The *Drosophila* Kinesin-like Protein KLP3A Is a Midbody Component Required for Central Spindle Assembly and Initiation of Cytokinesis

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Abstract. We describe here a new member of the kinesin superfamily in *Drosophila*, *KLP3A* (*Kinesin-Like-Protein-at-3A*). The KLP3A protein localizes to the equator of the central spindle during late anaphase and telophase of male meiosis. Mutations in the *KLP3A* gene disrupt the interdigitation of microtubules in spermatocyte central spindles. Despite this defect, anaphase B spindle elongation is not obviously aberrant. However, cytokinesis frequently fails after both meiotic divisions in mutant testes. Together, these findings strongly suggest that the KLP3A presumptive motor protein is a critical component in the establishment or stabilization of the central spindle. Furthermore, these results imply that the central spindle is the source of signals that initiate the cleavage furrow in higher cells.

During late anaphase and telophase, antiparallel interdigitating microtubules in the region of the spindle between the separating chromosomes form a conspicuous network variously termed the "central spindle," the "spindle midzone," or the "interzone." In many cell types, electron micrographs show that microtubules at the equator of the central spindle are surrounded by an amorphous, electron dense matrix material which we denote here by the term "midbody" (McIntosh and Landis, 1971; Mullins and Biesele, 1977). The midbody blocks the staining of microtubules with anti-tubulin antibodies, resulting in the appearance of a dark equatorial bar dividing the central spindle when it is visualized by immunofluorescence (Saxton and McIntosh, 1987; Sellito and Kuriyama, 1988).

Despite the prominence of these structures, the functions of the central spindle and midbody remain ill-defined. Most attention has focused on the potential role of the central spindle as the source of forces pushing the spindle poles during anaphase B. In diatom spindles isolated in vitro, interactions between overlapping microtubules presumably mediated by motor proteins clearly power the sliding apart of the two half-spindles, leading to overall spindle elongation (Cande and McDonald, 1985; Hogan et al., 1993). In other cell types, however, even though molecules with biochemical properties suggestive of a role as an anaphase B motor have been found in the midzone (Nislow et al., 1992; Liao et al., 1994), it is less certain that the sliding of interzonal microtubules powers anaphase B movements. Instead, several investigations have suggested that the poles may be pulled apart, rather than pushed, by forces at the cell cortex acting upon astral microtubules (reviewed by Ault and Rieder, 1994). It is thus possible that in many types of cells, the spindle midzone regulates the rate of anaphase B spindle elongation rather than providing the underlying force.

In this paper, we describe a genetic approach to analyzing the function of the central spindle in *Drosophila* spermatocytes, which is based upon our identification of a component of the midbody which is essential for the structural integrity of the central spindle during meiotic divisions in the male. This protein, KLP3A, is a member of the kinesin superfamily of microtubule-based mechanochemical motors known to be involved in diverse intracellular processes, including chromosome movement, karyogamy, spindle pole separation, and neuronal vesicle transport (reviewed by McIntosh and Pfarr, 1991; Endow and Titus, 1992; Goldstein, 1993). It has been estimated from in situ hybridization studies that there are at least 30 members of the kinesin superfamily in *Drosophila* (Endow and Hatsumi, 1991). Except for a small number of these KLPs, however, their specific intracellular functions remain unknown.

We have identified several mutations in the gene encoding the KLP3A protein. Our findings indicate that *KLP3A* function is not essential for *Drosophila* viability, but is required for fertility in both sexes. Females homozygous for mutations in the *KLP3A* gene are completely sterile, laying eggs that are fertilized but that arrest development at some stage prior to completion of the first mitotic division. Males carrying *KLP3A* mutations are only weakly fertile, and examina-

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tion of their testes shows that a high frequency of abnormalities in spermatogenesis has occurred. As we have not yet completely characterized the maternal effect lethal phenotype associated with *KLP3A* mutations in females, we focus here only upon the consequences of these mutations in spermatogenesis.

Our observations show that these *KLP3A* mutations affect the formation of the central spindle during late anaphase and telophase of the male meiotic divisions. Chromosome segregation appears unaffected by these *KLP3A* alleles, suggesting that these effects are specific for interzonal microtubules. This specificity is explained by immunolocalization experiments showing that the KLP3A protein is normally found associated with microtubules exclusively in the spindle midbody from late-anaphase through telophase.

Interestingly, our analysis of *KLP3A* mutant cells indicates that loss of the midzone has no significant effect on anaphase B spindle elongation. Instead, the most obvious consequence of this defect is a subsequent disruption of cytokinesis. This result suggests that the spindle midzone or midbody might be an important component of the signal generated by the mitotic apparatus that directs the initiation and positioning of cleavage furrow formation.

Materials and Methods

Stocks

Mutant alleles (*14-835* and *12-1611*) of the *Drosophila* X-linked female sterile mutation *fs(l)M4* were induced by ethylmethane sulfonate (EMS)[1] in a *y cv v f* background (Mohler, 1977; Mohler and Carroll, 1984), and were provided to us by Kevin Cook and Rod Nagoshi (University of Iowa, Iowa City, IA). Since *fs(l)M4* mutations have been shown to lie at the *KLP3A* locus (see Results), they are abbreviated as $KLP3A^{835}$ and $KLP3A^{1611}$, respectively. Mutations at the *l(l)zw4* locus were represented by the *63e4*, *GA107*, *9pp4*, *62dl8*, *DF944*, and *65h6* alleles (Lindsley and Zimm, 1992). Mutations and rearrangements were maintained in females over either of the X chromosome balancers *FM7a* or *FM0*, or in males covered by the Y chromosome derivative w^+Y that carries a duplication of the *z-w* region (see Lindsley and Zimm, 1992 for further explanation of chromosomes and genetic symbols used). To obtain *KLP3A/Y* mutant larvae and pupae, *KLP3A* mutant males were mated with *C(l)DX, yf/Y* females. For $KLP3A^{e4}$ larvae and pupae, $KLP3A^{e4}/FM7$ virgin females were mated with *FM7* males and the progeny scored for Malpighian tubule coloration (Williams et al., 1992).

