

fumble Encodes a Pantothenate Kinase Homolog Required for Proper Mitosis and Meiosis in *Drosophila melanogaster*

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ABSTRACT

A number of fundamental processes comprise the cell division cycle, including spindle formation, chromosome segregation, and cytokinesis. Our current understanding of these processes has benefited from the isolation and analysis of mutants, with the meiotic divisions in the male germline of *Drosophila* being particularly well suited to the identification of the required genes. We show here that the *fumble* (*fbl*) gene is required for cell division in *Drosophila*. We find that dividing cells in *fbl*-deficient testes exhibit abnormalities in bipolar spindle organization, chromosome segregation, and contractile ring formation. Cytological analysis of larval neuroblasts from null mutants reveals a reduced mitotic index and the presence of polyploid cells. Molecular analysis demonstrates that *fbl* encodes three protein isoforms, all of which contain a domain with high similarity to the pantothenate kinases of *A. nidulans* and mouse. The largest Fumble isoform is dispersed in the cytoplasm during interphase, concentrates around the spindle at metaphase, and localizes to the spindle midbody at telophase. During early embryonic development, the protein localizes to areas of membrane deposition and/or rearrangement, such as the metaphase and cellularization furrows. Given the role of pantothenate kinase in production of Coenzyme A and in phospholipid biosynthesis, this pattern of localization is suggestive of a role for *fbl* in membrane synthesis. We propose that abnormalities in synthesis and redistribution of membranous structures during the cell division cycle underlie the cell division defects in *fbl* mutant cells.

CYTOSKELETAL dynamics have an essential role in all stages of cell division. During the S and G phases of the cell cycle both the chromosomal and cytoplasmic contents of the cell increase to allow progression of the cell through mitosis. Subsequent segregation of the chromosomes during mitosis takes place on a bipolar microtubule spindle. At prophase, a pair of centrosomes and their associated microtubule asters separate to opposite sides of the dividing cell (reviewed in KASHINA *et al.* 1997). At metaphase, centrosomes initiate the bipolar microtubule spindle. The chromosomes are captured to the spindle structure via their kinetochores. Following the movement of chromosomes toward opposite poles during anaphase, a dense microtubule structure forms at the midzone of the spindle. This central spindle structure is thought to have a role in assembly and positioning of the acto-myosin contractile ring that mediates cytokinesis (reviewed in FIELD *et al.* 1999). All of these cell cycle events must be coordinated with biosynthesis of cellular components such as macromolecules and membrane phospholipids (reviewed in JACKOWSKI 1996).

Despite increasing knowledge about the mechanisms and pathways regulating cell division, many questions

remain unanswered, in part because not all components have been identified. Genetics has proven to be a powerful tool for addressing this problem. In *Drosophila*, null mutations for genes involved in cell division result in zygotic lethality in either larval or pupal stages, reflecting the time at which maternal stores of the affected protein are exhausted (GATTI and GOLDBERG 1991). Examination of dividing cells, such as neuroblasts, from mutant larvae can reveal which step in cell division is defective (KARESS and GLOVER 1989; GONZÁLEZ *et al.* 1994).

A number of essential cell division genes have been identified on the basis of mutations conferring a partial, rather than complete, loss of function. Flies homozygous for such hypomorphic mutations reach adulthood, but often exhibit defects during the proliferative stages of gametogenesis. *P* elements have proven useful in this regard, since the strong tendency of these transposons to insert in gene regulatory regions often causes a disruption in germline expression, resulting in female or male sterility (COOLEY *et al.* 1988; CASTRILLON *et al.* 1993).

The meiotic divisions in the male germline are especially useful for the analysis of defects caused by mutations in cell division loci. Spermatocytes, which will undergo meiosis, are particularly large cells in which each of the meiotic divisions is visible by phase contrast microscopy. As a consequence of these two divisions, a spermatocyte produces four haploid spermatids, each containing a single nucleus and a mitochondrial aggregate of a size comparable to the nucleus. Disruptions

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in chromosome segregation are reflected in alterations in nuclear size and number among the four daughter spermatids, whereas aberrant cytokinesis generates four nuclei in association with an abnormally large mitochondrial aggregate (reviewed in FULLER 1993).

Here we report the genetic and molecular characterization of *fumble* (*fbl*). Depletion of Fumble protein results in defects in spindle assembly, chromosome segregation, and cytokinesis. Nevertheless, the cell cycle does not arrest at metaphase, the central spindle and contractile ring form, and chromosome segregation proceeds in the absence of aster separation. Cloning of *fbl* revealed that it encodes three protein isoforms, all of which contain a domain homologous to the pantothenate kinase (PanK) in *Aspergillus nidulans* and mouse (CALDER *et al.* 1999; ROCK *et al.* 2000). In embryonic cells, an epitope-tagged version of the largest Fumble isoform associates with the microtubule spindle at metaphase and concentrates at the cleavage site during telophase. In addition, Fumble accumulates at the sites of membrane ingression during syncytial nuclear division and cellularization. These results demonstrate an essential role for PanK in both mitosis and meiosis in *Drosophila* and probably reflect the critical function of membrane assembly in cell division.

MATERIALS AND METHODS

Drosophila stocks and genetic manipulations: All crosses were performed at 20° on yeasted cornmeal-molasses agar. The original *fumble* allele (*fbl*¹) has been described previously (CASTRILLON *et al.* 1993). All genetic markers and balancers have been described (LINDSLEY and ZIMM 1992).

Remobilization of P element to generate new alleles: The P-element insert at the *fbl* locus was remobilized by crossing *fbl*¹/*TM3* flies to flies carrying *P* [*ry*⁺, Δ 2-3] as a transposase source (ROBERTSON *et al.* 1988). In the following generation the *P* [*ry*⁺, Δ 2-3] chromosome was crossed out and the *fbl*¹ derivative lines were selected on the basis of loss of the *ry*⁺ eye color marker.

Aside from obtaining revertants to male fertility, we selected new alleles of *fbl* from among the *ry* lines generated on the basis of the following criteria: (1) Both the *fbl*¹ allele and the deficiency *Df*(3L)*rdgc*(4), which deletes the entire *fbl* region, failed to complement these mutations, and (2) these lines failed to complement each other, indicating that they carry mutations in the same locus. We retained alleles that appeared stronger than the original *fbl*¹ mutation, *i.e.*, showed more severe defects in the testis as homozygotes (three alleles) or were homozygous lethal (two alleles, *fbl*² and *fbl*³).

