* locus, a gene encoding a *Drosophila* cofilin (Gunsalus et al. 1995), but the abnormal actin structures observed in *chic* testes appear to have a different origin from those in *tsr* mutants. Mutations in *tsr* produce two types of abnormal actin aggregates. The first type forms near the centrosomes of each primary spermatocyte and rapidly grows within the cytoplasm during aster formation; this actin cluster persists through prometaphase and metaphase I and disintegrates during the first meiotic anaphase. The second type of actin aggregates seen in *tsr* mutants results from the failure to disassemble meiotic contractile rings. The abnormal F-actin masses derived from the contractile ring of the first meiotic cytokinesis disintegrate only as late as the second meiotic anaphase, whereas the actin clusters originated from the second division rings persist in early spermatids, disintegrating slowly during spermatid elongation. In contrast, the actin aggregates caused by *chic* mutations are observed primarily in mature spermatocytes at the S5 stage, are not associated with centrosomes, and disintegrate during the first meiotic division. These aggregates have the appearance of fusome remnants; this assignment is supported by the observations that they contain  $\alpha$ -spectrin and are

almost always associated with ring canals. Because in wild-type mature spermatocytes at the S5 stage the fusome is almost completely disintegrated (Gunsalus et al. 1995), we suggest that degeneration of this structure is partially blocked in *chic* mutants, leading to the persistence of actin-enriched aggregates in S5 spermatocytes.

Our results clearly show that mutations in the *chic* locus suppress assembly of the contractile ring and disrupt meiotic cytokinesis. An involvement of *chic* in *Drosophila* cytokinesis was anticipated by Verheyen and Cooley (1994), who found binucleate nurse cells in mutant egg chambers. There is considerable precedent for the participation of profilin in cytokinesis of other organisms. Deletion of the profilin-encoding gene *PFY1* disrupts cytokinesis in *S. cerevisiae* (Haarer et al. 1990). *S. pombe* with mutations in the profilin-encoding *cdc3* gene do not form an actin ring and are defective in cytokinesis (Balasubramanian et al. 1994). In *Dictyostelium*



**Figure 9.** Effects of mutations in the *KLP3A* and *dia* loci, and of cytochalasin B treatment, on meiotic divisions in *Drosophila* males. (A) Wild-type control telophase I showing a regular actin ring around the central spindle midzone. Telophase I figures from *KLP3A<sup>e4</sup>* (B), *dia<sup>3</sup>* (C), and cytochalasin B-treated testes (D), all with severely defective central spindles and no actin rings. Bar, 10  $\mu$ m.



**Figure 10.** Effects of cytochalasin B on aster behavior and cytokinesis. A prometaphase I primary spermatocyte stained for tubulin (A) and chromatin (B), showing irregular aster positioning and migration. (C) Cytochalasin B-induced aberrant spermatids containing a large Nebenkern associated with four nuclei. Bar, 10  $\mu$ m.

amoebae carrying mutations in both of their two profilin genes, cytokinesis is severely impaired, causing the formation of cells up to 10 times larger than wild type (Haugwitz et al. 1994). Finally, consistent with a role in assembly of the contractile ring, the Cdc3 profilin of *S. pombe* concentrates in an equatorial band during mitotic cell division (Balasubramanian et al. 1994), and profilin is enriched in the cleavage furrow of *Tetrahymena* cells (Edamatsu et al. 1992). We have similarly observed here that the *chic*-encoded profilin accumulates at the equator of meiotic ana-telophases during *Drosophila* male meiosis.

Interestingly, in *S. cerevisiae*, *S. pombe*, and *Dictyostelium*, cells lacking profilin are not only defective in actin ring assembly and cytokinesis, but also exhibit abnormal actin accumulations (Haarer et al. 1990; Balasubramanian et al. 1994; Haugwitz et al. 1994). Thus, both in these organisms and in *Drosophila* male meiosis, mutations in profilin genes inhibit F-actin polymerization in the contractile ring and promote the formation of abnormal actin aggregates. The biochemical properties of profilin explain, at least in part, how reduction of the intracellular levels of profilin can elicit both negative and positive effects on actin polymerization (for review, see Machesky and Pollard 1993; Theriot and Michison 1993; Sohn and Goldschmidt-Clermont 1994). Early biochemical studies showed that profilin can bind and sequester G actin monomers, thus preventing F-actin polymerization and inducing F-actin depolymerization (Carlsson et al. 1977; Pollard and Cooper 1984). More recent studies, however, indicate that profilin can also

promote actin polymerization through two different mechanisms. First, under proper conditions, actin-profilin complexes polymerize into filaments more efficiently than G-actin monomers alone, so profilin can lower actin monomer critical concentration (Pantaloni and Carlier 1993). Second, it has been suggested that profilin can promote actin polymerization by catalyzing ADP/ATP exchange on actin. In the presence of excess ATP, profilin would generate ATP-actin, which polymerizes into F-actin faster than ADP-actin (Goldschmidt-Clermont et al. 1992).

