

The Kinesin-like Protein KLP61F Is Essential for Mitosis in *Drosophila*

Margarete M. S. Heck,*[‡] Andrea Pereira,[§] Patty Pesavento,[§] Yvonne Yannoni,[§] Allan C. Spradling,[‡] and Lawrence S. B. Goldstein[§]

*Johns Hopkins University, School of Medicine, Department of Cell Biology and Anatomy, Baltimore, Maryland 21205; [‡]Howard Hughes Medical Institute Research Laboratories, Carnegie Institution of Washington, Department of Embryology, Baltimore, Maryland 21210; and [§]Harvard University, Department of Cellular and Developmental Biology, Cambridge, Massachusetts 02138

Abstract. We report here that disruption of a recently discovered kinesin-like protein in *Drosophila melanogaster*, KLP61F, results in a mitotic mutation lethal to the organism. We show that in the absence of KLP61F function, spindle poles fail to separate, resulting in the formation of monopolar mitotic spindles. The resulting phenotype of metaphase arrest with polyploid cells is reminiscent of that seen in the fungal *bimC* and *cut7* mutations, where it has also been shown that spindle

pole bodies are not segregated. KLP61F is specifically expressed in proliferating tissues during embryonic and larval development, consistent with a primary role in cell division. The structural and functional homology of the KLP61F, *bimC*, *cut7*, and *Eg5* kinesin-like proteins demonstrates the existence of a conserved family of kinesin-like molecules important for spindle pole separation and mitotic spindle dynamics.

THE existence of microtubule-dependent force generating molecules has been known for nearly thirty years (reviewed in Vallee and Shpetner, 1990). The intrinsic polarity of the microtubule suggests there should be two classes of molecules capable of transducing force in either direction along the fiber. In general, dyneins move organelles along microtubules in the minus-end direction, whereas kinesins have been implicated in plus end-directed movement (reviewed in Endow, 1991; Goldstein, 1991; McIntosh and Pfarr, 1991; Sawin and Scholey, 1991; Vallee, 1991). The matriarch of the kinesin superfamily (kinesin) was discovered in squid axoplasm and as such, is likely to function in axonal transport (Allen et al., 1985; Brady, 1985; Vale et al., 1985). As expected for this role, mutation of the kinesin heavy chain in *Drosophila melanogaster* results in lethality with associated disruption of neuromuscular function (Gho et al., 1992; Saxton et al., 1991).

Since the initial identification of the kinesin heavy chain, a number of studies have led to the conclusion that a superfamily of kinesin-like proteins (KLPs)¹ plays diverse roles in cellular functions in all single- and multi-cellular eukaryotes examined to date (reviewed in Endow, 1991; Goldstein, 1991). These KLPs all share homology within the motor domain of the protein which is involved in ATP hydrolysis,

microtubule binding, and force generation. Two PCR-based screens (using primers to conserved sequences within the mechanochemical region) in *Drosophila melanogaster* have identified six, and probably more, genes encoding potential KLPs (Endow and Hatsumi, 1991; Stewart et al., 1991). Functional analysis is incomplete at best, and awaits the discovery of mutations in these putative KLP genes.

In addition to axonal transport, what other cellular processes may require microtubule-based motility? In the cell, the minus-ends of microtubules are embedded in the centrosome or microtubule organizing center, while the plus ends extend into the cytoplasm. The most dramatic cellular rearrangements occur during cell division. A mitotic spindle is first constructed from the duplicated centrosomes and the disassembled interphase microtubule array, and then used to segregate sister chromatids and separate spindle poles during cell division. One would expect these rearrangements to use molecules capable of generating directed movements of centrosomes, of chromosomes along microtubules, and of microtubules with respect to one another.

Key breakthroughs in the analysis of mitotic spindle dynamics have resulted from genetic analyses in fungal systems. Kinesin-like molecules have been implicated in the proper assembly and maintenance of the mitotic spindle (Enos and Morris, 1990; Hagan and Yanagida, 1990, 1992; Hoyt et al., 1992; O'Connell et al., 1993; Roof et al., 1992; Saunders and Hoyt, 1992). In *Aspergillus nidulans*, the *bimC* KLP is necessary for the separation of duplicated spindle pole bodies (Enos and Morris, 1990). Similarly, alteration of the kinesin-like *cut7* protein in *Schizosaccharomyces pombe* also blocks spindle formation, again owing to the inability of spindle pole bodies to separate (Hagan and Yana-

Margarete Heck's present address is Johns Hopkins University, School of Medicine, Department of Cell Biology and Anatomy, 725 Wolfe Street, Baltimore, MD 21205.

Yvonne Yannoni's present address is Brandeis University, Department of Biology, 415 South Street, Waltham, Massachusetts 02154.

1. Abbreviations used in this paper: KLP, kinesin-like proteins; NGS, normal goat serum.

gida, 1990). The resulting phenotype in both these cases is that polyploid cells arise due to repeated DNA replication in the absence of chromosome segregation and cell division. In *Saccharomyces cerevisiae*, the CIN8 and KIP1 gene products appear to be redundant with one another, performing functions similar to that required of the bimC and cut7 KLPs (Hoyt et al., 1992; Roof et al., 1992). CIN8 and KIP1 additionally appear to be required for maintenance of an established spindle; assembled preanaphase bipolar spindles collapse when these both of these molecules are inactivated (Saunders and Hoyt, 1992). The recent discovery that mutations in the KAR3 gene, also encoding a KLP, can suppress the defects in CIN8 and KIP1 mutants, and the discovery that *k1pA* can act as a suppressor of *bimC* in *A. nidulans* emphasizes the interplay among these KLPs in spindle dynamics (O'Connell et al., 1993).

Potentially analogous molecules have also been identified in nonfungal systems. The *Xenopus* Eg5 gene encodes a kinesin-like protein that acts as a plus end-directed motor, and appears to be important for bipolar spindle assembly in vitro (Le Guellec et al., 1991; Sawin et al., 1992). Strikingly, the bimC, cut7, CIN8, KIP1, and Eg5 proteins all have motor domain sequences more similar to each other than to other members of the kinesin superfamily (Hoyt et al., 1992; Roof et al., 1992), suggesting that they may form a subfamily of related proteins and functions. Two KLPs have additionally been identified in human tissue culture cells. The CENP-E centromere protein is a large (312 kD) KLP that appears to be required for the progression from metaphase to anaphase (Yen et al., 1991, 1992). In addition, human MKLP1 may be involved in sliding anti-parallel microtubules during spindle elongation (Nislow et al., 1992).

Although two *Drosophila* KLPs whose function had been analyzed genetically in *Drosophila*, *ncd* (nonclaret disjunctional) and *nod* (no distributive disjunction), appeared to play a role in meiotic chromosome segregation and spindle organization (Endow et al., 1990; McDonald and Goldstein, 1990; Zhang et al., 1990), these and kinesin heavy chain mutants exhibited no obvious defects in mitosis. In addition, whether any of the many *Drosophila* KLPs identified by PCR played a role in mitosis, analogous to that played by the KLPs discovered genetically in fungi, was unclear. We report here that a lethal mitotic mutation in *Drosophila* is a result of a transposon insertion in one of the KLPs identified molecularly. This KLP, KLP61F, bears a striking structural and functional resemblance to the bimC/cut7/Eg5 family of KLP genes, and thus clearly demonstrates the existence of a highly conserved family of KLPs important for mitotic spindle dynamics.

Materials and Methods

P Lethal Insertion Lines

The mutants described in this paper were derived from a large scale (22,000 crosses) single P element mutagenesis screen previously carried out in 1989 (described in (Karpen and Spradling, 1992)). The 15-kb mutator P element (PZ) carried the *Drosophila rosy*⁺ gene (allowing for eye color selection), the bacterial β -galactosidase (*lacZ*) gene (serving as a reporter gene), and a bacterial origin of replication and kanamycin resistance gene (Mlodzik and Hiromi, 1992). All 7,825 independent insertions of the PZ element were homozygosed and characterized with respect to phenotype (e.g., lethality, female sterility, male sterility). 958 lethal insertion lines on chromosome II or III were balanced and formed the collection used in these experiments.

Identification of Mitotic Mutants

555 chromosome III lethal insertions were rebalanced over TM6B in order to use the dominant Tubby larval marker (Tb). Larvae homozygous for the PZ insertion were Tb⁺, and therefore could be distinguished from their heterozygous Tb siblings. To determine the time of lethality during development, the viability of Tb⁺ homozygous larvae was determined in the culture vials. 140 of the 555 third chromosome lethals were found to die late in larval or pupal development. These late lethals were subsequently studied to ascertain the status of mitosis. Third instar homozygous larvae from each late lethal stock were selected and washed of adherent food in EBR (129 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 10 mM Hepes, pH 6.9). Brains and imaginal discs were dissected and fixed in 45% acetic acid for 3 min at room temperature. Brains were squashed in 45% acetic acid by pressure between a microscope slide and a siliconized glass coverslip. The preparation was frozen on dry ice for 10 min. The coverslip was then flicked off with a razor blade and the slide rehydrated in PBS (150 mM NaCl, 10 mM NaH₂PO₄, pH 7.2) to remove the acetic acid. Slides were then stained for 10 min in PBS containing 0.05% TX-100 and 0.1 μ g/ml DAPI (4',6-diamidino-2-phenylindole). After rinsing in PBS, the preparations were sealed under coverslips in Mowiol (Calbiochem-Behring Corp., San Diego, CA). Neuroblast spreads were subsequently examined by fluorescence microscopy and photographed on a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Excision of the PZ element to test for phenotypic reversion was conducted by crossing the P lethal lines to the P[Δ 2-3]99B transposase source (Robertson et al., 1988) as described previously (Cooley et al., 1988). Sequences flanking a PZ element insertion site were cloned by virtue of the bacterial origin of replication and Kanamycin resistance gene engineered into the P element also as published (Cooley et al., 1988).

Immunofluorescence on Neuroblast Squashes

Third instar homozygous (Tb⁺) and heterozygous sibling (Tb) larvae were washed of adherent food in EBR. Brains and imaginal discs were dissected and incubated in PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, pH 6.8) until the required number of brains were collected. No more than three brains were then gently squashed in PHEM containing 4% paraformaldehyde (EM Science, Gibbstown, NJ) between a Con A-treated glass cover slip (Heck and Earnshaw, 1986) and a square of Parafilm. Preparations were fixed in this sandwich for 6 min at room temperature. The parafilm was gently peeled off and the cover slip placed (cells up) in one well of a six-well multi-dish containing KB- (150 mM NaCl, 10 mM Tris, pH 7.7, 0.1% BSA). When all preparations were fixed, the cells were permeabilized in KB (KB- with 0.1% TX-100) for 10 min (with one change of solution). After a rinse in KB-, primary antibody was added for 30 min at 37°C (mouse monoclonal anti- β tubulin; Amersham Corp., Arlington Heights, IL; rabbit polyclonal anti- γ tubulin; generous gift of Dr. Harish Joshi, Emory University, Atlanta, GA). A moistened paper towel on the inside of the top of the dish prevented evaporation. After three washes in KB- for 2, 5, and 3 min, secondary antibody was added for 30 min at 37°C (FITC-conjugated goat anti-mouse; Cappel Laboratories, Cochranville, PA; biotinylated goat anti-rabbit; Vector Laboratories, Burlingame, CA). After three more washes in KB- for 2, 5, and 3 min, cover slips were incubated in Streptavidin/Texas red (GIBCO-BRL, Gaithersburg, MD) for 30 min at 37°C. Coverslips were then washed for 2 min in KB-, 5 min in KB- containing 0.5 μ g/ml DAPI, and then in KB- two more times for 3-min each. Coverslips were mounted in Slowfade (Molecular Probes). Digital images were acquired directly from an Olympus Vanox microscope equipped with a DAGE-MTI SIT-66 camera (detailed in Mackay et al., 1993).