Molecular cloning and sequence analysis of transcripts in the *fbl* region: The *fbl*¹ allele was generated by insertion of the *P* [*lacZ*, *ry*⁺] element (MLODZIK *et al.* 1992). Genomic sequences flanking the *fbl* region were isolated by first performing plasmid rescue from *Xba*I-digested genomic DNA and then by using the plasmid rescue DNA to probe an EMBL3 *Drosophila* genomic library (TAMKUN *et al.* 1992). The 40-kb genomic fragment encompassing the *fbl* locus was used to prepare a probe for screening a *Drosophila* testis cDNA library (provided by Tim Karr). Sequences from candidate cDNAs were analyzed using BLASTP (ALTSCHUL *et al.* 1990).

Transformation and phenotypic rescue: Full-length cDNAs

representing the transcripts flanking the P-element insert were each subcloned into pBUD (a gift from Jeff Sekelsky, UNC at Chapel Hill); this derivative of the Bluescript cloning vector (Stratagene, La Jolla, CA) contains a ubiquitin promoter and Flag-epitope tag upstream of the multiple cloning site. The resulting plasmids contain the Flag-epitope and the encoded proteins in the same reading frame. The promoter-tag-cDNA fragments were subsequently subcloned between the *Kpn*I and *Not*I sites of the pCasPeR4 transformation vector (PIRROTTA 1988). P-element germline transformation was performed as described (SPRADLING 1986), except that the plasmid carrying the Δ 2-3 transposase source was coinjected with the transformation plasmid.

Cytological analyses: Aceto-orcein squashes of larval neuroblast cells were performed as described (KARESS and GLOVER 1989). Testes were dissected and fixed as described (GUNSALUS *et al.* 1995), except that 3% bovine serum albumin was used as a block during the Triton X-100 incubation. Primary antibodies were diluted in PBS as follows: monoclonal antitubulin (Sigma, St. Louis), 1:500 and monoclonal anticentrosomin (a gift from Thom Kaufman), 1:10,000. The mitotic index was determined as described (GONZÁLEZ *et al.* 1988).

Immunostaining: The embryo immunostaining was as described (AFSHAR *et al.* 1995), except that the fixation was performed in a 1:1 solution of heptane and 3.5% formaldehyde in PBS for 5 min, the embryos were hand devitelinated, and embryos were mounted in Fluoromount.

RESULTS

Inactivation of *fbl* disrupts chromosome segregation and cytokinesis: The P-element-induced *fbl*¹ allele exhibits defects in chromosome segregation and cytokinesis in the male germline. In addition, *fbl*¹/*fbl*¹ flies are female sterile and uncoordinated. These phenotypes are more severe *in trans* to a deficiency, suggesting that the *fbl*¹ allele is hypomorphic. To generate a more severe loss of gene function, we remobilized the P-element insert at the *fbl* locus. We obtained five new alleles of *fbl* resulting from imprecise excision events. Three are viable with more severe male-sterile phenotypes than *fbl*¹; two are pupal lethal. The lethal alleles (*fbl*² and *fbl*³) each remove a substantial portion of the *fumble* gene (see below) and enhance the *fbl*¹ phenotype to the same extent as a chromosomal deficiency. We conclude that *fbl*² and *fbl*³ are null mutations and that *fumble* is an essential gene.

Examination of neuroblasts from third instar larval brains revealed that homozygotes for the null alleles of *fbl* have ~80% fewer mitotic figures than the wild type. In addition, among the cells with detectable mitotic figures, a large number of cells are aneuploid, polyploid, or have abnormal anaphase figures (Figure 1). The metaphase figures in wild-type third instar larval brains stained with aceto-orcein display four pairs of condensed chromosomes (Figure 1A). In contrast, ~35% of the aceto-orcein-stained mitotic figures from *fbl*² and *fbl*³ homozygotes contain more than the wild-type set of four chromosomal pairs (Figure 1, B and C; compare to 1A).

Anaphase figures are also abnormal in *fbl* null mu-

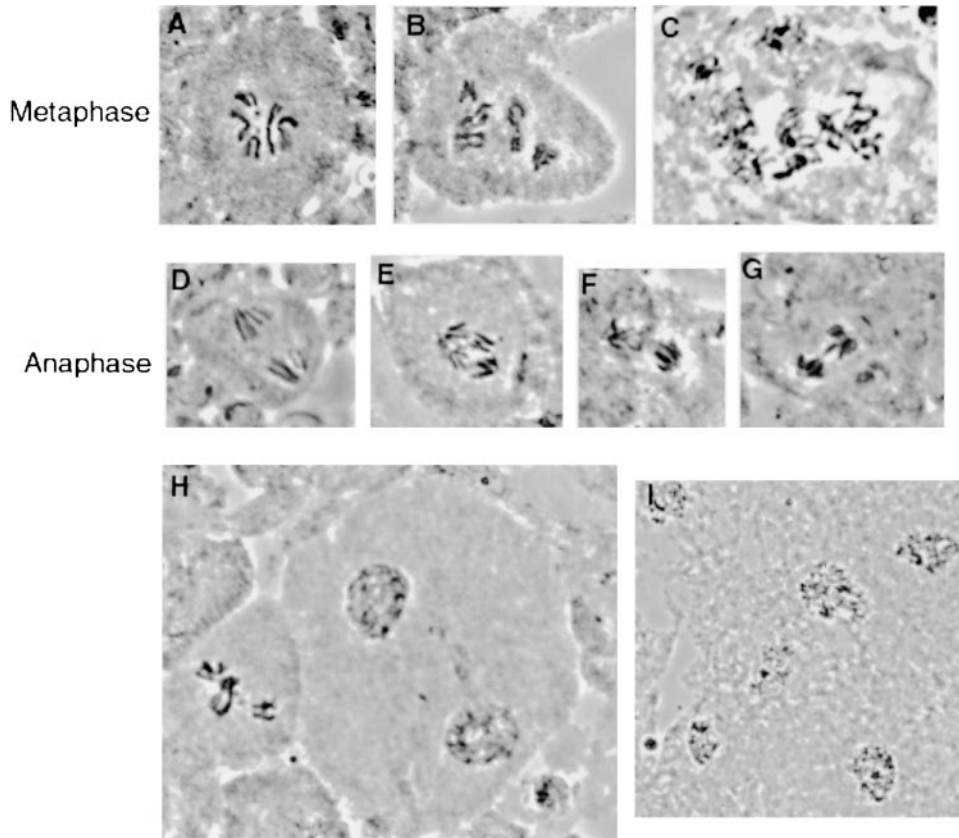


FIGURE 1.—Cell division defects in dividing larval neuroblasts. Aceto-orcein-stained mitotic chromosomes from wild-type (A and D) and *fbl* (B, C, E–I) neuroblasts are shown. (A) Wild-type mitotic figure with three pairs of condensed chromosomes, and a fourth pair of chromosomes appearing as two dots. (D) Wild-type anaphase figure showing separation of chromosomes toward opposite poles. (B and C) Mitotic figures from *fbl^l/fbl^l* larvae. More than three pairs of major chromosomes are readily detectable in these cells. (E–G) Anaphase figures from *fbl^l/fbl^l* larvae. Note the presence of the lagging chromatids (E and F) and chromosomal bridge (G). (H and I) Mitotic figures from *fbl^l/fbl^l* neuroblasts showing a binucleate cell (H), and a field of cells with high chromosomal content (I).