Nucleic Acids

Recombinant DNA from plasmids, cosmids, and bacteriophage lambda vectors, and genomic DNA from *Drosophila* adults were prepared by standard procedures (Sambrook et al., 1989) and as previously noted (Williams et al., 1992). Genomic clones were isolated either from an EMBL4 genomic library (Gunaratne et al., 1986), or from a cosmid library kindly provided by J. Tamkun (University of Colorado, Boulder, CO). Restriction fragments were subcloned into the polylinker of Bluescript KS^+ (Stratagene Corp., La Jolla, CA). Poly(A)+ RNA was isolated from staged wild-type *Drosophila* (Dombrádi et al., 1989). Electrophoresis of glyoxalated poly(A)+ RNA on agarose gels, transfer to Hybond-N membranes, and hybridization of Northern blots with labeled probes was also carried out as detailed by the same authors. Radiolabeling of purified DNA fragments, transfer of DNA to membranes, and hybridization techniques were performed as described (Sambrook et al., 1989; Williams et al., 1992).

Cloning of Deletion Breakpoints

The $zw4^{63e4}$ deletion breakpoint was cloned as follows: total genomic DNA of $zw4^{63e4}/w^+Y$ flies was completely digested with EcoRI and XhoI. DNA fragments including those containing the breakpoint, of the predicted size (~1.6 kb) were isolated from low melting temperature agarose (SeaPlaque; FMC Marine Colloids, Rockland, ME) and cloned into the Lambda-Zap phage vector (Stratagene) which had also been digested with EcoRI and XhoI. After packaging, infection, and amplification, the $zw4^{63e4}$ genomic library was screened by hybridization with *KLP3A*-containing fragments. Positive clones were purified and their lysates used for transformation of *XLl-Blue* cells according to Lambda-Zap manuals (Stratagene). The plasmid clones in Bluescript were then sequenced using the T3 and T7 primers.

1. *Abbreviation used in this paper*: EMS, ethylmethane sulfonate.

Isolation and Sequencing of KLP3A cDNA

A 4.4 kb cDNA, corresponding to the full-length *KLP3A* message, was the longest of several cDNA clones isolated from screening an imaginal disk cDNA library (Brown, 1988). The cytological location of the cDNA insert was confirmed by in situ hybridization to polytene chromosomes using the Detek kit (Enzo Inc., New York, NY) as described (Williams et al., 1992). A 4,008-bp EcoRI fragment from this cDNA clone (nucleotides 81 to 4089), comprising the majority of *KLP3A* cDNA sequences, was subcloned into the EcoRI site of Bluescript $SKII^+$ (Stratagene) in two opposite orientations. Both clones were separately digested with SalI and KpnI for production of deletions generated by partial ExoIII digestion (Henikoff, 1984) using reagents in the Erase-a-Base system (Promega Biotech, Madison, WI). Sequential deletions were produced from both the 5′ end and 3′ ends of the cDNA, and both strands were completely sequenced using chain termination methods (Sanger et al., 1977) with the Sequenase system (U.S. Biochemical Corp., Cleveland, OH). The terminal 5′ and 3′ ends of the cDNA (bases 0–80 and 4089–4395), which were not contained in the 4.0-kb EcoRI fragment (see above), were sequenced from the original cDNA plasmid based in pNB40 (Brown, 1988) using the SP6 and T7 primers, respectively. To eliminate compressions due to GC-rich regions, oligonucleotide primers were synthesized (Cornell Biotechnology Synthesis Facility) corresponding to regions 35–45 bp 5′ of the area giving rise to the compression, and 7-deaza-dGTP mixes and Mn^{2+} buffer (Stratagene) were both used in the sequencing reaction according to Sequenase manuals. Sequence analysis and searches of sequence databases were carried out using the Genetics Computer Group (GCG) programs (Devereux et al., 1984) and MacVECTOR software (International Biotechnology Inc., New Haven, CT).

Germline Transformation

Two fragments from *cosB*, a cosmid-based genomic clone containing the *KLP3A* region, were subcloned into the *Drosophila* transformation vector pw8 (Klemenz et al., 1987): a SalI-XhoI fragment from the distal end (called fragment A; Fig. 1) and a ScaI-SalI piece from the central region (called fragment B; Fig. 1) and used in P element-mediated germline transformation. DNA was microinjected into early embryos (from the *w*; *Δ2-3,Sb/TM6* stock) containing endogenous transposase supplied by the *Δ2-3* P-element (Robertson et al., 1988). Two separate transformants were obtained for fragment A: 18XS.1 (integrated on the X chromosome) and 18XS.2 (on chromosome 2). These fragments rescued the lethality caused by *zw4* mutant alleles *62dl8*, *63e4*, *GA107*, *9pp4*, and *65h6* (our unpublished data). Fragment A-rescued *zw4* mutants homozygous for alleles *62dl8*, *GA107*, *9pp4*, and *65h6* were fertile. Fragment A-rescued $zw4^{63e4}$ homozygotes, however, were female sterile and male semisterile, because the $zw4^{63e4}$ lesion also disrupts *KLP3A* (see Results). Fragment A-rescued $zw4^{63e4}$ homozygotes were thus termed $KLP3A^{e4}$. SCA9 (fragment B), as present in two independent transformant lines on chromosome 2 ($[B]^1$ and $[B]^2$) did not rescue *zw4* mutations but were each able to restore the fertility and cytological phenotypes of *KLP3A* mutant females and males (see Results). All transformant lines were positively identified for the presence of the integration of the appropriate DNA fragment by whole genomic Southern analysis (see above).