The regulatory role of profilin on actin polymerization may also depend on the interaction between profilin and two other ligands of this protein: the polyphosphoinositides and poly-L-proline stretches (for review, see Machesky and Pollard 1993; Theriot and Michison 1993; Sohn and Goldschmidt-Clermont 1994). Polyphosphoinositides such as phosphatidylinositol-4,5 biphosphate (PIP2) can interfere with the formation of profilin-actin complexes (Lassing and Lindberg 1985; Goldschmidt-Clermont et al. 1991). Proteins containing poly-proline stretches such as the product of the *cdc12* gene of *S. pombe* (see above) bind to profilin and contribute to actin polymerization at the contractile ring (Chang et al. 1996, 1997). Finally, the functions of profilin in vivo are also likely to reflect its different effects on actin filaments with barbed ends that are either capped or uncapped (Pantaloni and Carlier 1993). This complex biochemical scenario certainly allows sufficient flexibility for profilin to play diverse biochemical functions in living cells, such as its roles in degradation of the F-actin-enriched male fusome and in the assembly of the actomyosin ring that have been documented here. Understanding how all of these potential inputs are integrated to determine profilin activity at particular intracellular locations presents a major challenge for the future.

#### *The actin cytoskeleton and centrosome behavior in spermatocytes*

During prophase/prometaphase of both meiotic divisions in *chic* and *dia* mutants, and in cytochalasin B-treated testes, the asters fail to associate properly with the nuclear envelope. Moreover, separation of the asters and their movement to opposite poles of the spindle is significantly delayed in all cases, although a bipolar spindle is eventually established. There are several precedents for these findings that alterations of the actin cytoskeleton can affect centrosome behavior. Drugs that interfere with F-actin assembly disrupt normal spindle/centrosome movements in both budding yeast and nematodes (Hyman and White 1987; Palmer et al. 1992). In *S. cerevisiae*, a conditional lethal mutation in the actin gene *ACT1* causes spindle misorientation (Palmer et al. 1992), whereas spindle orientations during the early embryonic divisions of *Caenorhabditis elegans* are determined by the attachment of microtubules to a specific actin containing cortical site (Hyman 1989; Waddle et al. 1994).

The abnormal aster behavior seen in *chic* and *dia* mu-

tants is very similar to that observed previously in *tsr* mutant spermatocytes (Gunsalus et al. 1995). Previously, we hypothesized that problems in aster localization and migration in *tsr* mutants could result from either of two mechanisms. First, *tsr* mutations might disrupt connections between the actin cytoskeleton and the astral microtubules analogous to those involved in embryonic nuclear rotations in *C. elegans*, although we have no direct evidence for an involvement of specific cortical or perinuclear F-actin foci in spermatocyte aster behavior. The second possibility is suggested by the presence of large aggregates of F-actin in the vicinity of centrosomes in *tsr* mutants, which might physically block centrosome movements in mutant spermatocytes. The results presented in this paper show that aster movements are defective in testes treated with cytochalasin or mutant for either *chic* or *dia*, even though no actin accumulations are seen at the centrosome in any of these cases. Thus, although we cannot formally exclude that the abnormal aster behavior seen in *tsr* mutants is a secondary effect of the unusual actin aggregates associated with the centrosomes, we regard the present findings as strong evidence that the actin cytoskeleton normally plays a direct and crucial role in promoting correct aster positioning and migration during prophase/prometaphase of both meiotic divisions in *Drosophila* males.