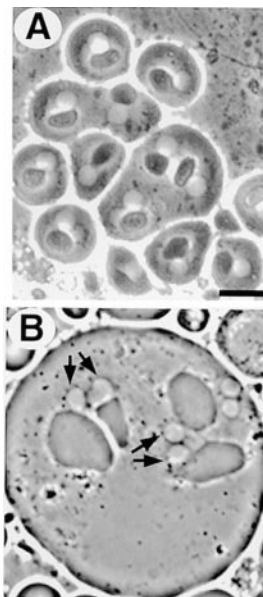


FIGURE 2.—Cell division defects in *fbl* testes. Squashes of live secondary spermatids from a wild-type (A) or *fbl* (B) testis examined by phase contrast microscopy are shown. (A) A portion of a cyst of 64 secondary spermatids at the onion stage before differentiation (FULLER 1993). Each nucleus (white) is paired with a major mitochondrial derivative (black). (B) Secondary spermatids at onion stage in *fbl^l/fbl^l* testis. An abnormal nebenkern structure associated with two or more nuclei of different size (arrows). Bar, 5 μ m.

tants. In wild-type cells all the chromosomes move synchronously to the poles during anaphase, such that at any point segregated sister chromatids are positioned equidistant from the midzone (Figure 1D). In *fbl* mutants at anaphase, we often detect lagging chromatids (Figure 1, E and F) or anaphase bridges (Figure 1G). These abnormalities can be detected among 60% of the anaphase figures from *fbl* null cells. In addition to these defects we can sometimes detect cells with two or more nuclei (Figure 1H) and nuclei with high chromosomal contents (Figure 1I). On the basis of these results, we conclude that *fumble* has an essential role in chromosome segregation.

To examine in more detail the effects on cytokinesis and chromosome segregation, we turned to an analysis of the meiotic divisions executed by spermatocytes. Defects in both cytokinesis and chromosome segregation are apparent when spermatids from a hypomorphic allele of *fbl* are examined by phase contrast microscopy (Figure 2). In wild type, each secondary spermatid contains a dark mitochondrial aggregate associated with a clear nucleus of the same size, indicating proper, equal segregation of chromosomes and cytoplasmic material into haploid spermatids during the two meiotic divisions (Figure 2A). In testes from males homozygous for *fbl^l* ~10% of the cysts contain spermatids with mitochondrial aggregates of abnormal size and shape and multiple nuclei of different size (Figure 2B). In the *fbl^l/fbl^l* mutant, >90% of spermatids have undergone aberrant