Cytological Preparations

Live testes were examined as described by Cenci et al. (1994). Briefly, larval testes were dissected in testis isolation buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl, pH 6.8), placed in a small drop of testis isolation buffer on a cover slip, and squashed very gently with an inverted glass slide. Live specimens were examined by phase contrast. Slides containing testes selected for fixation were frozen in liquid nitrogen. After removal of the cover slip, testes were fixed by methanol and acetone exactly as described

(Pisano et al., 1993; Cenci et al., 1994). Alternatively, squashed testes were fixed in cold methanol alone (−20°C for 7 min), a standard procedure used for the fixation of mammalian cells (Neighbors et al., 1987). The testes were then incubated in several changes of PBT (PBS, 2.6 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, 0.02% NaN_3, + 0.1% Triton-X) for 1 h before antibody staining. An additional fixation method used in examining the distribution of the KLP3A antigen was a formaldehyde treatment based on previously published protocols (Kremer et al., 1986) and was performed exactly as described for the fixation of *Drosophila* brain tissue (Williams and Goldberg, 1994). This formaldehyde fixation protocol has been demonstrated to preserve the distribution of both tubulin and the *zw10* antigen (Williams and Goldberg, 1994), which associates with the spindle and kinetochores in *Drosophila* testes (our unpublished results).

Preparation of KLP3A Polyclonal Antibodies

Creation of the expression clone pQEklp involved directional cloning of a 2.9-kb SphI-EcoRI fragment from the full length *KLP3A* cDNA clone (see above) into pQE31, which contains a histidine repeat (Qiagen Inc., Chatsworth, CA). This construct was transformed by electroporation (*Escherichia coli* pulser; Bio-Rad Laboratories, Cambridge, MA) into bacterial strain M15 (Qiagen). A large-scale culture was inoculated from overnight cultures and grown at 37°C with vigorous shaking to log-phase. Protein expression was induced by adding IPTG to 2 mM and grown for an additional 6 h at 37°C. Cells were harvested by centrifugation (4,000 *g*, 10 min) and the pellets resuspended in buffer B (8 M urea, 100 mM $NaPO_4$, 10 mM Tris-HCl, pH 8.0) on ice. After lysing the cells by sonication, the cellular debris was removed by centrifugation and the supernatant was transferred to fresh tubes. 100 μl of a 30–50% slurry of Ni-NTA resin was added to each microfuge tube and incubated at room temperature for 30 min. The resin was pelleted by centrifugation for 10 s at 15,000 *g*. The resin was washed three times with buffer C (8 M urea, 100 mM $NaPO_4$, 10 mM Tris-HCl, pH 6.5). Partially purified native fusion protein was eluted by incubating the resin in buffer C + 100 mM EDTA for 10 min with gentle mixing and then centrifuged for 10 s at 15,000 rpm. Fusion protein from the remainder of the supernatant was isolated by preparative SDS-PAGE as described by Williams et al. (1992). Thus both native and denatured KLP3A fusion protein were isolated, and used separately for the production of polyclonal antibodies in rabbits. KLP3A antibodies were affinity purified against KLP3A fusion proteins immobilized on nitrocellulose filters according to Sambrook et al. (1989).

Immunoblotting

Embryos were collected on yeasted agar plates containing grape juice, dechorionated, ground in sample buffer, and processed for SDS-PAGE according to Williams et al. (1992). Protein was transferred to Immobilon-P nitrocellulose membranes (Millipore Corp., Bedford, MA) using a Semiphor transfer unit (Hoefer Scientific, San Francisco, CA). Blots were blocked, incubated in affinity-purified anti-KLP3A antibodies (1/50 dilution), and processed for enhanced chemiluminescence; Amersham Corp., Arlington, Heights, IL) exactly as described for the detection of *zw10* protein (Williams et al., 1992).

Immunostaining

For KLP3A localization, fixed testes were incubated in crude sera (1/700 dilution) or affinity-purified sera (1/5 dilution in PBT) at 4°C overnight, with the same results. Also, antibodies produced against either native or denatured protein epitopes (see above) gave identical results. For simultaneous visualization of microtubules, testes were also incubated with a monoclonal anti-α-tubulin antibody raised against native chick brain microtubules (Amersham Corp.) which was used at a dilution of 1/50 together with the KLP3A antibody. After the overnight incubation, slides were washed in three changes of PBT for a total of 15 min. The primary antibodies were then detected with FITC-conjugated anti-mouse IgG (1/50 dilution) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG (Jackson Laboratories, Bar Harbor, ME) for 3 h at room temperature in the dark. Testes were then rinsed in PBT for 10 min, stained with Hoechst 33258 at a concentration of 0.5 μg/ml in Hoechst buffer, and mounted in Hoechst citrate buffer (solutions for these steps are described by Cenci et al., 1994). The elimination of Triton-X from antibody incubations and washes resulted in higher backgrounds but had no effect on the observed distribution of the KLP3A antigen. Also, incubating testes with pre-immune sera (followed by incubation in secondary antibodies as above) or secondary antibodies alone (at the same dilutions) resulted in no detectable signals.