Isolation and Sequencing of KLP61F cDNAs

The KLP61F gene was identified in a PCR-based screen (using primers to conserved regions of the kinesin-like motor domain) for new members of the kinesin superfamily (Stewart et al., 1991), and was initially named KLP2 (the new name reflects the genomic position). Four KLP61F cDNA clones were isolated from a 0-4 hour *Drosophila* cDNA library (Brown and Kafatos, 1988) using a small genomic fragment of the KLP61F gene as a probe. The largest two of these were subcloned into pBlueScript and sequenced using standard methods. The longer clone was 3,709 nucleotides in length and terminated with a long polyA tract; the shorter one was truncated at the 5' end relative to the longer clone (beginning at nucleotide 207) and also ended with a long polyA tract. The longest clone was sequenced on both strands completely. The noncoding strand was sequenced using exo-

nuclease III generated deletions, the coding strand was sequenced using newly synthesized oligonucleotides at intervals of approximately 200 nucleotides. The sequence of this clone is presented in Fig. 1; it is the one analyzed in the text. The shorter clone was sequenced on only one strand using the oligonucleotides. We found 20 differences in nucleotide sequence between the two clones, only three of which change the predicted protein sequence. Relative to the sequence presented in Fig. 1, these three differences are G at 2081 (M595 to V), A at 2904 (R869 to K), and G at 3010 (H904 to Q). Given that the shorter clone is capable of rescuing the defects in KLP61F mutants, we currently regard it as the wild-type sequence, although the sequence changes in the longer clone are relatively conservative and may be natural polymorphisms.

P Element-mediated Germline Transformation

Construction of Pwum2. The Pwum2 vector allows transcription of a transgene in a constitutive, tissue-independent manner by placing it under the control of the *Drosophila* polyubiquitin promoter (Up) region (Lee et al., 1988). Contiguous with Up is a fragment encoding 12 amino acids of *c-myc* (Munro and Pelham, 1987) that can be used to immunolocalize or immunoprecipitate the protein of interest with an anti-*myc* antibody. This vector was constructed by taking a 2-kb BglII fragment containing the promoter and 5' leader sequence (Lee et al., 1988) and modifying it in pBlue-script so that the ubiquitin initiator methionine became part of an NcoI site. An NcoI-EcoRI fragment encoding the 12 amino acid *myc* epitope (Munro and Pelham, 1987) was then placed in frame with the ubiquitin initiator methionine. (There are also KpnI and NotI cloning sites between the *myc* and the EcoRI site; the sequence of this region is available upon request.) This final construct was then cloned into the BamHI-EcoRI sites of PW8 (Klemenz et al., 1987) to give Pwum2.

Construction and Transformation of Pwum2KLP61F. The shorter of the two KLP61F cDNA clones (described above) within the vector pNB40 (Brown and Kafatos, 1988) was digested with MunI (New England Biolabs, Beverly, MA) and the resulting 5' overhang was filled with Klenow. This MunI site is 12-bp upstream of the initiator methionine of KLP61F. After digestion with EcoRI, a 3.5-kb MunI-EcoRI fragment was isolated and ligated into PvuII- and EcoRI-digested pRsetB (Invitrogen) to give pRsetKLP61F. For construction of Pwum2KLP61F, pRsetKLP61F was digested with BamHI, filled with Klenow and digested with EcoRI; the resulting 3.5-kb fragment was then gel isolated. This fragment was then ligated into NotI-digested Pwum2, filled with Klenow, and subsequently digested with EcoRI. The resulting KLP61F fusion protein therefore contains 10 additional amino acids (encoded by the pRset vector) between the *myc* tag (see above) and the KLP61F initiator methionine. This 10 amino acid sequence is: DPSSRSAAGT. Pwum2KLP61F was transformed using the helper plasmid p π 25.7wc (Karess and Rubin, 1984) into the host *y* w strain. One *w*⁺ transformant was recovered from 100 G0's. This Pwum2KLP61F transgene maps to the second chromosome.

Detection of the β -galactosidase Reporter Gene in Larvae

The original mutator PZ element served as an enhancer trap because it carried the bacterial lacZ reporter gene under control of the weak P element promoter. Cellular enhancers have been shown to influence β -galactosidase expression, often mimicking the expression of the endogenous gene. In larvae, we detected β -galactosidase by either enzyme activity assays or immunolocalization of the polypeptide.

For activity assays, larvae were rinsed with EBR and pulled open at the mouth hooks to expose larval tissues. Preparations were fixed in 3.7% formaldehyde/0.1% glutaraldehyde in PBS for 10 min at room temperature. After three rinses for 5 min in PBS, preparations were rinsed in staining solution and then incubated in staining solution containing 0.27% X-Gal at 37°C. When staining was judged to be complete, preparations were rinsed twice in PBS, and then equilibrated in 70% glycerol in PBS. Desired tissues were dissected from the "whole mount" preparations and mounted in 70% glycerol in PBS. Preparations were observed using Nomarski optics and photographed on a Zeiss Axiophot microscope.

For immunodetection of lacZ polypeptide, larvae were similarly dissected, then fixed in 4% paraformaldehyde (EM Sciences) in PBS for 2 h at room temperature. Preparations were rinsed with at least six changes of PBS + 0.1% TX-100 (PTX) over 90 min, and then blocked for 2 h with 10% normal goat serum (NGS) in PTX. Preparations were incubated overnight at 4°C in mouse monoclonal anti- β -galactosidase (Promega Biotec, Madison, WI) in 3% normal goat serum/PTX. After washes in PTX (every 30 min for 4 h), preparations were blocked as above. Preparations were in-

cluded overnight at 4°C in HRP-conjugated goat anti-mouse (Jackson ImmunoResearch Labs, West Grove, PA) in 3% NGS/PTX. The next morning, preparations were washed in PTX (every 30 min for 4 h), then reacted with diaminobenzidine (0.4 mg/ml) and NiCl₂ (0.06%) in PBS containing 0.02% H₂O₂. When staining was complete, the reaction was stopped by washing in PTX. Tissues were equilibrated and mounted in 70% glycerol in PBS. Preparations were observed using Nomarski optics and photographed on a Zeiss Axiophot microscope.

Detection of the β -galactosidase Reporter Gene in Embryos

Mouse monoclonal anti- β -galactosidase, followed by HRP-conjugated goat anti-mouse was used to detect the β -galactosidase polypeptide in whole mount embryos as described in (Patel et al., 1989).

Analysis of RNA Expression in Embryos

In situ hybridization was performed according to (Tautz and Pfeifle, 1989) with modifications as follows.

Fixation. After devitelination, embryos were transferred to 100% ethanol, and treated with xylene. After a rinse in 1:1 ethanol/xylene, embryos were soaked in xylene for 2 h. Another rinse in ethanol/xylene was followed by dehydration in 100% ethanol, which was replaced with 100% methanol. Embryos were hydrated into PBT (PBS, 0.2% BSA, 0.1% TX-100) through a series of graded MeOH/PBT steps. Proteinase K treatment (0.05 μ g/ml in PBT, 4 min) was followed by several quick washes in PBT and finally, the second 5% formaldehyde treatment.

Hybridization. Embryos were washed with several changes of PBT before treatment with 1:1 PBT/hybridization solution (50% formamide, 5 \times SSC, 50 μ g/ml heparin, 0.1 mg/ml sonicated salmon sperm DNA, 0.1% Tween-20). Embryos were then placed into hybridization solution at 55°C. After 2 h, most of the hybridization solution was removed, and probe was added. The probe was hybridized for 2 d at 55°C.

Probe. A Boehringer kit was used to synthesize the digoxigenin-UTP riboprobe (Boehringer-Mannheim Biochemicals, Indianapolis, IN), following the instructions provided with the kit. Anti-sense digoxigenin-UTP-labeled riboprobe was prepared from a KLP61F cDNA insert in the PBS-SK vector. Probe was reduced in size by treatment at pH 10.2 and then precipitated before being added to hybridization buffer. Sense KLP61F digoxigenin-UTP riboprobe was used as a control for non-specific hybridization (results not shown).

Probe Detection. After probe incubation, embryos were washed in six changes of fresh hybridization solution for a total of 3 h at 55°C. Embryos were then brought to room temperature and rehydrated by graded steps into PBT before being washed several times in PBT alone. Pre-absorbed (4-h incubation with fixed unprobed embryos at room temperature) alkaline-phosphatase-conjugated anti-digoxigenin was added. After 2 h, embryos were washed with five changes of PBT and then transferred into reaction solution (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M MgCl₂, and 0.1% Tween-20). BCIP and NBT solutions, provided in the kit, were used at the suggested concentrations. Embryos were staged according to (Campos-Ortega and Hartenstein, 1985).

Results

Identification of a Gene Essential for Mitosis in Drosophila

To identify genes required for mitosis, we searched through lethal mutations for disruptions in normal mitotic progression. This screen was motivated by two features of *Drosophila* development. First, the components for the early embryonic cleavage divisions are maternally supplied. Therefore the maternal genotype determines the nature of early embryonic cleavage divisions. Second, larval growth proceeds largely as a consequence of an increase in cell size and not cell number. Endoreduplication of the genome in the absence of cell division results in the large polyploid cells that comprise the vast majority of larval tissues. Mitosis is only required in the nervous system, abdominal histoblasts, and in the developing imaginal discs that are to become the adult