#### *The relationship between profilin and cofilin*

Cofilin accelerates the rate of actin filament turnover in living cells, presumably because of its actin severing and depolymerizing activity, as well as its ability to bind actin monomers (Lappalainen and Drubin 1997; Theriot 1997). Results of our previous investigations on mutants in the *tsr* gene of *Drosophila*, which encodes a cofilin homolog, are mostly congruent with this view of cofilin's role. In *tsr* mutants, the contractile ring forms at the right time and in the correct position. This ring manages to contract, but during telophase, it becomes progressively misshapen and more prominent than in wild type, and eventually fails to disassemble (Gunsalus et al. 1995). This phenotype is clearly anticipated from the decreased rate of actin filament turnover in the contractile ring, expected to accompany cofilin depletion. In contrast, in *chic* mutants there is a complete failure in contractile ring assembly. Epistasis of *chic* to *tsr* is thus easily explained: If there is no contractile ring, it cannot disassemble improperly. Thus, in terms of these two proteins, actin ring assembly is predominantly determined by the *chic* profilin, whereas subsequent actin ring disassembly is predominantly regulated by the *tsr* cofilin. Both of these proteins are nonetheless likely to play at least some role throughout the life history of the contractile ring. For example, the actin ring in *tsr* mutants does not appear morphologically normal, arguing that actin filament turnover aided by cofilin is important to fine-tune contractile ring structure during its assembly (Gunsalus et al. 1995).

Two other aspects of the epistasis of *chic* over *tsr* in double mutants are worthy of brief discussion. First, the

double mutants display the same partial failure in fusome disassembly seen in *chic* mutants alone, yet *tsr* mutants alone are not defective in fusome disassembly. Why profilin is of greater importance to this process than cofilin is currently unknown. A second intriguing finding is the fact that double-mutant spermatocytes do not contain the large actin aggregates associated with the centrosome seen in *tsr* mutant spermatocytes. One possibility is that the profilin function is required for the assembly of these F-actin aggregates in the absence of cofilin. Alternatively, it is possible that in double-mutant spermatocytes, the actin that would be used for aggregate formation remains trapped in the fusome remnants.

#### *Interdependence of the central spindle and the contractile ring*

Our previous studies established that mutations in *KLP3A*, a gene encoding a kinesin-like protein, disrupt the formation of the central spindle (Williams et al. 1995). In this paper we present the surprising finding that these same mutations also impair assembly of the actin-based contractile ring. Because kinesins act as microtubule-based motors, it is logical to assume that *KLP3A* mutations primarily affect the formation of the central spindle and that the absence of the contractile ring is a secondary consequence of this defect. On the other hand, our results in *chic* and *dia* mutants, and our observations on cytochalasin B-treated cells, strongly suggest the converse: that problems in contractile ring assembly can secondarily disrupt central spindle formation. Taken together, these results strongly suggest the existence of a cooperative interaction between elements of the actin-based contractile apparatus and the central spindle microtubules. When either of these structures is perturbed, the proper assembly of the other is disrupted.

The idea that spindle microtubules and the actin cytoskeleton may interact with each other during cytokinesis has long been proposed (White and Borisy 1983; Rapaport 1986, 1991), but has only recently received experimental support (Cao and Wang 1996; Fishkind et al. 1996; Wheatley and Wang 1996). The results described in this paper provide further, stronger evidence for such an interaction and raise the question of the underlying molecular mechanisms. The current view is that microtubules can interact with actin filaments through either microtubule-associated proteins (MAPs) (Griffith and Pollard 1978; Sattilaro et al. 1981; Pedrotti et al. 1994), or microtubule-based motor molecules (for review, see Schroer 1994). The microtubule/F-actin interactions involved in contractile ring and central spindle formation are likely to be mediated by one or more of the numerous proteins that accumulate in the central spindle midzone during late anaphase and telophase (for review, see Margolis and Andreassen 1993; Goldberg et al. 1997). These include the so-called passenger proteins (Earnshaw and Bernat 1990) such as the TD60 protein (Andreassen et al. 1991), the inner centromere protein (INCENP; Cooke et al. 1987; Earnshaw and Cooke 1991), and the kinesin-



like proteins CHO1/MKLP1, CENP-E, XKLP1, and chromokinesin (Sellitto and Kuriyama 1988; Yen et al. 1991, 1992; Nislow et al. 1992; Vernos et al. 1995; Wang and Adler 1995), which move from the chromosomes to the spindle midzone. Although impairment of INCENP, CHO1, and XKLP1 functions affect spindle formation and interfere with the completion of cytokinesis, it is not clear whether these effects are accompanied by problems in central spindle assembly (Nislow et al. 1990; Vernos et al. 1995; Eckley et al. 1997).