Microscopy

Specimens were examined either with a Zeiss Axioskop or a Zeiss III photomicroscope (Carl Zeiss, Oberkochen, Germany), equipped for epifluorescence with an HBO 100 W/2 mercury short arc lamp (Osram, Germany), using the 09 filter sets (BP 450-490, FT 510, LP 420). Photographs were recorded on TMax 100 and Technical Pan films (Kodak). Immunostained testes preparations were also observed using a charge-coupled device (Photometrics) connected to a Zeiss Axioskop microscope. Exposure times ranged from 0.2–1.0 s. Hoechst, FITC, and TRITC signals were collected separately using IP Lab Spectrum software, and programs written by E. Marchetti (University of Rome, Rome, Italy). Images were then converted to Photoshop format (Adobe Systems Inc., Mountain View, CA) and merged in pseudocolor. Final images were printed using a dye sublimation process.

Results

Identification of the KLP3A Gene

The existence of a kinesin-like gene in the *zeste-white* region (3A3-3C2) of the *Drosophila* X chromosome was first suggested by experiments of Endow and Hatsumi (1991). They found that a PCR amplified product containing sequences encoding the conserved mechanochemical motor domain of kinesin hybridizes in situ to numerous locations on *Drosophila* polytene chromosomes, including a site in bands 3A5-8 (Endow and Hatsumi, 1991). We established that a sample of this PCR product (kindly supplied by S. Endow, Duke University Medical Center, Durham, NC) hybridized to a previously cloned genomic contig containing the lethal loci *zw4*, *zw10*, and *zw13* (Williams et al., 1992; our unpublished data), as depicted in Fig. 1. Subsequent sequence analysis demonstrated that the sequences homologous to the PCR product indeed encode a member of the kinesin superfamily (see below), yet do not correspond to previously identified loci in the *zeste-white* region (Judd et al., 1972). We have named this gene *KLP3A* (*Kinesin-Like-Protein-at-3A*), in accordance with a generally-accepted nomenclature for *Drosophila* KLPs (Goldstein, 1993).

The *KLP3A* gene is transcribed into poly(A)+ RNAs ∼4.4 kb in length. Expression of this gene is developmentally regulated: transcripts are present at relatively high levels in male and female adults, in the embryo, and in the pupal stages, while very little KLP3A mRNA is present in the early larval stages (data not shown).

We have obtained a 4.4-kb KLP3A cDNA from an imaginal disc library. The sequence of this cDNA (Fig. 2) predicts a protein of 1,211 amino acids with a molecular weight of 138 kD. Computerized sequence analysis (Garnier et al., 1978; Devereux et al., 1984; Lupas et al., 1991) of this protein suggests the existence of three distinct domains (Fig. 2). Amino acids 1-340 at the NH_2 terminus share strong homology with the mechanochemical motor domain of the kinesin superfamily, particularly in the ATP-binding and microtubule-binding motifs. This is followed by a long stalk-like region (amino acids 341–1,000) predicted to have α-helical coiled coil characteristics (Fig. 2, *B* and *C*). This region is likely to be important for dimerization as demonstrated for kinesin heavy chain (Yang et al., 1989). Several motifs that conform to the consensus sequence for nuclear localization (Dingwall and Laskey, 1991) are found within this stalk-like

middle portion of the KLP3A protein. At the COOH terminus (amino acids 1001-1211), the sequence predicts a globular "tail" that may be of importance in attaching a cargo for transport along microtubules, as has been suggested for several KLPs (Yang et al., 1990; Yen et al., 1992; Heck et al., 1993).

Evolutionary relationships between the KLP3A protein and other previously characterized members of the kinesin superfamily have been presented elsewhere (Goldstein, 1993; *Dm3A7Kin* of Goodson et al., 1994). Briefly, several methods of sequence comparison suggested that the KLP3A motor domain is insufficiently related to other KLP motor domains to be regarded as belonging to any established kinesin family. Moreover, the remainder of the *KLP3A* sequence had no significant homology to other KLPs or to any other protein previously reported in available databases. Recently, however, two other KLPs have been discovered in *Xenopus* (XKLP1; Vernos et al., 1993) and mouse (KIF4; Sekine et al., 1994) that are more closely related to KLP3A than to other members of the kinesin superfamily. It has thus been proposed that KLP3A, XKLP1, and KIF4 define a new kinesin family (Sekine et al., 1994). Within this family, XKLP1 and KIF4 are more similar to each other than either is to KLP3A. XKLP1 and KIF4 show strong amino acid similarities in the motor, stalk, and tail regions (81, 68, and 44% identical, respectively). KLP3A is a more divergent member of this family; amino acid identity of KLP3A to either XKLP1 or KIF4 is only about 50, 30, and 20% in motor, stalk, and tail regions, respectively.

Mutations in KLP3A Affect Fertility in Females and Males

The *KLP3A* gene is adjacent to the lethal locus *zw4* (Judd et al., 1972; see Fig. 1). In the course of otherwise unrelated investigations on *zw4*, we found that the X-ray induced allele $zw4^{63e4}$ was associated with a 2.4-kb deletion that would be expected to remove information from the 3′ ends of both the *zw4* and *KLP3A* genes (Figs. 1 and 2). As described in Materials and Methods, we determined the sequence of the $zw4^{63e4}$ breakpoints, which verified that the deficiency would in fact delete sequences encoding 82 amino acids at the COOH terminus of the KLP3A protein, information encoding the COOH-terminal part of the *zw4* gene product, and presumably transcription termination signals for both genes. The $zw4^{63e4}$ rearrangement fuses *KLP3A* coding sequences to noncoding sequences in an intron of *zw4* (Fig. 2 and data not shown). Although this fusion could theoretically result in the replacement of the COOH-terminal amino acids of *KLP3A* with at least 34 unrelated residues encoded by the intron, it appears likely that the deletion would abolish the presumptive cargo-carrying functions dependent upon this part of the KLP3A protein.