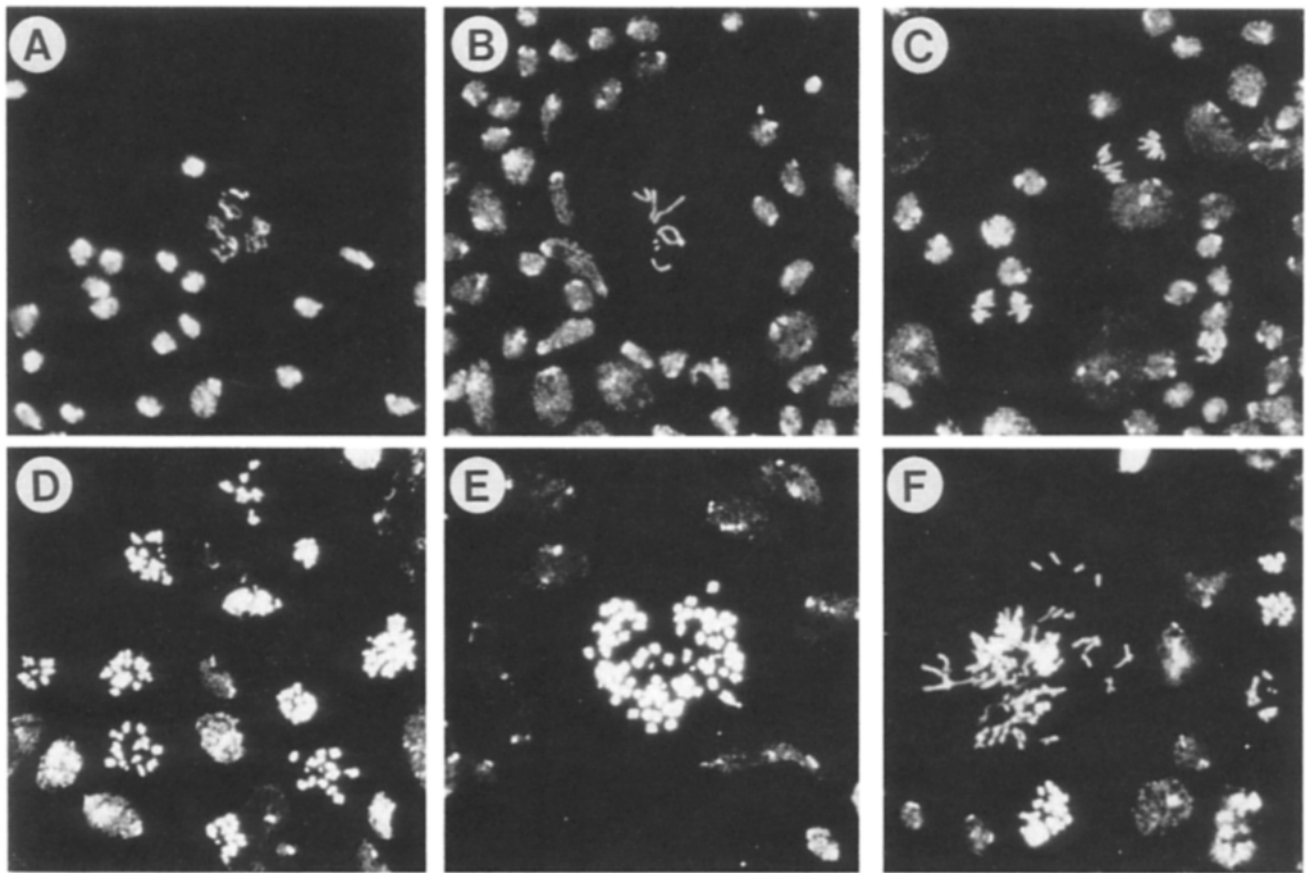


Figure 1. Wild type (A–C) and mutant (D–F) mitotic figures in neuroblast spreads. Brains isolated from crawling third instar larvae were fixed, squashed, and stained with the DNA-binding dye, DAPI. (A) Prophase, (B) metaphase, (C) anaphase figures from Oregon R (P2) larvae. (D–F) Three examples of mitotic figures from *KLP61F³* brains.

structures upon metamorphosis. In the absence of these tissues, the animal survives until it attempts to metamorphose. Gatti and Baker demonstrated previously that mutations in zygotically active genes encoding essential cell cycle functions in *Drosophila* cause death late in larval or pupal development (Gatti and Baker, 1989).

Because of the relative ease of cloning and identification of genes using transposon-tagged mutations, a collection of ~1,000 P element-induced autosomal lethal mutations served as our starting source of mutations (Karpen and Spradling, 1992). To determine which of the lethal mutations caused death late in larval or pupal development, all chromosome III lethal lines were rebalanced to take advantage of the dominant larval marker, Tubby. After rebalancing, heterozygotes were Tubby, while homozygotes for the P lethal chromosome appeared Tubby⁺ (or phenotypically normal). For 142 of the 555 (26%) chromosome III P lethals, dying Tubby⁺ larvae or pupae were observed in the culture vials, a value that corresponded well to the previously observed frequency for late larval lethality of 28% (Shearn, 1978). The other lethal lines died earlier in development—either as embryos or first or second instar larvae (the phase was not precisely determined). The 142 chromosome III late larval lethals then constituted the pool of mutations to be examined for mitotic phenotype. Since development of the nervous system is dependent on mitotic activity, dividing cells can be examined in larval brains.

To identify those lines with defects in mitosis, brains were isolated from homozygous larvae, and neuroblast spreads were prepared from them. Mitotic figures in neuroblast spreads were observed in the fluorescence microscope following staining of the chromatin with DAPI. Normal prophase, metaphase, and anaphase figures from a wild type culture are shown in Fig. 1 (A–C). Among 70 late lethal lines whose mitotic figures were initially examined, three showed the striking mitotic defect described below. The three lines failed to complement, and thus appeared to define a single genetic locus. Fig. 1 (D–F) shows three examples of mitotic figures from larvae homozygous for the *KLP61F³* allele. (By this assay, the phenotypes of the three alleles were indistinguishable.) The mutant phenotype is characterized by a metaphase arrest, as evidenced by an increased mitotic index (16.6% relative to 6.0% for wild type larval brains) and the complete absence of anaphase figures. One consequence of the metaphase arrest is the observed hypercondensation of the chromosomes as compared to wild type, similar to that seen when cells are blocked in mitosis with colchicine. Highly polyploid cells are also a common feature of the mutant phenotype. The observed polyploidy suggests that mutant cells can reenter the cell cycle to replicate the genome repeatedly without chromosome segregation and cytokinesis. Only normal mitotic figures were observed in neuroblast spreads from heterozygous sibling larvae (data not shown).

Polyploid cells could result because of a defective spindle

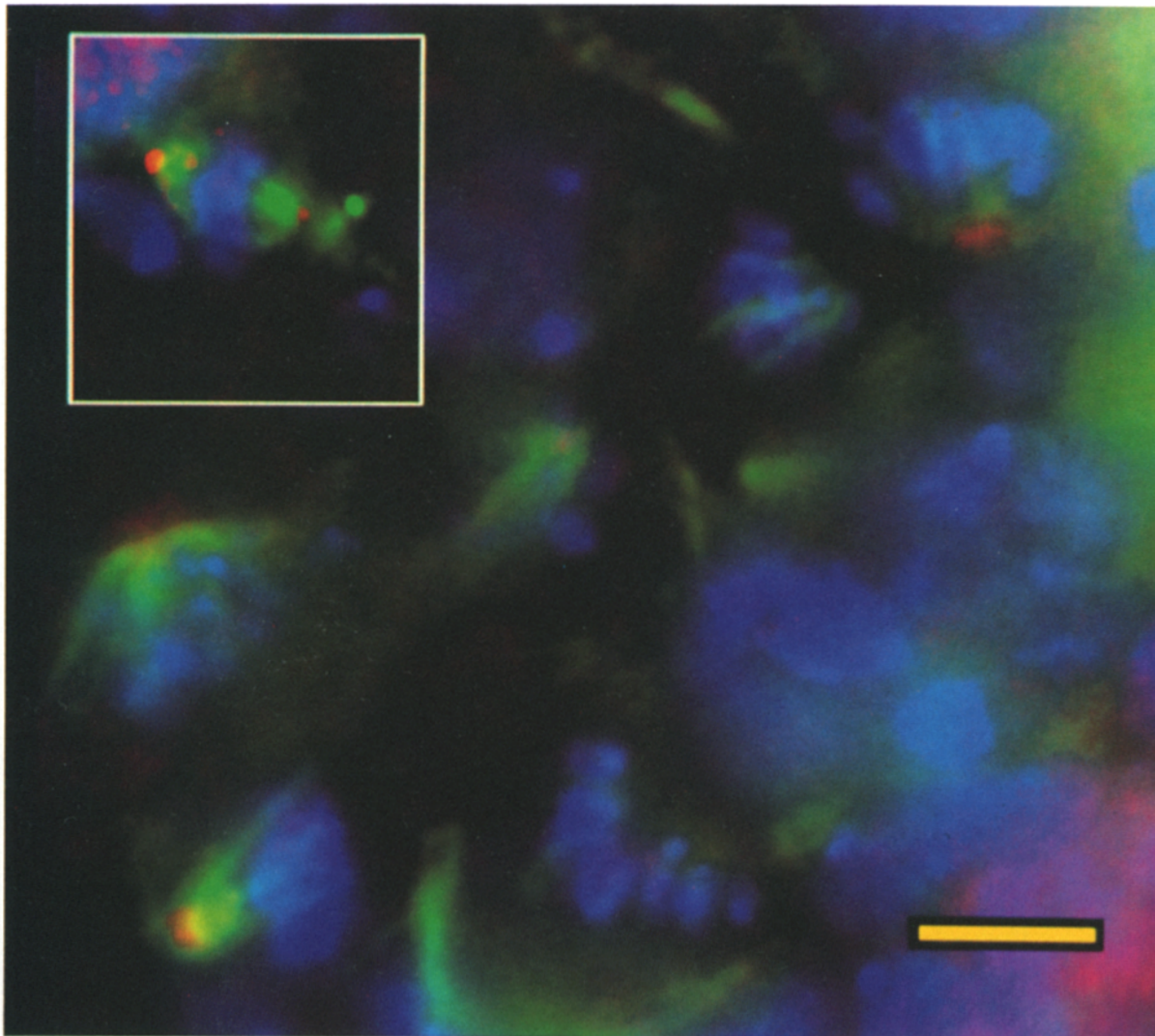


Figure 2. Immunofluorescent detection of mitotic spindles in neuroblast spreads from *KLP61F*³ heterozygous (normal) and homozygous (mutant) larvae. Fixed neuroblast spreads were reacted with antibodies to β - and γ -tubulin to visualize microtubules (*green*) and centrosomes (*red*), and counterstained with DAPI to visualize the chromatin (*blue*). The inset in the upper left shows a normal bipolar spindle from a heterozygous larva, while the large field shows monopolar spindles with unseparated centrosomes observable in homozygous larva. Bar, 5 μ m.

or because of defective kinetochore attachment to an otherwise normal spindle. Drs. Wilson and Fuller (personal communication) have observed that centrosomes fail to segregate in the *urchin* alleles of the *KLP61F* gene. We have independently confirmed this observation using our P insertion alleles. To directly observe the mitotic apparatus in this mutation, we performed immunofluorescent localization of both β - and γ -tubulin (to visualize microtubules and centrosomes), along with DAPI fluorescent visualization of the chromatin. In the inset of Fig. 2, a normal bipolar spindle (with two centrosomes) from a larva heterozygous for the *KLP61F*³ allele is shown. The large field of Fig. 2, on the other hand, shows six monopolar spindles observed in one neuroblast spread from a larva homozygous for *KLP61F*³. The localization of γ -tubulin to only one diffuse pole in the mutant suggests that duplicated centrosomes fail to separate, resulting in the formation of monopolar spindles. Because of the defective spindles, cells cannot segregate chromosomes

and complete cell division and thus become polyploid. From this result alone, we are unable to ascertain the structural integrity of the centrosomes. This phenotype is strikingly similar to that observed for mutations in the *bimC* and *cut7* KLPs in *A. nidulans* (Enos and Morris, 1990) and *S. pombe* (Hagan and Yanagida, 1990).