In *Drosophila*, the best candidate at present for mediating interactions between the central spindle and cortical actin, at least during male meiosis, is the KLP3A kinesin-like protein. This protein could interact directly with both the central spindle microtubules and components of the contractile ring. Alternatively, KLP3A could transport to the spindle midzone molecules that mediate F actin-microtubule interactions. At the moment, we cannot discriminate between these possibilities, nor we have any information on the proteins that bind to or might be transported by KLP3A. We believe, however, that the isolation and characterization of additional mutations causing cytological phenotypes similar to those of *KLP3A*, *chic*, and *dia*, will eventually provide substantial insight into the mechanisms underlying microtubule-actin interaction during cytokinesis.

## Materials and methods

### *Drosophila stocks*

The *chic* alleles *chic<sup>R1</sup>*, *chic<sup>8A</sup>*, *chic<sup>13E</sup>*, and *chic<sup>35A</sup>* were generated through the transposition of single, marked *P[lacW]* elements (Bier et al. 1989) as outlined by Gatti and Goldberg (1991). Mobilization of the P-element insertions associated with these mutant alleles was carried out as described by Gatti and Goldberg (1991). The alleles *chic<sup>37</sup>* and *chic<sup>1320</sup>* (Cooley et al. 1992; Verheyen and Cooley 1994) were the kind gift of Dr. Lynn Cooley (Yale University, New Haven, CT); the deficiencies *Df(2L)c1ot7* and *Df(2L)GpdhA* were obtained from Dr. Ross MacIntyre (Cornell University, Ithaca, NY). Mutations in *chic* and deletions were usually maintained as heterozygotes balanced by the second chromosome balancer *CyO*. To provide a marker to distinguish between homozygous mutant or heterozygous larvae and pupae, these mutations were also balanced over *ST*, a translocation between *SM6a* and the third chromosome balancer *TM6B* that carries the dominant larval marker *Tubby (Tb)* (the kind gift of Dr. Antonio Garcia Bellido, Universidad Autonoma, Madrid, Spain); see also Gatti and Goldberg (1991) for further explanation.

The *KLP3A<sup>e4</sup>* allele has been described previously (Williams et al. 1995). To obtain *KLP3A<sup>e4</sup>/Y* male larvae, *KLP3A<sup>e4</sup>/FM7* virgin females were mated to *FM7/Y* males and their progeny were scored for Malpighian tubule coloration (Williams et al. 1995). The *dia<sup>1</sup>* and *dia<sup>3</sup>* alleles (Castrillon and Wasserman 1994) were the kind gift of Dr. Steven Wasserman (University of Texas, Dallas, TX). *dia<sup>1</sup>* homozygotes are viable but male sterile, whereas homozygosity for *dia<sup>3</sup>* causes lethality during the early pupal stages. Both mutations were balanced over *In(2LR)*, *Gla Bc*; the dominant marker *Black Cells (Bc)* allows recognition of the desired homozygous *dia* mutant larvae.

The *tsr<sup>1</sup>* mutation has been described by Gunsalus et al. (1995). To construct *chic<sup>R1</sup> tsr<sup>1</sup>* double mutants, *chic<sup>R1</sup>/tsr<sup>1</sup>* fe-

males were crossed to *CyO/Sco* males; putative *chic<sup>R1</sup> tsr<sup>1</sup>* recombinant chromosomes were recovered and balanced over *CyO*. These chromosomes were then tested for complementation with both *chic<sup>R1</sup>* and *tsr<sup>1</sup>* to identify double mutants. As a final step, *chic<sup>R1</sup> tsr<sup>1</sup>* chromosomes were rebalanced over *ST* to allow identification of the double-mutant larvae. All the markers and special chromosomes mentioned above, but not otherwise specified, are described in detail by Lindsley and Zimm (1992).

### Identification of P-element insertion sites

DNA adjacent to the P-element insertion sites of the *chic* alleles *chic<sup>R1</sup>*, *chic<sup>8A</sup>*, *chic<sup>13E</sup>*, and *chic<sup>35A</sup>* was cloned by plasmid rescue (Pirrota 1986). This was possible because the *P[lacW]* element causing these mutations contains a bacterial origin of replication and a selectable marker for ampicillin resistance (Bier et al. 1989). Genomic DNA from adults homozygous for these mutations was prepared, digested with either *EcoRI* or *PstI*, and ligated as described by Gunsalus et al. (1995). The ligation mix was transformed directly into XL-1 Blue cells by electroporation and desired clones were selected for ampicillin resistance. Double-stranded plasmid DNAs containing parts of the P element and adjacent genomic DNA were sequenced by the dideoxy method (Sanger et al. 1977) by use of Sequenase version 2.0 (U.S. Biochemical Corp.) or the Ladderman kit (Takara Biochemical Inc.), [<sup>35</sup>S]dATP (Amersham Corp.), 6% Long Ranger (AT Biochem) gels, and sequence-specific primers from the P-element ends made by the Biotechnology Synthesis and Analytical Facility at Cornell University.