For our studies of the *zw4* gene, we reintroduced a fragment of genomic DNA containing the *zw4* transcriptional unit (fragment A in Fig. 1) into the *Drosophila* genome by P element–mediated transformation. Transduced copies of fragment A as expected rescued the viability of animals hemizygous or homozygous for all tested alleles of the lethal *zw4* complementation group, including $zw4^{63e4}$ (data not shown). Because of the small deletion associated with the $zw4^{63e4}$ allele, $zw4^{63e4}$ individuals with fragment A would be expected to lack normal KLP3A gene product (as fragment

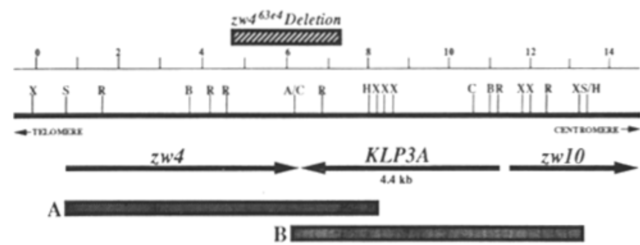

Figure 1. Genetic and molecular map of the klp3a region. Transcriptional units in the KLP3A region, corresponding to the genes *zw4*, *KLP3A*, and *zw10*, are shown by arrows indicating their 5′ to 3′ transcriptional polarity. Introns in these genes are not shown; the length of the *KLP3A* mRNA is 4.4 kb. Fragment A ($zw4^+$) and fragment B ($KLP3A^+$) were used for P-element-mediated germline transformation (see Materials and Methods). The extent of the $zw4^{63e4}$ deletion is represented by a hatched box above the map. Restriction enzyme sites are abbreviated as follows: *H*, *HindIII*; *X*, *XhoI*; *B*, *BamHI*; *R*, *EcoRI*; *C*, *SacI*; *S*, *SalI*. The linear scale is in kilobases (kb).

A would supply *zw4* but not *KLP3A* activity). This combination of genes ($zw4^{63e4}$; *[fragment A]*) will subsequently be abbreviated as $KLP3A^{e4}$. The survival of these flies shows that the *KLP3A* gene is not required for *Drosophila* viability.

Remarkably, however, these KLP3A-deficient flies are completely sterile. $KLP3A^{e4}$ females lay eggs that are fertilized with sperm, but the large majority of these eggs arrest at a very early stage of development prior to the completion of the first embryonic mitotic division. We are currently in the process of characterizing the aberrations in these eggs, but will not report here further on this maternal effect lethal phenotype. In males, lack of the KLP3A product is also associated with a loss of fertility. In tests with individual $KLP3A^{e4}$ males, more than 50% are completely sterile, and the remainder produce a smaller number of progeny than controls.

To search for additional alleles of the *KLP3A* gene, we concentrated on the female sterile phenotype because of its complete penetrance and because large-scale screens for female sterile mutants on the X chromosome have previously been mounted. We discovered that two EMS-induced mutations in the formerly described locus *fs(l)M4* (Mohler, 1977; Mohler and Carroll, 1984) were allelic to $zw4^{63e4}$, as females heterozygous for $zw4^{63e4}$ and *fs(l)M4* mutations were viable but sterile. We subsequently found that males hemizygous for these *fs(l)M4* mutations exhibited low fertility. Typically, as for $KLP3A^{e4}$ males, well over half of *fs(l)M4/Y* mutant males are completely sterile, while the remainder vary in fertility. These results indicate that these mutations are in fact alleles of the *KLP3A* gene, and suggest that the female sterile and male sterile phenotypes are both properties of several different lesions in the *KLP3A* gene.

Mutations in KLP3A Cause Cytokinesis Defects during Male Meiosis

To better understand how a lack of $KLP3A^+$ activity would disrupt male fertility, we examined living preparations of mutant testes by phase contrast microscopy. All premeiotic stages of spermatogenesis appeared normal by this technique. However, a high proportion of postmeiotic spermatids showed obvious aberrations (Table I). Normally, after the

Figure 2. Sequence of the *KLP3A* cDNA. (*A*) The sequence of the coding strand of the longest available *KLP3A* cDNA is depicted with the predicted 1,211-residue amino acid sequence of its product. An ATG at position 218 probably represents the site of translation initiation, based on KLP superfamily homologies (Endow and Hatsumi, 1991) and on the *Drosophila* translational start consensus (Cavener and Ray, 1991). The putative kinesin motor domain is underlined (amino acids 1-343); regions shared by many KLP motor domains are shown in bold type. The $zw4^{63e4}$ deletion (see Fig. 1) eliminates *KLP3A* sequences downstream of base 3601 (*dashed arrow*). (*B*) Structural predictions of the KLP3A polypeptide from the UWGCG Pepstructure program based on the algorithms of Garnier et al. (1978). Note particularly the region of high α-helical content between residues 350 and 1,000, suggestive of a stalk-like structure involved in the dimerization of KLPs (Goldstein, 1993). (*C*) Probabilities of the KLP3A protein forming α-helical coiled coils as predicted from the algorithms of Lupas et al. (1991). Regions between amino acids 400 to 1,000 have a high probability of forming α-helical coiled coil domains. This sequence is available under GENBANK accession number L19117 as DRO3AKLP.

completion of meiosis II at the so-called "onion stage" of early spermatid differentiation, spermatids contain a spherical, phase-clear nucleus and a spherical phase-dense mitochondrial derivative called the Nebenkern (Fig. 3 *A*). The sizes of the Nebenkern and nuclei in any one spermatid are roughly similar, and the sizes of Nebenkern or nuclei in different spermatids appear to be equal. In contrast, a substantial percentage of *KLP3A* mutant spermatids have an abnormally large Nebenkern associated with two nuclei of normal and equal size (Fig. 3, *B* and *C* and Table I). In addition, a high proportion of spermatids from males with the $KLP3A^{e4}$ allele have even larger mitochondrial derivatives in association with three or four normal sized nuclei. Males carrying other mutant alleles have lower but above-background levels of these stronger types of aberration (Fig. 3, *C* and *D* and Table I).