The PZ Insertions Are Localized at the Site of One of the Drosophila KLP Genes

To use the PZ element as a molecular tag to the flanking genomic DNA, we first needed to show that the PZ element caused the mutation. For all three PZ insertions, we found that excision of the PZ element was accompanied by reversion of lethality and the abnormal mitotic phenotype. Therefore, the PZ element caused these mutations. We then localized the site of PZ insertion by performing *in situ* hybridization to salivary gland polytene chromosomes. All

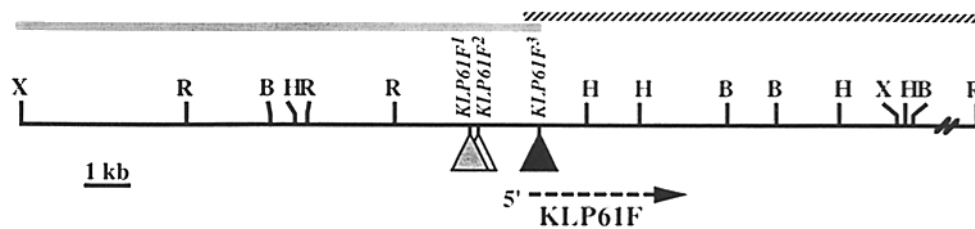


Figure 3. Genomic map of PZ insertions in the KLP61F gene. The three PZ insertions spanning 1.5 kb are depicted as triangles (the PZ insertion within the KLP61F transcription unit [dashed line] is shown as the solid triangle). Genomic DNA upstream of the KLP61F gene was cloned

by plasmid rescue, and is indicated by the shaded line on the left of the figure. The DNA downstream of, and including, the KLP61F gene was cloned by screening of a *Drosophila* genomic library, and is depicted by the striped line on the right of the figure.

three PZ elements were found to hybridize to 61F3-4, close to the distal tip of the left arm of chromosome III, near the previously reported site for one of the KLP genes (Stewart et al., 1991). That all three PZ elements hybridized to the same site provided further evidence that insertion of the PZ element caused the mutation.

Homozygous larvae were examined carefully for the phase of lethality. All three P element-induced alleles obtained in this screen die at the late third larval instar/early pupal stage. To determine if a more severe phenotype (i.e., earlier lethality) existed for mutations at this locus, stocks heterozygous for the PZ chromosome and a deficiency for this region (Df[3L]61C3-4;62A8) were created. The transheterozygotes also exhibited a late larval/early pupal phase of lethality that could not be distinguished from the original homozygotes. Thus, it appeared likely that the phenotype observed was representative of either a null, or severely hypomorphic allele. However, we have recently isolated six EMS-induced alleles that all have an earlier lethality, dying as embryos (Pereira, A., unpublished results). This raises the possibility that the P-insertion alleles are not nulls, and that this gene may function earlier in development. Therefore, in addition to being required for mitotic activity during larval development, this gene may also be required for embryonic cell divisions. This suggestion is supported by our expression data presented below.

The PZ Insertions Disrupt KLP61F Function

61F3-4 corresponded, interestingly, to the site for the KLP61F gene (KLP61F was originally named KLP2) (Stewart et al., 1991). Since the mitotic arrest phenotype we observed was reminiscent of the original fungal *bimC* and *cut7* mutant phenotypes, and since *bimC* and *cut7* had been shown to be KLPs, we wanted to determine whether our mutations were actually in the KLP61F gene. Genomic DNA flanking the PZ insertions were recovered by exploiting the fact that the PZ element contains a bacterial origin of replication and drug resistance marker. Digestion of genomic DNA containing the PZ insert, followed by ligation and transformation of bacteria to kanamycin resistance thus allowed the cloning of genomic sequences flanking the PZ element. Construction of a genomic restriction map surrounding the three sites of insertion showed that the three PZ elements were all independent insertions within a region spanning 1.5 kb (Fig. 3). The DNA immediately flanking the downstream-most PZ insertion (KLP61F³) was labeled on Southern blots of genomic DNA by a KLP61F cDNA probe. Analysis of the DNA sequence adjacent to KLP61F³ indicated that this in-

sertion indeed occurred within the 5'-untranslated region of the KLP61F transcription unit between nucleotides 62 and 63 of the sequenced cDNA (236 nucleotides upstream of the ATG at nucleotide 299; see arrowhead in Fig. 4).

These mapping data suggested that the mutant phenotype observed was due to disruption of the KLP61F gene. To rule out the possibility that a nearby gene was affected by the insertions, we used P element-mediated germline transformation to confirm that the mutated gene was in fact KLP61F. We generated a KLP61F minigene in which the KLP61F cDNA was placed under the control of the constitutive tissue-independent ubiquitin promoter. This minigene, Pwum2-KLP61F, was introduced into the germline by injection into embryos and transposase-mediated integration. Progeny bearing one copy of the transgene and two mutant alleles of the KLP61F gene developed as headless adults; the headless defect appeared to be due to the inability of the transgene to fully rescue eye-antennal imaginal disc proliferation. All of the other imaginal discs appeared to be fully rescued. Two copies of the Pwum2KLP61F transgene in a mutant background allowed normal head development with a low frequency of incomplete eye development. These animals were fertile and healthy. Our rescue data demonstrated that the mitotic arrest phenotype arose from disruption of the KLP61F gene.

The Sequence of KLP61F Reveals Striking Homology to the *bimC* Family of KLPs

A small fragment of the KLP61F gene was first identified in a search for genes encoding kinesin-like proteins in *Drosophila* (Stewart et al., 1991). To determine the complete primary sequence of the KLP61F protein, the original fragment was used as a probe to isolate two cDNA clones that were full-length for the protein coding region (Fig. 4A). Sequence analysis revealed that the longest clone was 3,709 nucleotides in length; one long open reading frame beginning at the first ATG at position 299 was found. This open reading frame predicted a protein of 1,066 amino acids in length, with a pI of 6.54. A second slightly shorter clone was sequenced on one strand; the predicted amino acid sequence was identical with the exception of three residues in the nonmotor region (M595 to V, R869 to K, H904 to Q). Searches of current data bases revealed no obvious sequence similarity to proteins other than already recognized members of the kinesin superfamily.

Further analysis of the KLP61F sequence revealed that it was composed of three domains. The first 354 amino acids shared significant similarity with the NH₂-terminal 331 amino acids within the head/motor region of *Drosophila*

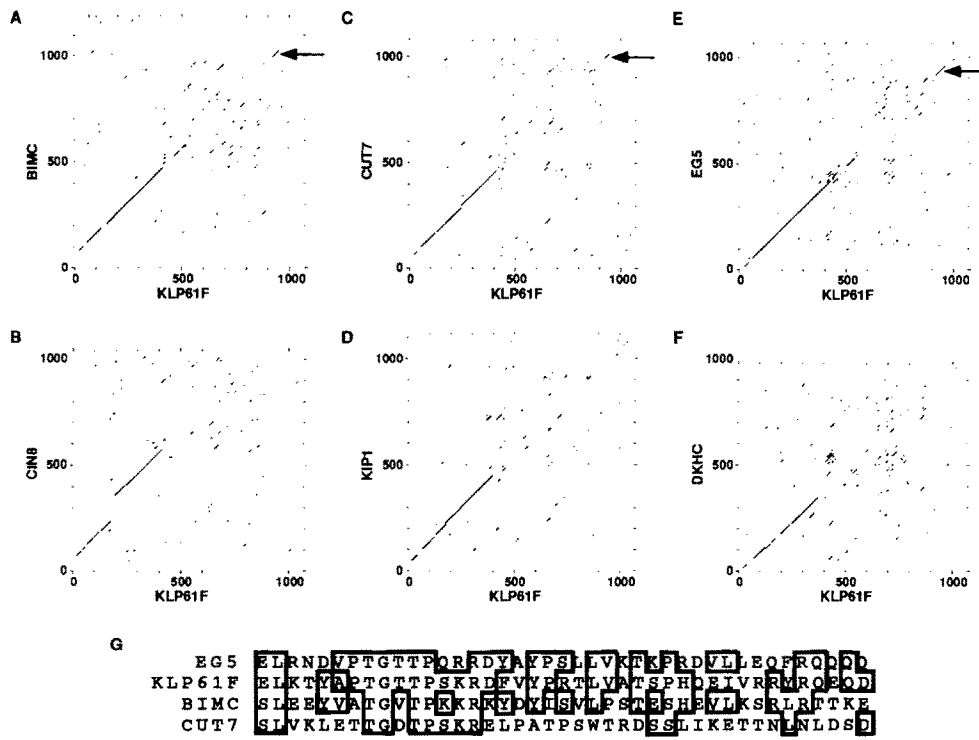


Figure 5. Dotplot comparison of KLP61F sequence to other kinesin superfamily members using the UWGCG programs COMPARE and DOTPLOT. Comparisons are to bimC (A), cut7 (B), Eg5 (C), CIN8 (D), KIP1 (E), and *Drosophila melanogaster* kinesin heavy chain (F). The horizontal arrows in A–C indicate a short region in the tail that is well conserved among these proteins. The region is aligned in G using the UWGCG program PILEUP. Residues identical between two or more proteins are boxed. The regions of sequence shown are amino acids 920–960 (EG5), 923–963 (KLP61F), 996–1036 (BIMC), and 989–1029 (CUT7).

determine if this region truly forms an α -helical coiled coil. Finally, there was a 111 amino acid tail region that may be globular, with a pI of 6.3.

Comparison of the KLP61F sequence to other kinesin superfamily members indicated that it was a member of the *bimC* family of kinesin-like proteins. First, sequence comparisons of two different nested regions of the presumptive KLP61F motor domain (either amino acids 94 to 323 or amino acids 19 to 347) to comparable regions of other kinesin superfamily members indicated that the motor region of KLP61F was most closely related to bimC, cut7, Eg5, CIN8, and KIP1, and rather more distantly related to all other known kinesin superfamily members. Second, KLP61F, bimC, cut7, and Eg5 all had a long central region predicted to be either α -helix or α -helical coiled coil (Enos and Morris, 1990; Hagan and Yanagida, 1990; Le Guellec et al., 1991). Third, KLP61F, bimC, cut7, and Eg5 all had a short region of shared sequence similarity within their tail domains (Fig. 5, arrows and alignment). This region of conserved sequence was centered around a conserved motif, TGXTPXX/RR, which could be a phosphorylation site for either the proline-dependent protein kinase (Vulliet et al., 1989) or kinases of the ERK family (Crews et al., 1992), or for an as yet unidentified kinase. These data, in combination with the genetic results presented in this report, indicate that