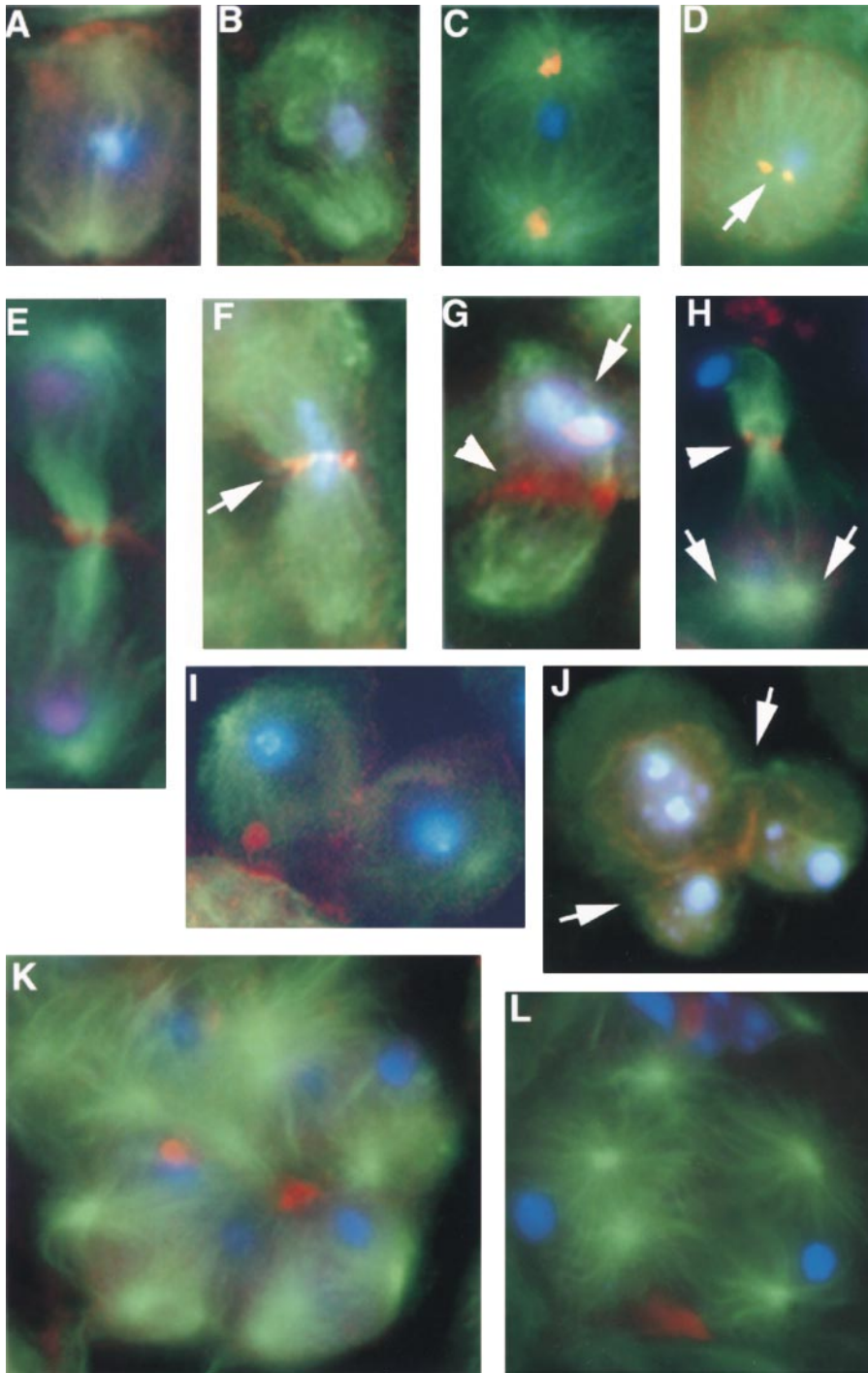


FIGURE 3.—Requirement for Fumble in spindle organization and cytokinesis. Testes were stained with antitubulin antibody (green) to examine the structure of the spindle and either phalloidin to detect actin structures (red; A, B, E–L) or anticentrosomin (red; C and D) for detection of centrosomes. DAPI staining (blue) was used to visualize chromosomes. (A) Wild-type primary spermatocyte at metaphase, containing a bipolar spindle and a single chromosomal mass. A dot-like actin structure is positioned close to one of the poles. (B) Primary spermatocyte at metaphase of the first meiotic division in *fbl¹/fbl¹* testis, revealing an asymmetric bipolar spindle. (C) Spindle structure in a wild-type spermatocyte, containing one centrosomal structure at each pole, marked by an anticentrosomin antibody (red). (D) Spermatocyte in *fbl¹/fbl¹* testes; although the chromosomes have been condensed, the centrosomes are located next to each other and have not separated (arrow). (E) Wt primary spermatocytes at anaphase stage of meiosis. Note the segregating masses of chromosomes and the presence of an aster at each pole of the spindle structure. The central spindle and acto-myosin ring are apparent at the midzone, as revealed by an intense microtubule staining and actin staining, respectively. (F and G) Spermatocytes at anaphase stage in *fbl* testis. Note the presence of a contractile ring over the mass of chromosomes (F, arrow) and the absence of chromosomes from one pole on the microtubule spindle (G, arrowhead). (H) A primary spermatocyte at anaphase stage in *fbl¹/fbl³* testis. Note that despite the lack of aster separation (arrow), segregation of chromosomes, formation of a central spindle, and assembly of a contractile ring (arrowhead) have proceeded. (I) Wild-type primary spermatocyte being pinched into two equal daughter cells during cytokinesis at late telophase. (J) Cells in *fbl* testes at late telophase, revealing division of two or more cells (arrow) with unequal chromosomal and cytoplasmic contents. (K and L) Cell from a cyst of primary spermatocytes from *fbl¹/fbl²* (K) and *fbl¹/fbl³* (L) testes. Note the presence of multiple centrosomes in these cells and lack of chromosomal association with asters.

cell division and spermiogenesis (postmeiotic spermatid differentiation) is grossly defective (data not shown).

Structure of the bipolar spindle is defective in *fbl* mutants: To understand the basis for the cell division defects in *fbl* flies, we stained testes with 4′6-diamidino-2-phenylindole (DAPI), antitubulin antibody, and phalloidin for simultaneous observation of chromosomes,

microtubule spindles, and the actin contractile ring, respectively (Figure 3).

At meiotic prophase in wild-type testes, the centrosomes nucleate microtubule asters, which subsequently separate to opposite poles of the nuclear envelope (CENCI *et al.* 1994). At metaphase, the microtubule arrays projected by the centrosomes assemble a bipolar

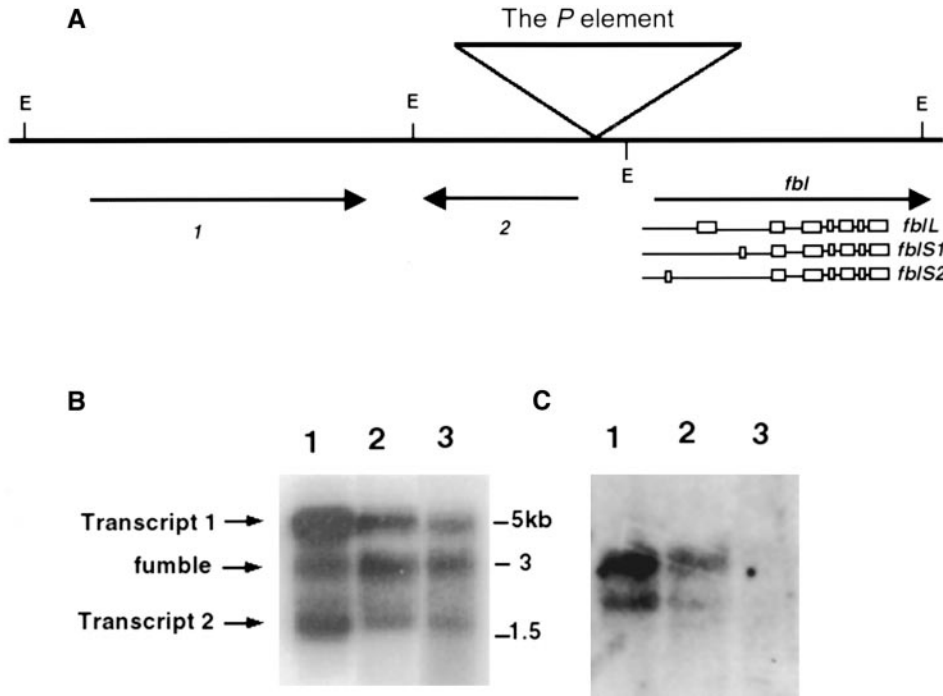


FIGURE 4.—(A) Physical map of the *fbl* genomic region, showing the transcription units flanking the *P* insertion site. Arrows indicate the transcription units at and flanking the *fumble* locus (CG5725). Three splice variants of *fumble* are shown below the corresponding transcription unit and genomic region. The alternate splice forms of *fumble* are designated as *fblL*, *fblS1*, and *fblS2*. *fblS1* and *fblS2* are the same as CT17969 and CT37729 transcripts in GadFly. The open boxes indicate the position of exons and the lines between the boxes show the introns. The position of the *P*-element was determined by Southern blot analysis and sequence alignment of the cDNAs with the genomic sequences adjacent to the *P*. The GenBank accession numbers for the cDNAs corresponding to transcripts 1 and 2 are AAF34654 and AAD38623, respectively. Restriction site, E-*EcoRI*. (B) Northern blot analysis using a 40-kb *fbl* genomic fragment as

probe. Poly(A)⁺ RNA was prepared from embryonic (lane 1), larval (lane 2), and adult (lane 3) tissues. The three bands represent transcripts marked on A. The sizes of the transcripts were determined relative to GIBCO-BRL RNA molecular markers. (C) The same blot probed with the *fumble* cDNA.