Table I. Cytokinesis Failure in KLP3A Mutants and Rescue with the [KLP3A⁺] Transgene

	Number of spermatids	Percent spermatids				Percent abnormal
		1	2	3	4	
Wild-type	875	99.5	0.5	0.0	0.0	0.5
e4	1638	43.6	23.2	3.2	30.0	56.4
e4;[B][1]	3400	98.7	1.1	0.1	0.1	1.3
e4;[B][2]	866	99.7	0.3	0.0	0.0	0.3
835	1491	73.6	23.8	0.9	1.7	26.4
835;[B][1]	269	100.0	0.0	0.0	0.0	0.0
1611	1185	69.6	28.5	0.8	1.1	30.4
1611;[B][1]	684	100.0	0.0	0.0	0.0	0.0

The post-meiotic onion stage spermatid cysts from wild-type and designated *KLP3A* mutant alleles were examined, and the number of spermatids containing one, two, three, or four nuclei was determined. The frequencies of defective spermatids in the *KLP3A* mutants are compared with those in rescued males containing either of two independent insertions of the [B] transgene.

These unusual spermatids are the result of defects in cytokinesis during the meiotic divisions. During meiosis I and meiosis II in males, the mitochondria line up parallel to the spindle axis; these organelles are then partitioned into daughter cells at cytokinesis (Fuller, 1993). If cytokinesis does not occur at the end of both meiosis I and meiosis II, the result is the generation of spermatids with a single large Nebenkern and four nuclei. A failure of cytokinesis at only one of the two meiotic divisions would yield cells with intermediate-sized mitochondrial derivatives and two nuclei. The formation of spermatids with three nuclei would require failure of cytokinesis following meiosis I. Depending upon the orientation of the four hemispindles in the resultant secondary spermatocyte, the mitochondria associated with these hemispindles could fuse into two Nebenkerns of different size, one associated with three nuclei and the other with a single nucleus. It should be noted that the equality in nuclear size observed in all these aberrant spermatids indicates that chromosome segregation is not affected by *KLP3A* mutations, as spermatid nuclear size is related to chromosome number (González et al., 1990). Similar spermatogenic defects have been seen in the testes of flies with a variety of other male-sterile mutations affecting cytokinesis (Fuller et al., 1987; Castrillon et al., 1993; our unpublished observations), and when testes are treated with the microfilament inhibitor cytochalasin (S. Bonaccorsi, G. Cenci, and M. Gatti, unpublished results).

The data in Table I are presented as an average that masks considerable variability in the appearance of aberrant spermatids in *KLP3A* mutant strains. In some testes, virtually all spermatids appear normal, while in other testes from males with identical genotypes, almost all of the onion stage spermatids are defective. In most cases, however, both normal and aberrant cells can be found in a single cyst of spermatids. This is reflected in the incomplete sterility of mutant flies. Clearly, the mutations in *KLP3A* we have studied show neither complete penetrance nor expressivity in males, and are probably quite sensitive to genetic modifiers. In spite of this variability, we are convinced that the phenotype we observe is a property of lesions in *KLP3A*. Not only are a variety of independent mutations in the gene associated with similar