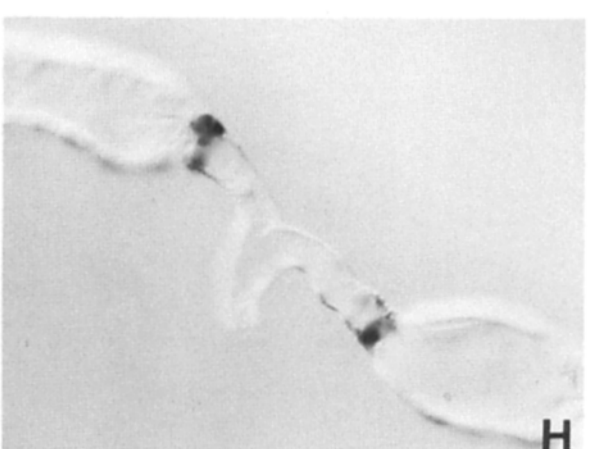
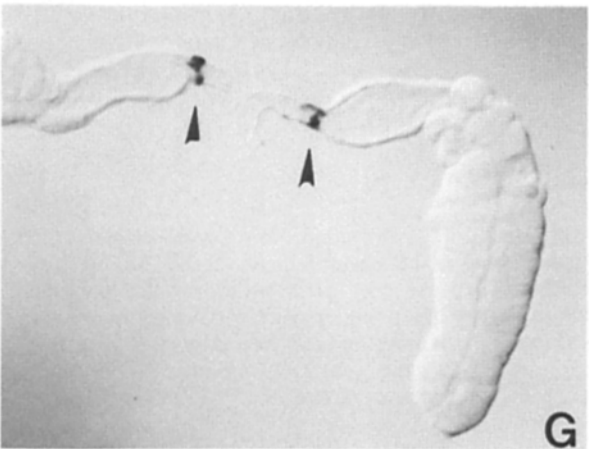
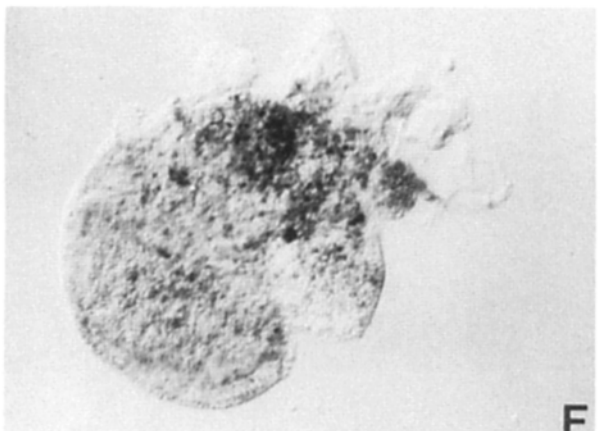
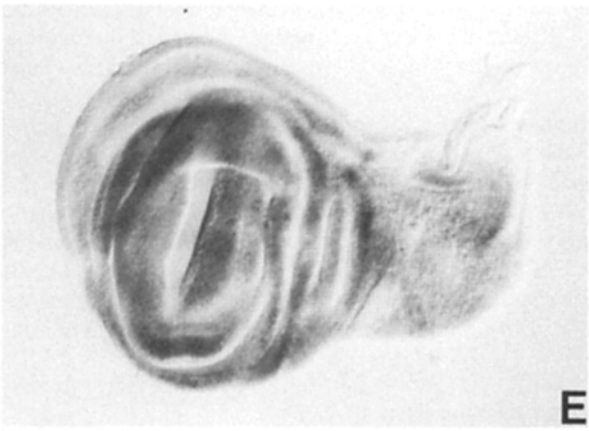
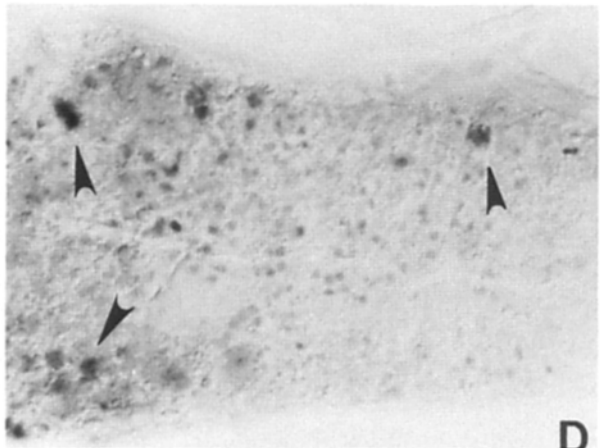
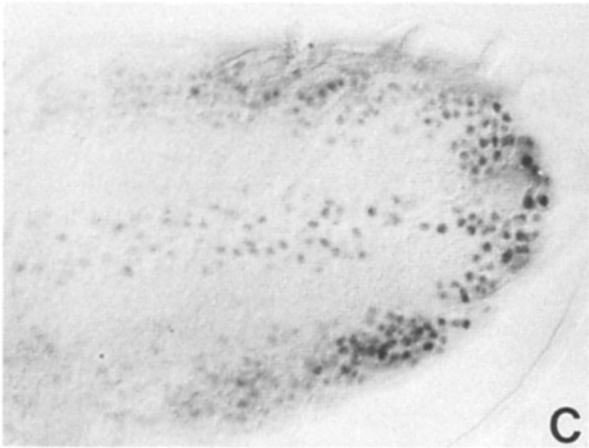
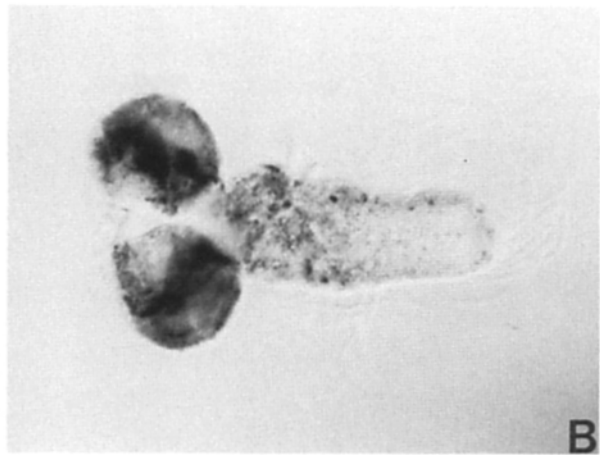
KLP61F is likely to be the *Drosophila* homologue of bimC, cut7, and Eg5.

KLP61F Is Expressed in and Required for Proper Development of Actively Proliferating Tissues

Larval Expression and Development. Transcriptional enhancers “trapped” by the PZ element control the expression of *lacZ*, frequently mimicking the expression of the endogenous gene (Wilson et al., 1989). As demonstrated above, the PZ element in *KLP61F³* was inserted in the 5-untranslated region of the KLP61F transcription unit. Therefore, the KLP61F promoter may be used to drive the expression of the β -galactosidase (*lacZ*) reporter gene within the PZ element. Nuclear localization of the *lacZ* polypeptide is observed (resulting from the translational fusion of P transposase sequences containing a nuclear localization signal with the *lacZ* sequences). We thus examined KLP61F expression by activity assays and by antibody detection of the *lacZ* polypeptide (antibody detection having the advantage of finer resolution).

Not unexpectedly, mitotically active tissues, such as the larval brains and imaginal discs (precursors of the adult tissues), exhibited *lacZ* expression (Fig. 6). In brains (Fig. 6, A–D), we observed expression both in the two optic lobes

Figure 6. Detection of the β -galactosidase reporter polypeptide in KLP61F³ heterozygous and homozygous larval brains and imaginal tissues. Crawling third instar larvae were isolated from culture vials, and the internal tissues were processed for detection of β -galactosidase immunologically (A–F) or by activity assay (G–H). Brains from larvae heterozygous (A and C) and homozygous (B and D) for the PZ insertions are shown. C and D are fourfold higher magnifications of the ventral ganglion; many larger, β -galactosidase-expressing nuclei are present in the homozygous brain (D, arrowheads). Wing discs from heterozygous (E) and homozygous (F) larvae are shown. A salivary gland from a larva homozygous for the KLP61F³ mutation is shown at two magnifications in G and H. The nondividing polytene cells of the salivary gland do not express the β -galactosidase reporter gene, whereas expression of the β -galactosidase gene is detected in the imaginal ring, the dividing precursors of the adult salivary gland (H, arrowheads).



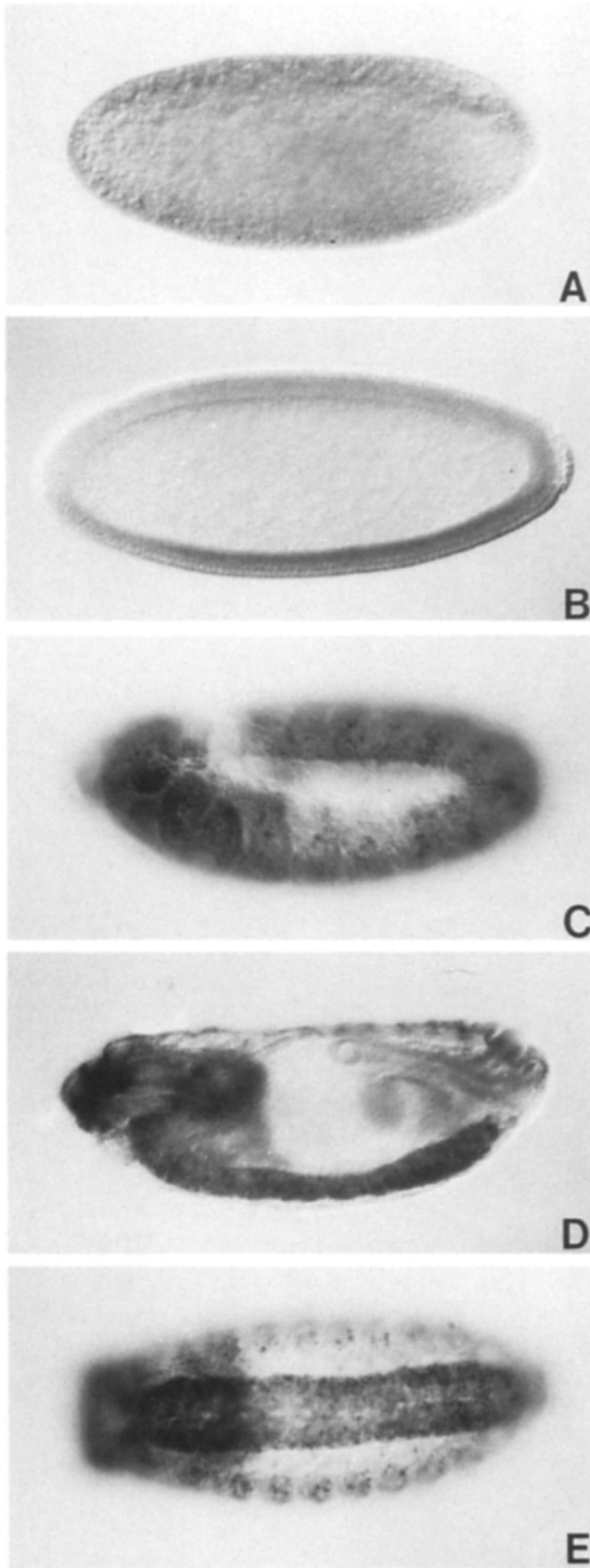


Figure 7. Immunodetection of the β -galactosidase reporter polypeptide in *KLP61F₃* embryos. Embryos at five sequential stages of development are shown. (A) Early cleavage (precellularization). (B) Syncytial blastoderm (pole cells at the posterior end have been

and in the ventral nerve cord. The nonrandom distribution of lacZ expressing cells in the wild type ventral nerve cord (Fig. 6, A and C) corresponded closely to the observed pattern of BUdR-incorporating cells (Truman and Bate, 1988). Thus it appeared that the lacZ-positive cells were cycling, as opposed to quiescent. We observed many large, more intensely stained nuclei in the homozygous brain that were not apparent in the heterozygous brain (Fig. 6 B and arrows in D). These most probably represent the large polyploid cells we previously noted in the DAPI-stained brain squashes (Fig. 1, D-F).