spindle structure (Figure 3A). The centrosomes are located at each pole of the spindle and can be detected by staining with anticentrosomin antibody (Figure 3C), whereas the chromosome bivalents congress at the equatorial region of the spindle (metaphase plate; Figure 3, A and C). During both metaphase and early anaphase a dot-like actin structure appears close to one of the poles (Figure 3A; GUNSALUS *et al.* 1995). At anaphase, the bivalent chromosomes segregate toward opposite poles of the spindle (Figure 3E), and at the midbody a high density of microtubule staining marks the central spindle, which is associated with the acto-myosin contractile ring (Figure 3E).

Testes homozygous for *fbl* show abnormalities in spindle structure and cytokinesis. During prometaphase and metaphase, a fraction (~10%) of the primary spermatocyte cysts have asymmetric and/or apolar microtubule structures and lack any actin structures (Figure 3B). There is also a failure in centrosomal separation, as apparent in dividing cells stained with an anticentrosomin antibody (Figure 3D). Defects in organization and assembly of the bipolar spindle are more severe in cells from *fbl^l/fbl^l* or *fbl^l/fbl^p* testes. The frequency of spindle structure abnormalities reaches 80% in these cells. Moreover, the majority of spindles are multipolar, presumably due to a failure in cytokinesis in the preceding mitotic divisions and the accumulation of multiple centrosomes in one cell (Figure 3, K and L). In some *fbl^l/fbl^l* spermatocytes, chromosomes are associated with only one-half of the spindle (Figure 3G, arrow). Al-

though an actin ring is apparent at the midzone of the half spindle, the intense tubulin-staining characteristic of the central spindle is absent (Figure 3G, arrowhead). In some instances, the actin contractile ring forms around the chromosomes at the midbody, indicating a defect in the timing of contractile ring formation or in chromosome segregation (Figure 3F, arrow).

Anaphase and telophase stages were difficult to define in *fbl^l/fbl^l* or *fbl^l/fbl^p* testes, most likely as a result of the severe defects in metaphase spindle organization. However, in some less severely affected cells, analysis of anaphase figures revealed two dots of intense tubulin staining, and hence two asters, at a single pole (Figure 3H, arrow). Such cells contain a contractile ring positioned over a central spindle (Figure 3H, arrowhead), demonstrating that chromosome separation and cytokinesis can proceed in the absence of aster separation.

At late telophase in *fbl^l/fbl^l* testes, we detect cells containing two or more actin rings and associated cleavage furrows situated between distinct masses of chromosomes (Figure 3J; contrast with Figure 3I), suggesting unequal partitioning of chromosomal masses and cytoplasm. Such abnormalities in number and positioning of the actin contractile ring during anaphase/telophase can be due to abnormalities in the number of asters and spindle structures at earlier stages of meiosis. The observation that various cellular processes are affected during the division cycle in *fbl* mutants suggests that the Fumble protein acts at a global level.

Molecular cloning of *fumble*: Genomic sequences

FblS1	1	-----
FblS2	1	-----
FblL	1	MKVPTNRNSKYSFNLKIFKHLNDKKPSTSPPTSPVISAKRNSWRASWRSALGSKSKSIAM
mouse	1	-----
yeast	1	-----
FblS1	1	-----MPEALHFTSIFDLIIKSAMPWFGMDIG
FblS2	1	-----MTEDEQINLRNQLARAMPWFGMDIG
FblL	61	ASDSSSDPIDLEAEPVFFFEHSRCNSYCSSSAFTELPHPAIQIPQGDLEMSMPWFGMDIG
mouse	1	-----MKLVNGRKQTFPWFGMDIG
yeast	1	-----MPRITQESYNCDYGDNTFNLAIDIG
Fbl	121	GTLITKLVYFEPKDIPTDEQDRFAGILRNIRNYLTKNSAYGKTGHRDTHLQFDNVEIRKRK
mouse	20	GTLVTLVYFEPKDIPTAEEQDFVENLKSIRNYLTSNAYGKTGIRDVHLEKNEITCCGR
yeast	27	GTLAKVYFS-P-----IHSRLMFYTIETE
Fbl	181	GSLHFIREFQTTDMGNFESLAKQGMALVTVCATGGGAFKFEQDFRQVNV--MKLAKFD
mouse	80	GNLHFIREFPTICAMHLFIQMGSEKFNSSLHTTLCATGGGAFKFEEDFRMIAD--LQLHKLD
yeast	51	KIDKFMELLHS-----ILKEHNNGCYRMLHIIATCGGAFKFDLLEYLFPQIKGISRF
Fbl	239	ELDTLIKGLFAD---LHNPTCEYYYENARDLTKSEKQQNFNSOPHPELILVNVGSGVSIL
mouse	138	ELDCLTQCLLYVDSVGFNGKPECYFENPTNPELCOKPYCLDNYPYMLLVNVGSGVSIL
yeast	105	EMEGLIHGLDFFIH-EHPDEVFTYNDODGERTIIPSSGTMDSKATYPVLLVNVGSGVSIL
Fbl	296	AVYGPDNYKRISGTSLGGGTFLGLCCLLTGCTSFEEAQLQATKGDNRKVDKLVKDIYGGD
mouse	198	AVYSKDNKYKRVITGTSLGGGTFLGLCCLLTGCTSFEEALDMAAKGDSINVDKLVKDIYGGD
yeast	164	KVTEPNNEISRVGGSSSLGGGTFLGGLSLITCAQTYDOMLLDWAQEGDNSVDMLVGDYIGTD
Fbl	356	YNRFGLPGLDLVASSFCQMLNDKRVSSSRE-----DLANAT
mouse	258	YERFGLQGSVAVASSFCNMMSKEKREBSSSKE-----DLARAT
yeast	224	YNIIGLKSALVASSFGKVFONRMTSNKSLNENNKLYSSHESIEKNNGQMFKNPDIICKSL
Fbl	392	LVTITNNGISIARMCALNEKIDRVVFGNFLRVNPIISMKLLAYAMFWSNGTMRKLFLEH
mouse	294	LVTITNNGISIARMCALNENIDRVVFGNFLRVNPIISMKLLAYAMFWSKQQLKALFLEH
yeast	284	LFATSNNGICQAYIQAKINNIQNYFEGGSYTRGHITIMNTLSYALNFWSCGSKQAFELKH
Fbl	452	EGYFGALGCLLQFNGLAAALNDGVEHSIHTESDSASEAAQTSSTADEPPEKAPTSKHST
mouse	354	EGYFGAVGALLELFKMDITQ-----
yeast	344	EGYLGAMGAFLSASRHSSTIKKTST-----
Fbl	512	R
mouse	-	
yeast	-	