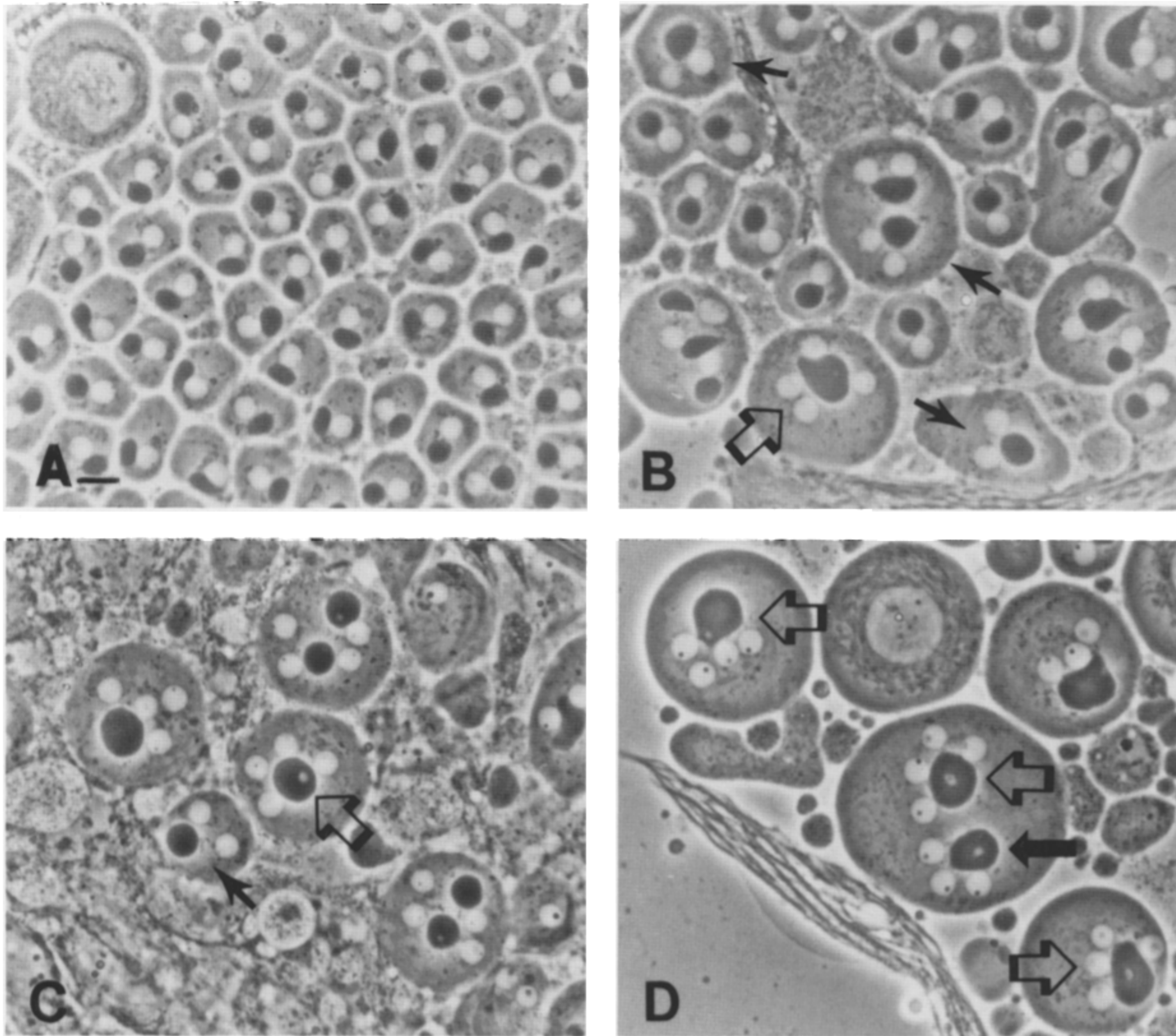

Figure 3. Failure of cytokinesis in *KLP3A* mutants. Live testes were viewed by phase contrast microscopy to examine spermatid defects in post-meiotic onion stage cysts. (*A*) Wild-type cyst showing spermatids each of which contain a phase-dark mitochondrial derivative (the Nebenkern) and a phase-clear nucleus. Note the one-to-one association between the Nebenkern and nucleus, and the uniform size of both organelles. (*B–D*) Partial onion stage cysts from (*B*) $KLP3A^{835}$, and (*C–D*) $KLP3A^{e4}$ mutant testes showing abnormally large Nebenkerns each associated with two, three, or four nuclei (represented respectively by arrows of increasing width). These abnormalities indicate that cytokinesis has failed during the meiotic divisions (Fuller, 1993). Spermatids normally develop within a cyst; adjacent spermatids are interconnected by cytoplasmic bridges. During squashing of testes, adjacent cells can fuse together, explaining the appearance of more than one Nebenkern (with its associated nuclei) sharing the same cytoplasm. Bar, 10 μm.

effects, but all these phenotypes are corrected when flies carrying *KLP3A* mutant alleles also contain a $KLP3A^+$ transgene (fragment B in Fig. 1; see Table I).

Mutations in KLP3A Disrupt the Central Spindle

To investigate the primary cause of cytokinesis failure in *KLP3A* mutants, we visualized DNA and microtubules throughout meiosis in fixed mutant testes. Meiotic spindle assembly and chromosome behavior in $KLP3A^{e4}$ mutants appear normal through early anaphase (from stage Mla through stage M4a according to the stage terminology of Cenci et al., 1994; data not shown). Thus, events such as centrosome duplication and migration, the establishment of the bipolar spindle, chromosome condensation, and chromosome congression to the metaphase plate do not seem to be influenced by the mutation. Instead, the first visible defects in *KLP3A* mutant spindles occur in late anaphase/early telophase, when the two chromosome complements appear well-separated (M4c stage). At this time in wild-type, a prominent central spindle, composed of a dense network of interdigitated microtubules, is established (Fig. 4, *A* and *B*). In contrast, this central spindle is conspicuously absent in most $KLP3A^{e4}$ late anaphases and telophases (stages M4c-M5), although it appears that there may be some minor equatorial interaction between microtubules emanating from the two hemispindles (Fig. 4, *C–E*; Table II). We do not observe an obvious midbody structure forming in this region of mutant cells. The central spindle also fails to form during the second meiotic division (data not shown). Surprisingly, the absence of the central spindle during late anaphase/early telophase has little effect on anaphase B spindle elongation. In $KLP3A^{e4}$ mutants, the pole-to-pole distance at the M4c-M5 stages of meiosis I was comparable to wild-type (Table II).