Though expression of the lacZ reporter gene was homogeneous throughout the normal wing disc in Fig. 6 E, clusters of large, intensely stained nuclei were also present in the disorganized imaginal discs from homozygous larvae (compare Fig. 6 E with F). With the exception of the eye imaginal disc in which we observed lacZ expression in and posterior to the morphogenetic furrow (thus correlating with the wave of mitosis; data not shown), all other wild type imaginal discs showed homogeneous expression of the lacZ reporter gene. In contrast, all polyploid tissues examined (e.g., salivary glands, the ring gland attached to the brain) were devoid of any lacZ expression (Fig. 6 G). The only detectable expression of lacZ in the salivary gland was in the imaginal ring (Fig. 6 H, arrow), a set of dividing, diploid cells destined to become the adult salivary gland.

Polyploid tissues (developing normally in the absence of cell division), such as the salivary glands and the ring glands, appeared normal (Fig. 6, G and H). The morphology of the polytene chromosomes by DAPI staining in these tissues appeared unaffected as well (data not shown). On the other hand, the size and morphology of mitotically active tissues in homozygous larvae was sensitive to disruption of the *KLP61F* gene. This was most easily visible in a direct comparison of brains from wild type heterozygous (Fig. 6 A) and mutant homozygous (Fig. 6 B) larvae. By measurement of the diameter of the two optic lobes and the ventral nerve cord, the size of the mutant brain was only 75–80% that of the wild type. However, upon conversion to volume (assuming spherical shape for the optic lobes and cylindrical shape for the ventral nerve cord), the volume of the homozygous brain was only 55% that of the heterozygous brain. Therefore, the loss of *KLP61F* function (and the ability to undergo cell division) significantly reduced the ability to form normal tissues.

Embryonic Expression of *KLP61F*

In earlier Northern blotting studies, *Drosophila* embryos were shown to contain a pool of maternally deposited *KLP61F* mRNA (Stewart et al., 1991). We analyzed the expression of the *KLP61F* gene in wild type embryos both by immunolocalization of the lacZ polypeptide (Fig. 7) and by

pinched off, but cellularization has not yet been completed). (C) Gastrulating, germband extended embryo. (D and E) Gastrulating, germband-retracted embryo. All views (except E) are lateral, shown with anterior-left, posterior-right, dorsal-top, ventral-down. (E) A ventral view of germband-retracted embryo. No expression of the β -galactosidase gene is observed until gastrulation, with expression being most prominent in the central and peripheral nervous system.

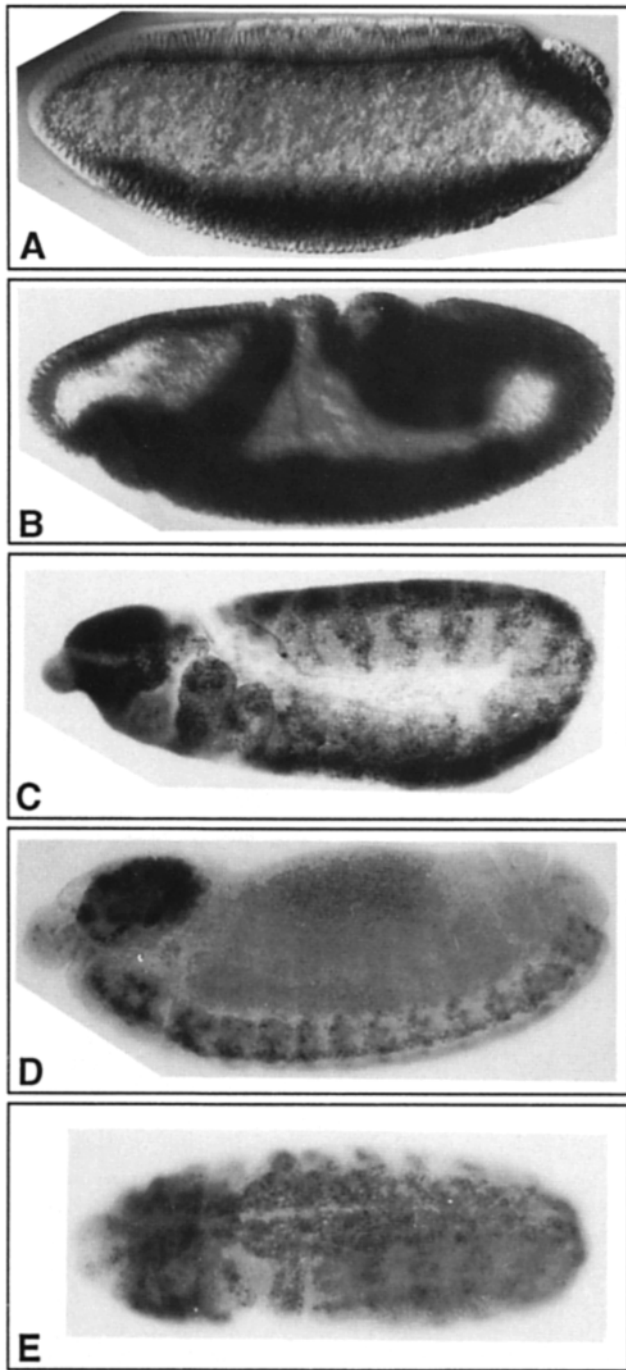


Figure 8. Whole mount in situ detection of the KLP61F mRNA in embryos. Digoxigenin-labeled probes were hybridized to fixed embryos and detected by alkaline phosphatase-conjugated antibody to digoxigenin. (A) Early gastrulating, ventral furrow formation; (B) gastrulating, cephalic furrow formation; (C) gastrulating, germband extended embryo; (D) lateral; and (E) ventral views of a germband retracting embryo. Expression of the KLP61F gene becomes restricted to the central and peripheral nervous system, with intense expression observed in the proliferative zone of the brain cortices.

in situ hybridization to the KLP61F mRNA (Fig. 8). No lacZ polypeptide was detected early in embryonic development, before and including the stages of pole cell (the future germ line) formation and cellularization (Fig. 7, A and B). How-

ever, lacZ expression was observed once gastrulation and organogenesis initiated. In particular, we observed intense expression in the developing nervous system in both germ band extended and germband retracted embryos (Fig. 7, C and D). We also detected expression in the peripheral nervous system (Fig. 7 E, ventral view). As in larval tissues, KLP61F expression in embryos appeared to correlate with regions of high mitotic activity.

By in situ hybridization, we found the maternal store of KLP61F mRNA to be distributed throughout the cytoplasm of unfertilized eggs (data not shown). This maternal complement of KLP61F message persisted, with decreasing intensity, until the onset of cellularization. No KLP61F mRNA was detectable in the newly formed pole cells, similar to what we observed with the lacZ reporter gene. KLP61F message was however detectable by in situ hybridization once gastrulation began (Fig. 8).

During gastrulation, KLP61F mRNA was first detected in a wide band of cells along the ventral midline, corresponding to the invaginating cells of the presumptive mesoderm. The KLP61F mRNA was also seen in a thinner, dorsal strip of cells, as well as cells beneath the shifting pole cells (Fig. 8 A). Embryos in later stages showed staining in extensive areas of the head, along the cephalic furrow, along the ventral midline, and within the posterior region (Fig. 8 B). The patterns of expression were more discernible as germ band elongation ended (Fig. 8 C), and hybridization became restricted to regions of the head, cells along the ventral midline, and segmentally repeated units of cells in the extended germ band. The expression of the endogenous KLP61F gene correlated well with the embryonic expression of β -galactosidase (compare the views of the germband extended embryos in Figs. 7 C and 8 C).

KLP61F expression became limited to cells of the head and ventral neurogenic region once the germband retracted. Lateral and ventral views of a germband retracting embryo are shown in Fig. 8 D and E. Lateral clusters of KLP61F expressing cells along the ventral midline alternated in their expression to first include cells in a wide band immediately surrounding the ventral midline (Fig. 8 E), then to bilateral domains of cells bracketing the midline (not shown). KLP61F expression then became restricted to the ventral region once more, where expression remained along with the proliferative zone of the brain cortices (Fig. 8 D). This expression of KLP61F mRNA during gastrulation showed a remarkable correlation with domains of cell division (Foe, 1989).

A Potential Germline Role for KLP61F?

We have shown that KLP61F is required for mitosis in somatic tissues in *Drosophila*. Is KLP61F also utilized in germline cells? KLP61F mRNA was detectable by Northern blotting in adult testes and ovaries (Stewart et al., 1991). Using the lacZ reporter gene, we examined gonadal tissue for potential KLP61F expression (Fig. 9). We observed lacZ activity in both larval (Fig. 9, A and B) and adult (C and D) ovaries (A and C) and testes (B and D). The activity detected was strikingly restricted to germline cells, and not found in somatic cells. The expression pattern within region 2 of the germarium (Fig. 9 C, arrowheads) suggests that KLP61F may be involved in oocyte determination (a process known to be sensitive to colchicine) (Koch and Spitzer, 1983). (The

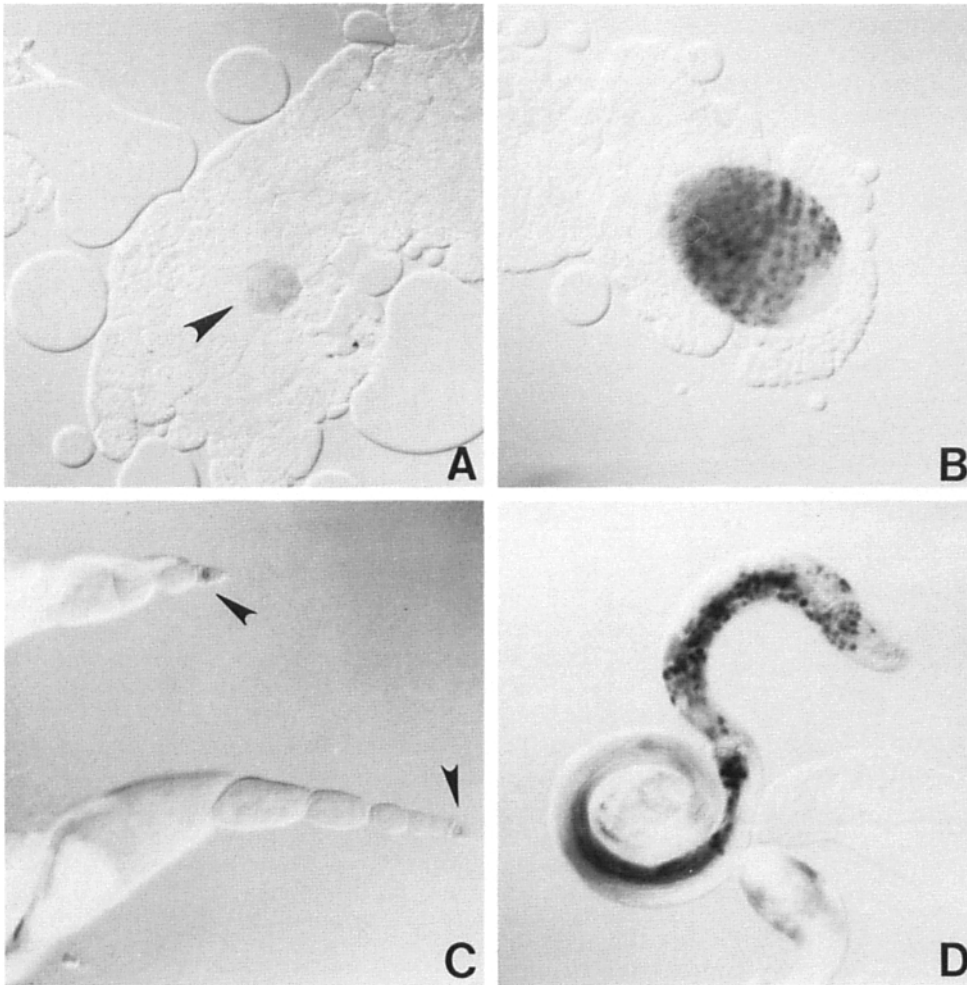


Figure 9. Activity of the β -galactosidase reporter gene in *KLP61F*³ heterozygous gonadal tissue. β -galactosidase activity was assayed following fixation of tissues and incubation with the chromogenic substrate XGal. A larval ovary (A) and testis (B) is shown. Adult ovarioles from an ovary (C) and an adult testis (D) are shown. Expression in the gonads is limited to the germline-derived cells.