FIGURE 5.—Alignment of amino acid sequences for FblL, FblS1, and FblS2 (GenBank accession nos. AAF34653, AAF51587, and AAF51588), mouse PanK (GenBank accession no. AAF23952), and yeast PanK (GenBank accession no. YDR531W). Amino acid residues identical among the sequences are shaded. In the PanK domain, the amino acid sequence for the three Fbl isoforms is identical. The lysine residue that binds to ATP in pantothenate kinases is indicated with an asterisk. Within a stretch of 368 amino acids, Fumble shares 59% identity with mouse PanK and 30% identity with yeast PanK.

flanking the *P* [*lacZ*, *ry*⁺] insert were isolated and used as a probe to screen a *Drosophila* genomic library. Northern blot analysis revealed the presence of three transcription units in the 40 kb of genomic DNA surrounding the insertion site (Figure 4A). All transcripts are expressed during embryonic, larval, and adult stages (Figure 4B). Further Northern blot analysis using different genomic DNA fragments as a probe revealed that one of the transcription units produces at least two RNA splice variants (see below). Screening of testis and embryonic cDNA libraries using the 40-kb genomic fragment as a probe led to the isolation of cDNA clones representing three genes. We determined the relative posi-

tion of the three transcription units to each other and to the position of the *P* element by aligning the cDNA sequence with that of the genomic DNA (Figure 4A).

The gene proximal to the *P*-element insert is *fumble*. Our evidence is as follows: we obtained an *ep3* line (RØRTH *et al.* 1998) with a *P*-element insert that interrupts the coding region of transcript 1 (Figure 4A). This mutant complemented *fb*^l, indicating that this transcript does not encode Fumble. We determined which of the other two transcripts is *fumble* by two criteria. First, we subcloned cDNAs for the two transcripts into a transformation vector under control of the ubiquitin promoter. We then performed phenotypic rescue ex-

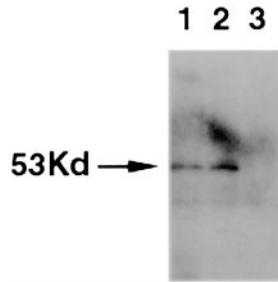


FIGURE 6.—Immunoblot analysis on Flag-Fumble transformant lines. M5 anti-Flag antibody was used to detect Flag-Fumble protein. Lanes 1 and 2 represent embryonic extracts from two independent Ubiquitin-Flag-*fbl* cDNA transgenic lines, revealing the presence of a single band migrating at an apparent molecular size of ~53,000, the predicted size for Flag-FumbleL protein. Lane 3 represents extract from nontransgenic embryos.

periments with several independent transformant lines for each construct. The cDNA that we have designated *fumble* fully rescued both the sterile and lethal phenotypes of the *fbl* mutations, whereas the cDNA corresponding to transcript 2 provided no detectable rescuing activity. Furthermore, Southern blot analysis of the *fbl^L* and *fbl^P* mutants revealed that each of these alleles is deleted for a portion of the genomic DNA encoding the cDNA capable of rescuing *fumble* (data not shown).

Analysis of Fumble protein sequence: Using a *fumble* testis cDNA as probe, two sizes of RNA were detected on a Northern blot (Figure 4C). These correspond to three splice variants of *fumble* that differ in their 5' exons as evident in the expressed-sequence-tagged database (www.fruitfly.org/index.html). We refer to the one long and two short *fumble* cDNA variants as *fblL*, *fblS1*, and *fblS2*. The *fblL* cDNA encodes a protein of 512 amino acids with a predicted molecular weight of 56,755. The *fblS1* and *fblS2* cDNAs encode proteins of 412 amino acids and 417 amino acids, respectively (Figure 4A). The *fblL* cDNA was used in subsequent experiments.

The *fbl* gene product contains a putative ATP binding site and other polypeptide motifs that are conserved among all eukaryotic pantothenate kinases (Pan K; CALDER *et al.* 1999). The striking sequence similarity between Fumble and eukaryotic PanK proteins strongly suggests that *fbl* encodes a functional *Drosophila* pantothenate kinase. We note, however, that the Fumble isoform for which we have demonstrated *in vivo* activity and characterized subcellular localization contains an N-terminal domain absent from other known PanK proteins. This additional sequence may specifically regulate Fumble's enzymatic activity or confer a novel role to this isoform. For instance, through these novel sequences, Fumble could directly regulate some of the cell division processes that are affected in *fbl* mutants.

The domain common to all Fumble isoforms is homologous to the characterized PanK of *A. nidulans*, *Saccharomyces cerevisiae*, and *Mus musculus* (Figure 5; CALDER