### Cytology and indirect immunofluorescence

All techniques for cytological preparations of larval testes, for staining of DNA, microtubules, and F-actin, and for fluorescence microscopy recorded with a cooled charge-coupled device (CCD) have been described previously in detail (Cenci et al. 1994; Gunsalus et al. 1995).

For visualization of microtubules, testes were incubated for 45 min with a 1:50 dilution in PBS of a mouse monoclonal anti- $\alpha$  tubulin antibody (Amersham International). After two 5-min washes in PBS, the slides were incubated for 50 min with the secondary antibody [sheep anti-mouse IgG, F(ab)<sub>2</sub> fragment, conjugated with 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester (FLUOS), (Boehringer) diluted 1:15 in PBS].

For anillin localization, we used a rabbit antiserum raised against amino acids 401–828 of anillin (Field and Alberts 1995), diluted 1:300 in PBT (PBS with 0.1% Triton X-100) also containing 1% BSA. After overnight incubation at 4°C, the slides were washed twice in PBT and once in PBS, for a total of 15 min. The primary antibody was detected by incubation for 2 hr with FITC-conjugated anti-rabbit IgG (Cappel), diluted 1:15 in PBT.

For  $\alpha$ -spectrin visualization, we used a rabbit polyclonal antibody (rabbit 675; Byers et al. 1987) diluted 1:100 in PBS. Fixed testes were incubated with the primary antibody overnight at 4°C and then washed twice in PBT (5 min each) and once in PBS (10 min). They were then incubated with the secondary antibody (FITC-conjugated anti-rabbit IgG, Cappel), diluted 1:15 in PBT.

For profilin localization, fixed testes were incubated overnight in a humid chamber at 4°C with an undiluted antiprofilin monoclonal antibody from cell line 6F (Verheyen and Cooley 1994). After three 5 min washes in PBS, the slides were incubated for 50 min at room temperature with the secondary antibody [sheep anti-mouse IgG, F(ab)<sub>2</sub> fragment, conjugated with FLUOS, (Boehringer), diluted 1:15 in PBS].



Actin staining was usually performed after immunostaining. 300 units of rhodamine-labeled phalloidin (Molecular Probes) were dissolved in 1.5 ml of methanol. One hundred microliters of this stock solution was vacuum dried and resuspended in 200  $\mu$ l of PBS. Testis preparations were incubated with 20  $\mu$ l of this solution for 1 hr and 30 min at 37°C.

In all cases, the final staining procedure was treatment with Hoechst 33258. After immunostaining, or immunostaining plus actin staining, testis preparations were rinsed in PBS (10 min), air dried, and stained with Hoechst 33258 according to Cenci et al. (1994).

Unless otherwise specified, all the immunostaining and staining procedures described above were performed at room temperature.

### Fluorescence microscopy

All preparations were examined with a Zeiss Axioplan microscope equipped with an HBO 50 W mercury lamp for epifluorescence and with a CCD (Photometrics Inc.). Hoechst 33258, FLUOS, and rhodamine fluorescence were detected by use of the 0.1 (BP 365/11, FT 395, LP 397), 10 (BP 450/490, FT 510, LP 515/565), and 15 (BP 546, FT 580, LP 590) Zeiss filter sets, respectively. Gray-scale digital images were collected separately with the IP Lab Spectrum software (Signal Analytics). They were then converted to Photoshop 2.5 format (Adobe Systems), and either used as black and white images or pseudocolored and merged. Final images were printed by a dye sublimation process.

### Cytochalasin B treatment

Larval testes, dissected in saline (0.7% NaCl), were incubated with 1  $\mu$ g/ml of cytochalasin B (Sigma) dissolved in the same saline solution for 2 hr at 25°C. Testes were then fixed and stained as described above.

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## Cooperative interactions between the central spindle and the contractile ring during *Drosophila* cytokinesis

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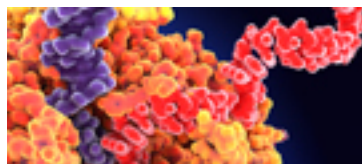
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