postmitotic 16-cell cysts within region 2 of the germarium in adult ovaries are differentiating into 15 nurse cells and one oocyte, with the two pro-oocytes forming synaptonemal complexes.) In adult testes, the primary spermatocytes expressed the lacZ reporter gene, suggesting a possible involvement of KLP61F in meiosis I. Further genetic tests will be required to ascertain what function(s), if any, KLP61F may have in germline cells.

Discussion

Our discovery of the essential nature of the KLP61F kinesin-like protein in *Drosophila* is significant on a number of grounds. KLP61F is the first mitotic kinesin for which mutants are available in an organism easily amenable to both genetic and cytological analysis. Therefore, future studies focusing on detailed morphological aspects of spindle assembly or chromosome movement will be possible. In addition, though at least five kinases and phosphatases have been shown genetically to play regulatory roles in the cell cycle in *Drosophila* (Axton et al., 1990; Biggs et al., 1990; Edgar and O'Farrell, 1989; Llamazares et al., 1991; Mayer-Jaekel et al., 1993), KLP61F represents one of perhaps only three *Drosophila* proteins (aside from tubulin) identified to date as having important structural roles in mitosis. The other relevant structural proteins that have been genetically analyzed

are the regulatory light chain of nonmuscle myosin (Karess et al., 1991), and the *l(1)zw10* gene product (Williams et al., 1992). Our results have confirmed that structural proteins, perhaps the targets of regulatory kinases or phosphatases, are also mutable in *Drosophila*, and therefore may be subjected to rigorous genetic and functional characterization.

We have shown that KLP61F is required for normal progression through the cell cycle. In its absence, dividing, diploid cells are blocked in metaphase with hypercondensed chromosomes. Aberrant mitoses in the absence of KLP61F often result in the production of large polyploid cells, which presumably are due to the failure of chromosome segregation and/or cytokinesis. The block in mitosis has important consequences for the development of the organism. Tissues dependent on mitotic activity, such as the larval brain and imaginal discs, are affected both in their size and gross cellular organization. Homozygous individuals lacking the precursors of adult tissues die at the time when metamorphosis from larval to pupal stages should occur. KLP61F is therefore not only essential for mitosis, but also for normal development as well. Thus the analysis of this KLP in a metazoan has revealed aspects of its function not apparent from studying unicellular eukaryotes.

Our analysis of the developmental expression of KLP61F demonstrated that KLP61F was expressed in all proliferating tissues. The comparison of the expression pattern in larval

brains of a lacZ reporter gene under control of the KLP61F promoter with the BUdR incorporation studies of Truman and Bate suggested that KLP61F may be expressed specifically in cycling cells (Truman and Bate, 1988). This hypothesis was also consistent with the pattern of KLP61F mRNA expression observed in whole-mount embryos. KLP61F mRNA distribution correlated with regions that were among the first to divide in the gastrulating embryo. In addition, the patterns of mRNA distribution closely followed the domains of mitotic activity documented in gastrulating embryos (Foe, 1989). Expression of the KLP61F gene in these domains was transient during embryogenesis, and in at least some of the domains appeared to precede mitosis. These findings are all consistent with our expectations for a protein required for mitosis.

The observation that KLP61F mRNA may be present only in cells about to divide has two other implications. First, it is likely that KLP61F functions only in mitosis, and does not function in nonmitotic microtubule-based transport events. Thus it will be interesting to examine protein distribution as antibodies become available. Second, it is possible that KLP61F mRNA is regulated at the level of transcription or stability by cell cycle cues. What the cues might be is obscure at present, but another mRNA, *string* (the *Drosophila* homolog of *cdc25*) appears also to be expressed in spatial patterns that anticipate the mitotic domains (Edgar and O'Farrell, 1989). These genes may thus use similar regulatory strategies for their cell cycle-dependent expression. Incidentally, CENP-E protein, a kinesin-like component of the kinetochore corona (Cooke, C. A., T. J. Yen, and W. C. Earnshaw, personal communication) thought to be involved in chromosome movement and/or spindle elongation, accumulates specifically in the G2 phase of the cell cycle (Yen et al., 1992).

The patterns of KLP61F expression suggest an additional role for this protein in germline cells. Previously, the analysis of two female-specific, recessive meiotic mutants in *Drosophila* revealed a role for two other KLPs in meiosis (reviewed in Carpenter, 1991; Endow, 1992). Mutations at either the *ncd* (nonclaret disjunctional) or *nod* (no disjunction) locus exhibited high frequencies of nondisjunction during meiosis I, implying the existence of multiple motor molecules in the meiotic spindle (Endow et al., 1990; McDonald and Goldstein, 1990; Zhang et al., 1990). LacZ expression in primary spermatocytes suggested a possible role for KLP61F in meiosis I. We also observed lacZ expression early in oogenesis once the 16-cell cysts have formed, but not in the final maturation stages of oogenesis when meiotic spindle assembly is occurring. KLP61F may thus additionally be required for oocyte determination which includes the accumulation of centrioles (from the nurse cells) in the oocyte. In light of this possibility, it is interesting to note that the spindle poles of mature oocytes appear to lack a number of centrosomal antigens, though the antigens are present at spindle poles early in oogenesis (Theurkauf and Hawley, 1992).

The *Drosophila* KLP61F gene encodes a protein that is a member of the bimC/cut7/Eg5 family of KLPs; this family may also include the CIN8 and KIP1 proteins from *S. cerevisiae* although these latter two kinesins lack the COOH-terminal tail motifs found in the others. The finding that the KLP61F protein has a structure and a function similar to the

bimC, cut7, Eg5, and CIN8/KIP1 proteins is intriguing for several reasons. In particular, this finding suggests that metazoans may use a machinery similar to that of fungi for separating spindle poles/centrosomes during mitosis. As the minus ends of microtubules are embedded in the centriolar material, a plus end-directed motor such as Eg 5 (Sawin et al., 1992), and other members, could function to separate duplicated centrosomes, thus facilitating the establishment of a bipolar spindle. We have here reported that KLP61F is required for centrosomal segregation. Thus KLP61F appears to be analogous to the other family members, both in structure and in its specific role during mitosis.

In *S. cerevisiae*, CIN8/KIP1 also appear to function following spindle pole separation and spindle assembly in the maintenance of a bipolar metaphase spindle. Whether KLP61F also performs this role is unknown at this time. However, given that cut7, Eg5, and epitope-tagged CIN8 have been immunolocalized to metaphase spindles, and thus may have metaphase function, it is tempting to speculate that KLP61F may also play a role in bipolar spindle maintenance (Hagan and Yanagida, 1992; Hoyt et al., 1992; Sawin et al., 1992). Antibodies allowing us to address subcellular localization throughout the cell cycle are currently being prepared.

An intriguing aspect of the current work concerns the role of the tail domains of the bimC family members. It is striking that bimC, cut7, Eg5, and KLP61F all have a shared motif in the COOH-terminal segment of the predicted protein. Perhaps these tail motifs all interact with a conserved centrosomal or cytoskeletal cargo required for centrosomal separation. Additionally, this motif may be a kinase recognition sequence, and thus regulated by posttranslational modification. The CIN8 and KIP1 proteins, however, have tail sequences distinct from the other bimC family members. Possibly the bimC tail motif is found on another protein in *S. cerevisiae*. Whether the sequence differences reflect functional differences between these proteins or that the motif is not actually required for a common function remains to be elucidated.

KLP61F is essential in *Drosophila*, whereas CIN8 and KIP1 appear to be functionally redundant. This result can be explained by postulating that the NH₂-terminal heads of both CIN8 and KIP1 are able to carry out both mechanochemical activity and the binding of a component needed for centrosomal separation. To slide apart opposing microtubules, CIN8 and KIP1 must dimerize via their COOH-terminal tails (hetero or homo) to carry out the function of the single KLP61F molecule. Functional redundancy is thus explained by the ability of CIN8 and KIP1 to form functional homodimers in the absence of one of the two molecules.

Perhaps the most illuminating aspect of our results is the demonstration of KLP61F as a member of the conserved bimC/cut7/Eg5 family of kinesin-like proteins. The KLP61F gene is essential for mitosis and development in *Drosophila*, apparently without a redundant counterpart. The situation in *A. nidulans* and *S. pombe* is unclear as only ts, not true "knock-out," mutants of bimC and cut7 have been reported. It is not yet known whether these represent complete loss of function mutants. Why apparently homologous functions are redundant in some, but not other organisms, is not clear at present. Perhaps this reflects distinct structural requirements in different systems or merely evolutionary happenstance.

We would like to thank Dianne Stern and Dianne Thompson for their assistance during testing for the phase of lethality, Ron DuBrueil, Maggie deCuevas, and Eric Brandon for their contributions to the construction of Pwum2, and Steven Chen for sequencing the shorter KLP61F cDNA clone. In addition, we would especially like to thank Patricia Wilson and Margaret Fuller for communication of results before publication. M. Heck is particularly grateful to William Earnshaw and the members of his laboratory for expertise and help with fluorescence microscopy.

M. Heck was supported by a postdoctoral fellowship from the Jane Coffin Childs Memorial Foundation for Medical Research. Additional support came from the Howard Hughes Medical Institute (A. Spradling), a National Institutes of Health postdoctoral fellowship (A. Pereira), a National Institutes of Health grant (L. Goldstein), and an American Cancer Society Faculty Research Award (L. Goldstein).

Received for publication 1 April 1993 and in revised form 26 July 1993.