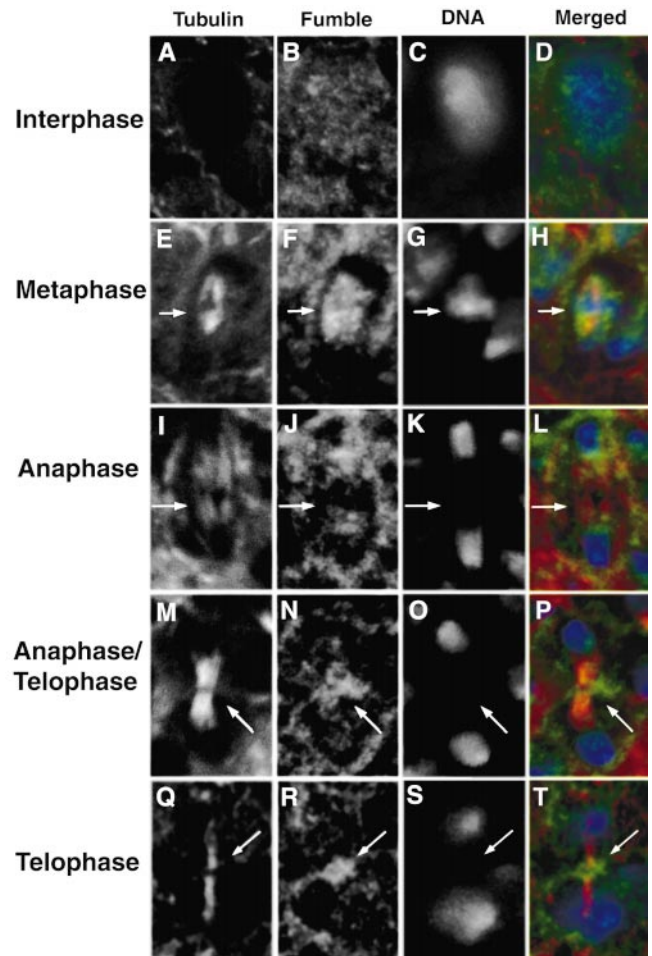


FIGURE 7.—Immunolocalization of Fumble during embryonic cell division. Wild-type embryos expressing Flag-Fumble protein were stained with antitubulin antibody (A, E, I, M, O), anti-Flag antibody (B, F, J, N, R), and DAPI (C, G, K, O, S) to detect microtubules, Fumble, and chromosomes, respectively. In D, H, L, P, and T the microtubule staining is shown in red, Fumble staining in green, and chromosomal staining in blue. (A–D) Cell at the interphase stage of the cell cycle, as evident by diffused DAPI staining (C), revealing dispersed Fumble staining at the cytoplasm. (E–H) Mitotic chromosomes are aligned at the midzone of a microtubule spindle at metaphase of cell division. Fumble staining is apparent and concentrated around the spindle apparatus (arrow in F). (I–L) Dividing cell undergoing anaphase. (M–P) Cell at late anaphase/early telophase, evident by the presence of a central spindle. Note that Fumble staining is concentrated primarily toward the midzone (arrow in N). (Q–T) Cell at late telophase, evident from the constricted central spindle. Fumble staining is restricted to the periphery of the cell and is more concentrated at the midzone (arrow in R).

et al. 1999; ROCK *et al.* 2000), as well as closely related gene products in worms, humans, and plants. In contrast, the amino-terminal extension found only in the longer isoform lacks sequence similarity to other pantothenate kinases or any other proteins in the GenBank database. Searches with both the Fumble and mouse PanK sequences indicate that *fumble* encodes the sole *Drosophila* PanK protein.

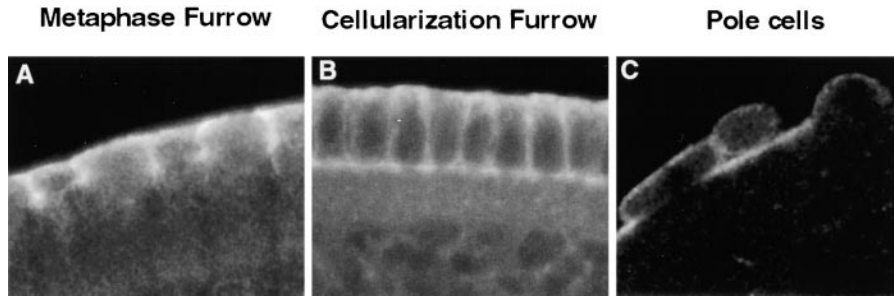


FIGURE 8.—Fumple staining in early embryonic development. Wild-type embryos expressing Flag-Fumple protein were stained with anti-Flag antibody. Fumple staining appears at (A) the metaphase furrow during embryonic nuclear division, (B) the cellularization furrow, and (C) the base of nascent pole cells.

Immunolocalization of Fumple: A Flag-epitope was engineered into the *fumple* cDNA construct used in the phenotypic rescue experiments (see above). We used an anti-Flag antibody to probe protein extracts from embryos transgenic for this construct by immunoblot analysis and detected a single species of the size predicted for the largest Fumple isoform (Figure 6). We then used the anti-Flag antibody to characterize the subcellular immunolocalization of this Fumple species.

We examined Fumple staining during the mitotic divisions of the cellular blastoderm. High expression of the *fumple* transcript during the embryonic stage suggests a functional requirement for *fumple* embryos (Figure 4B). In addition, germline clones of the *fbl* mutants do not survive, indicating a requirement for Fumple during oogenesis or embryogenesis (data not shown). Fumple localization during the cell cycle is dynamic. During interphase, Fumple localizes in the cytoplasm, with defined staining at the plasma membrane (Figure 7, A–D). At metaphase and early anaphase, Fumple becomes concentrated around the spindle (Figure 7, E–L). At late anaphase and early telophase, Fumple is concentrated at the cleavage furrow (Figure 7, M–T). At late telophase, Fumple is concentrated at the spindle midzone, the site of membrane addition during the last step of cytokinesis. We could not assess Fumple distribution during spermatogenesis because of cross-reactivity of the anti-Flag antibody with cellular structures in testes.

Fumple localization during early embryonic development exhibited patterns that corresponded to the active site of membrane addition. During the syncytial nuclear division, Fumple localized predominantly to the metaphase furrow, where a transient ingression membrane appears between the dividing nuclei (Figure 8A). During cellularization, Fumple staining appears at the cellularization furrow, where membrane addition between the nuclei creates individual cells (Figure 8B). Fumple also concentrates at the base of pole cells, where cytokinesis events separate the pole cells from the rest of the embryo (Figure 8C).

DISCUSSION

Inactivation of *fumple* causes a global defect during cell division: We have shown here that cells deficient for *fumple* function are defective for cytokinesis and

chromosome segregation. At the subcellular level, we find that these macroscopic effects are due to striking alterations in several key aspects of spindle organization: aster assembly, aster separation, and central spindle formation. We further find that in the wild type Fumple accumulates around the spindle and at the mid-body, suggesting that its activity is coupled directly to the function of these cellular organelles. Surprisingly, *fumple* encodes a fly homologue of pantothenate kinase, the first enzyme in the biosynthetic pathway for production of Coenzyme A.