References

- Allen, R. D., D. G. Weiss, J. H. Hayden, D. T. Brown, H. Fujiwake, and M. Simpson. 1985. Gliding movement of and bidirectional transport along single native microtubules from squid axoplasm: evidence for an active role of microtubules in cytoplasmic transport. *J. Cell Biol.* 100:1736-1752.
- Axton, J. M., V. Dombradi, P. T. W. Cohen, and D. M. Glover. 1990. One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis. *Cell.* 63:33-46.
- Biggs, J., E. Hersperger, P. S. Steeg, L. A. Liotta, and A. Shearn. 1990. A *Drosophila* gene that is homologous to a mammalian gene associated with tumor metastasis codes for a nucleoside diphosphate kinase. *Cell.* 63:933-940.
- Brady, S. T. 1985. A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature (Lond.)*. 317:73-75.
- Brown, N. H., and F. C. Kafatos. 1988. Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* 203:425-437.
- Campos-Ortega, J. A., and V. Hartenstein. 1985. The Embryonic Development of *Drosophila melanogaster*. Springer-Verlag, Berlin. 1-222.
- Carpenter, A. T. C. 1991. Distributive segregation: motors in the polar wind? *Cell.* 64:884-890.
- Cooley, L., R. Kelley, and A. Spradling. 1988. Insertional mutagenesis of the *Drosophila* genome with single P elements. *Science (Wash. DC)*. 239:1121-1128.
- Crews, C. M., A. Alessandrini, and R. L. Erikson. 1992. ERKs: their 15 minutes has arrived. *Cell Growth & Differ.* 3:135-142.
- Devereux, J., P. Haerberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.
- Edgar, B. A., and P. H. O'Farrell. 1989. Genetic control of cell division patterns in the *Drosophila* embryo. *Cell.* 57:177-187.
- Endow, S. A. 1991. The emerging kinesin family of microtubule motor proteins. *Trends Biochem.* 16:221-225.
- Endow, S. A. 1992. Meiotic chromosome distribution in *Drosophila* oocytes: roles of two kinesin-related proteins. *Chromosoma*. 102:1-8.
- Endow, S. A., and M. Hatsumi. 1991. A multimeric kinesin gene family in *Drosophila*. *Proc. Natl. Acad. Sci. USA*. 88:4424-4427.
- Endow, S. A., S. Henikoff, and L. Soler-Niedela. 1990. Mediation of meiotic and early mitotic chromosome segregation in *Drosophila* by a protein related to kinesin. *Nature (Lond.)*. 345:81-83.
- Enos, A. P., and N. R. Morris. 1990. Mutation of a gene that encodes a kinesin-like protein blocks cell division in *A. nidulans*. *Cell.* 60:1019-1027.
- Foe, V. 1989. Mitotic domains reveal early commitment of cells in *Drosophila* embryos. *Development*. 107:1-22.
- Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* 120:97-120.
- Gatti, M., and B. S. Baker. 1989. Genes controlling essential cell-cycle functions in *Drosophila melanogaster*. *Genes & Dev.* 3:438-453.
- Gho, M., K. McDonald, B. Ganetzky, and W. M. Saxton. 1992. Effects of kinesin mutations on neuronal functions. *Science (Wash. DC)*. 258:313-316.
- Goldstein, L. S. B. 1991. The kinesin superfamily: tails of functional redundancy. *Trends Cell Biol.* 1:93-98.
- Hagan, I., and M. Yanagida. 1990. Novel potential mitotic motor protein encoded by the fission yeast *cut7+* gene. *Nature (Lond.)*. 347:563-566.
- Hagan, I., and M. Yanagida. 1992. Kinesin-related cut7 protein associates with mitotic and meiotic spindles in fission yeast. *Nature (Lond.)*. 356:74-76.
- Heck, M. M. S., and W. C. Earnshaw. 1986. Topoisomerase II: a specific marker for cell proliferation. *J. Cell Biol.* 103:2569-2581.
- Hoyt, M. A., L. He, K. K. Loo, and W. S. Saunders. 1992. Two *Saccharomyces cerevisiae* kinesin-related gene products required for mitotic spindle assembly. *J. Cell Biol.* 118:109-120.
- Karess, R. E., and G. M. Rubin. 1984. Analysis of P transposable element functions in *Drosophila*. *Cell.* 38:135-146.
- Karess, R. E., X. Chang, K. A. Edwards, S. Kulkarni, I. Aguilera, and D. P. Kiehart. 1991. The regulatory light chain of nonmuscle myosin is encoded by *spaghetti-squash*, a gene required for cytokinesis in *Drosophila*. *Cell.* 65:1177-1189.
- Karpen, G. H., and A. C. Spradling. 1992. Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome *Dp1187* by single P-element insertional mutagenesis. *Genetics*. 132:737-753.
- Klemenz, R., U. Weber, and W. Gehring, J. 1987. The white gene as a marker in a new P-element vector for gene transfer in *Drosophila*. *Nucleic Acids Res.* 15:3947-3959.
- Koch, E. A., and R. H. Spitzer. 1983. Multiple effects of colchicine on oogenesis in *Drosophila*: induced sterility and switch of potential oocyte to nurse-cell developmental pathway. *Cell Tissue Res.* 228:21-32.
- Le Guellec, R., J. Paris, A. Couturier, C. Roghi, and M. Philippe. 1991. Cloning by differential screening of a *Xenopus* cDNA that encodes a kinesin-related protein. *Mol. Cell Biol.* 11:3395-3398.
- Lee, H., J. A. Simon, and J. T. Lis. 1988. Structure and expression of the ubiquitin gene from *Drosophila melanogaster*. *Mol. Cell Biol.* 8:4727-4735.
- Llamazares, S., A. Moreira, A. Tavares, C. Girdham, B. A. Spruce, C. Gonzalez, R. E. Karess, D. M. Glover, and C. E. Sunkel. 1991. *polo* encodes a protein kinase homolog required for mitosis in *Drosophila*. *Genes & Dev.* 5:2153-2165.
- Lupas, A., M. Van Dyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science (Wash. DC)*. 252:1162-1164.
- Mackay, A. M., D. M. Eckley, C. Chue, and W. C. Earnshaw. 1993. Molecular analysis of the INCENPs: separate domains are required for association with microtubules during interphase and with the central spindle during anaphase. *J. Cell Biol.* 123:373-385.
- Mayer-Jaekel, R. E., H. Ohkura, R. Gomes, C. E. Sunkel, S. Baumgartner, B. A. Hemmings, and D. M. Glover. 1993. The 55 kd regulatory subunit of *Drosophila* protein phosphatase 2A is required for anaphase. *Cell.* 72:621-633.
- McDonald, H. B., and L. S. B. Goldstein. 1990. Identification and characterization of a gene encoding a kinesin-like protein in *Drosophila*. *Cell.* 61:991-1000.
- McIntosh, J. R., and C. M. Pfarr. 1991. Mitotic motors. *J. Cell Biol.* 115:577-585.
- Mlodzik, M., and Y. Hiromi. 1992. The enhancer trap method in *Drosophila*: its application to neurobiology. Methods in Neuroscience (Gene Expression in Neural Tissues). Academic Press, Orlando, FL. 397-414.
- Munro, S., and H. R. B. Pelham. 1987. The C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899-907.
- Nislow, C., V. A. Lombillo, R. Kuriyama, and J. R. McIntosh. 1992. A plus-end-directed motor enzyme that moves antiparallel microtubules *in vitro* localizes to the interzone of mitotic spindles. *Nature (Lond.)*. 359:543-547.
- O'Connell, M. J., P. B. Meluh, M. D. Rose, and N. R. Morris. 1993. Suppression of the *bimC4* mitotic spindle defect by deletion of *klpA*, a gene encoding a KAR3-related kinesin-like protein in *Aspergillus nidulans*. *J. Cell Biol.* 120:153-162.
- Patel, N. H., E. Martin-Blanco, K. G. Coleman, S. J. Poole, M. C. Ellis, T. B. Kornberg, and C. S. Goodman. 1989. Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell.* 58:955-968.
- Robertson, H. M., C. R. Preston, R. W. Phillis, D. M. Johnson-Schlitz, W. K. Benz, and W. R. Engels. 1988. A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics*. 118:461-470.
- Roof, D. M., P. B. Meluh, and M. D. Rose. 1992. Kinesin-related proteins required for assembly of the mitotic spindle. *J. Cell Biol.* 118:95-108.
- Saunders, W. S., and M. A. Hoyt. 1992. Kinesin-related proteins required for structural integrity of the mitotic spindle. *Cell.* 70:451-458.
- Sawin, K. E., and J. M. Scholey. 1991. Motor proteins in cell division. *Trends Cell Biol.* 1:122-129.
- Sawin, K. E., K. LeGuellec, M. Philippe, and T. J. Mitchison. 1992. Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature (Lond.)*. 359:540-543.
- Saxton, W. M., J. Hicks, L. S. B. Goldstein, and E. C. Raff. 1991. Kinesin heavy chain is essential for viability and neuromuscular functions in *Drosophila*, but mutants show no defects in mitosis. *Cell.* 64:1093-1102.
- Shearn, A. 1978. Mutational dissection of imaginal disc development. The genetics and biology of *Drosophila*. Academic Press, London. 443-510.
- Stewart, R. J., P. A. Pesavento, D. N. Woerpel, and L. S. B. Goldstein. 1991. Identification and partial characterization of six members of the kinesin superfamily in *Drosophila*. *Proc. Natl. Acad. Sci. USA*. 88:8470-8474.
- Tautz, D., and C. Pfeifle. 1989. A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma*. 98:81-85.
- Theurkauf, W. E., and R. S. Hawley. 1992. Meiotic spindle assembly in *Drosophila* females: behaviour of nonexchange chromosomes and the effects of mutations in the *nod* kinesin-like protein. *J. Cell Biol.* 116:1167-1180.
- Truman, J. W., and M. Bate. 1988. Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev. Biol.* 125:145-157.
- Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39-50.
- Vallee, R. 1991. Cytoplasmic dynein: advances in microtubule-based motility. *Trends Cell Biol.* 1:25-29.

- Vallee, R. B., and H. S. Shpetner. 1990. Motor proteins of cytoplasmic microtubules. *Ann. Rev. Biochem.* 59:909-932.
- Vulliet, P. R., F. L. Hall, J. P. Mitchell, and D. G. Hardie. 1989. Identification of a novel proline-directed serine/threonine protein kinase in rat pheochromocytoma. *J. Biol. Chem.* 264:16292-16298.
- Williams, B. C., T. L. Karr, J. M. Montgomery, and M. L. Goldberg. 1992. The *Drosophila l(1)zw10* gene product, required for accurate mitotic chromosome segregation, is redistributed at anaphase onset. *J. Cell Biol.* 118:759-773.
- Wilson, C., R. K. Pearson, H. J. Bellen, C. J. O'Kane, U. Grossniklaus, and W. J. Gehring. 1989. P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes & Dev.* 3:1301-1313.
- Yang, J. T., R. A. Laymon, and L. S. B. Goldstein. 1989. A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analysis. *Cell.* 56:879-889.
- Yen, T. J., D. A. Compton, D. Wise, R. P. Zinkowski, B. R. Brinkley, W. C. Earnshaw, and D. W. Cleveland. 1991. CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1245-1254.
- Yen, T. J., G. Li, B. T. Schaar, I. Szilak, and D. W. Cleveland. 1992. CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature (Lond.)*. 359:536-539.
- Zhang, P., B. A. Knowles, L. S. B. Goldstein, and R. S. Hawley. 1990. A kinesin-like protein required for distributive chromosome segregation in *Drosophila*. *Cell.* 62:1053-1062.