How would depletion of Fumple (PanK) result in global defects in cell division? It has been reported that protein synthesis is severely affected in response to reduced cellular levels of Coenzyme A (JACKOWSKI and ROCK 1986). Thus, it is possible that the defects we observe in *fumple* mutants are a consequence of a reduction in protein synthesis. This might most easily explain why the strongest mutants have defects in spermatid differentiation, which relies on extensive protein synthesis. Similarly, defects in protein synthesis might affect mitosis in larval neuroblast cells, once stores of maternally synthesized proteins are depleted. Such a model is also consistent with the observation that a mutation in the *Drosophila* translation factor *sup35p* results in defects similar to those reported here for spindle organization in spermatocytes (BASU *et al.* 1998). However, defects in protein synthesis are unlikely to be the cause of most of the effects we observe in *fumple*-deficient cells, because flies harboring so-called “Minute” mutations, which drastically reduce protein synthesis, do not show phenotypes similar to *fumple*. Furthermore, the viable *fumple* alleles that we have isolated show neither the reduced body size nor the bristle shape alterations characteristic of Minutes. We therefore suggest that a protein synthesis decrease is unlikely to be the reason for the division defects observed in *fbl* mutant cells.

We favor a hypothesis that explains the cell division phenotype of *fbl* based on the requirement for Coenzyme A in the production of phospholipids (SONG and JACKOWSKI 1992). In this model, abnormalities in *fbl* mutant cells are consequences of defects in membranous structures during cell division. Strong, albeit circumstantial, evidence for this idea comes from the localization of epitope-tagged Fumple protein during the division cycle. Flag-Fumple concentrates around the mi-

otic apparatus at metaphase and the site of the cleavage furrow during anaphase and telophase. It also localizes to metaphase and cellularization furrows, where vesicle fusion and extensive membrane synthesis are taking place. This dynamic localization profile, the first known for a eukaryotic PanK enzyme, suggests a targeted role for this protein at sites requiring membrane synthesis or reorganization.

Membranous structures comprise a significant part of the mitotic apparatus and undergo major transformation and redistribution during mitosis (reviewed in HEPLER 1989). The endoplasmic reticulum (ER) membrane system is associated with the microtubule spindle and spindle poles and is thought to regulate the formation and function of the spindle fibers by controlling the local concentration of Ca^{++} . In some cells, such as *Drosophila* spermatocytes, laminar ER has been shown to encase the spindle apparatus, separating it from the rest of the cytoplasm (CHURCH and LIN 1982). The nuclear envelope, another membranous structure, also appears to participate in the organization of the mitotic spindle in animal cells, as evidenced by positioning and separation of the centrosomes within the grooves of the nuclear envelope (NADEZHINA *et al.* 1979).

The function of membranous structures is particularly important for the completion of cell division; the dynamic redistribution of the plasma and vesicle membranes is essential for cytokinesis. During cytokinesis new plasma membrane is added at the cleavage plane. There is growing evidence that the formation, targeting, and fusion of membrane vesicles to the cleavage furrow are the mechanisms for the completion of cytokinesis. In this respect, the central spindle and the contractile ring serve as structures to direct and arrange membrane addition (reviewed in HALES *et al.* 1999).

Considering the importance of membranous structures during cell division one can imagine that depletion of phospholipids, the basic structural component of membranes, in dividing cells would ultimately affect the integrity of the mitotic apparatus and cytokinesis. Compatible with this view, it has been shown recently that alterations in lipid composition affect cytokinesis. In particular, changes in the level of the lipid metabolite psychosine (galactosyl-sphingosine) interfere not only with the function of plasma membrane microdomains but also with the execution of cytokinesis (KANAZAWA *et al.* 2000). Similarly, the cellular level of the membrane phospholipid phosphatidylethanolamine is critical for completion of cytokinesis (UMEDA and EMOTO 1999; EMOTO and UMEDA 2000). Therefore, since depletion of *fumble* could alter phospholipid biosynthesis, it is easy to imagine how this would severely affect cytokinesis. Indeed, several studies have pointed to the presence of control mechanisms for coordination between phospholipid metabolism and cell cycle events (reviewed in JACKOWSKI 1996).

***fbl* phenotype during male meiotic divisions:** In most eukaryotic cells, bipolar attachment of sister chromatid spindle microtubules creates tension at the kinetochores. This tension is monitored by the spindle assembly checkpoint that regulates the metaphase to anaphase transition (reviewed in SORGER *et al.* 1997). The mechanism by which defects in spindle assembly feedback to the cell cycle oscillator is not clear, although several components in the signaling pathway, such as BUB1 and MAD1, have been identified in yeast (reviewed in WELLS 1996). The spindle assembly checkpoint appears to be present in at least some meiotic cells. For instance, in male meiosis in mantids, the presence of a chromosome attached to only one spindle pole delays anaphase (LI and NICKLAS 1996). However, the existence of a tension-mediated mechanism for exit from metaphase in *Drosophila* spermatogenesis has been questioned (CHURCH and LIN 1982). Although homologs of the conserved checkpoint proteins BUB1 and MAD1 localize as expected during *Drosophila* spermatogenesis, the presence of a chromosome attached to only one spindle pole does not delay cell cycle progression (BASU *et al.* 1999). In addition, analysis of the dividing neuroblast cells in the *asterless* mutants indicated that chromosome segregation can proceed in the absence of a conventional spindle structure (BONACCORSI *et al.* 2000).

The phenotype of *fbl* spermatids presents another example of cell cycle progression in the absence of tension on chromosomes. In *fbl* spermatids we detected defects in both aster separation and bipolar spindle assembly. As a consequence, chromosomes were probably not associated properly with the spindle. The nuclear division cycle nonetheless proceeded, resulting in chromosome missegregation, as evidenced by the appearance of small, variably sized postmeiotic nuclei in spermatids. Cytokinesis also proceeded with formation of an acto-myosin ring in the absence of chromosome segregation (Figure 3F) or aster separation (Figure 3H). These results indicate that, in *fbl*-deficient cells, the absence of a bipolar spindle did not lead to metaphase arrest. It remains possible that the cells were delayed in progression to the subsequent stages of cell division by initiation of a tension-mediated checkpoint.

Not only proper spindle assembly, but also aster separation, appears dispensable for formation of the central spindle and contractile ring in *Drosophila* spermatids (see Figure 3H). The central spindle is thought to be formed from interdigitation of microtubules projected from the opposite poles (reviewed in FIELD *et al.* 1999). In some *fbl* spermatocytes, however, we observe chromosome segregation on a bipolar structure despite a failure in aster separation. These observations are not without precedent, as previous studies have shown that kinetochores and chromosomes can function as a spindle organization center and that a bipolar spindle can be formed

in the absence of a spindle pole (CHURCH *et al.* 1986; DEBEC and ABBADIE 1989). It is possible that in *fbl* spermatocytes chromosomes can play a role in organization of a bipolar spindle and central spindle in the absence of aster separation. Nevertheless, we cannot exclude the possibility that a microtubule-organizing center is present at the anastral (asterless) pole of the spindle.

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