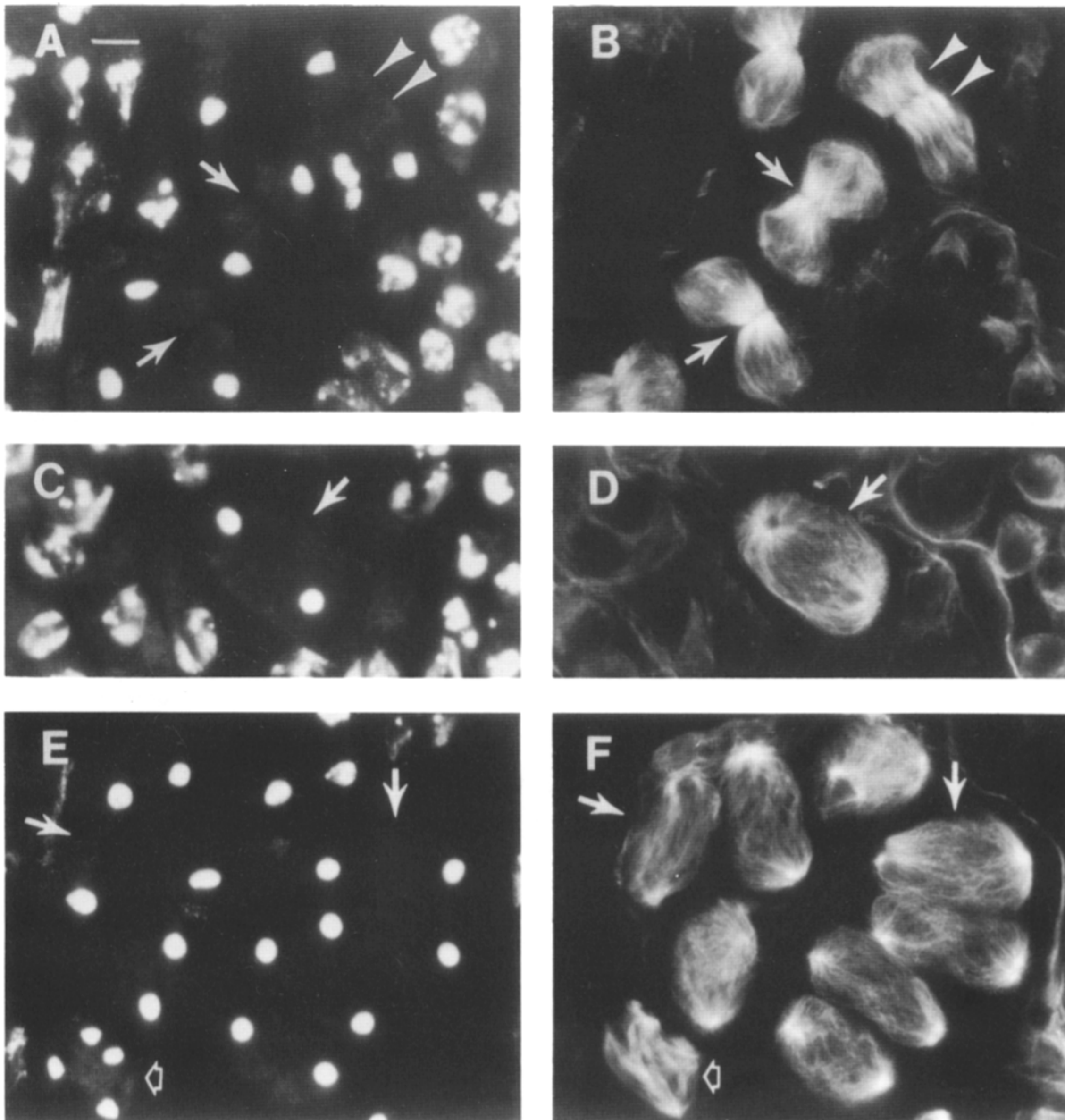

Figure 4. Central spindle defects in *KLP3A* mutant telophases. Wild-type testes (*A* and *B*) and *KLP3A^{e4}* mutant testes (*C–F*) were fixed and stained to visualize DNA (*A, C,* and *E*) and microtubules (*B, D,* and *F,* respectively). (*A* and *B*) First meiotic divisions in wild-type at late anaphase/early telophase showing the prominent central spindles (e.g., *arrowheads*) bisected by the midbody, a narrow relatively unstained band at the equator (*arrows*). Note that the spindle has constricted at the midbody, indicating that the cleavage furrow is present. (*C–F*) First meiotic late anaphase/early telophase figures in *KLP3A^{e4}* showing the markedly reduced density of microtubules in the central spindle and the absence of midbodies (*solid arrows*). Constrictions of the spindles as in wild-type are absent, indicating that the cleavage furrow has not formed. The result of cytokinesis failure during meiosis I is the appearance of two divisions in the same cell during meiosis II (*open arrow* in *E* and *F*). Bar, 10 μm.

Table II. Central Spindle Defects in KLP3A^{e4} Testes

Genotype	Number of Testes	Average spindle length (μm)		Central spindle		Percent abnormal
		Metaphase	Telophase	+	−	
Wild-type	5	18.0 ± 1.9	33.0 ± 5.8	55	0	0.0
KLP3A^{e4}	24	20.0 ± 3.4	30.3 ± 3.5	27	111	80.4

Meiosis I figures at the M4c-M5 (late anaphase–early telophase) stages were scored for the presence (+) or absence (−) of the prominent central spindle identified by anti-tubulin antibodies. Spindle lengths of metaphases (stage M3) and late anaphases (stage M4c) were measured from photographs and presented as the mean and standard deviation of these values. For this table, only mutant testes containing 80–100% of abnormal spermatids were scored.

Our observations of abnormal anaphases and telophases in *KLP3A^{e4}* mutants further suggest that aberrations occur during the earliest stages of cytokinesis. We see no indication of equatorial constrictions of the spindle or cell cortex during either meiotic division, indicating that the cleavage furrow is not forming (Fig. 4 and data not shown). This is consistent with the idea that the central spindle may be the source of signals dictating creation of the contractile ring (see Discussion).

In summary, our analysis of aberrant phenotypes associated with *KLP3A* mutations suggests that establishment of the prominent central spindle and midbody at late anaphase/telophase requires normal *KLP3A* activity. Although this structure appears to be at most minimally involved in anaphase B spindle elongation, it instead appears to be important for the initial stages of contractile ring formation during cytokinesis.

The KLP3A Protein Is Localized in the Spindle Midbody during Anaphase and Telophase

To visualize KLP3A protein within the cell, we have generated antibodies against a portion of bacterially-expressed KLP3A protein that excludes the conserved motor domain (Materials and Methods). Before and after affinity purification, these antibodies recognize a band of ~140 kD on immunoblots of wild-type *Drosophila* embryo extracts (Fig. 5). The size of this band is close to the value of 138 kD expected for the KLP3A protein based upon conceptual translation of the *KLP3A* open reading frame. Additional bands of lower intensity are also observed, probably reflecting some degree of protein degradation in the sample. The antibody does not detect the major 140-kD band in similarly-prepared extracts from embryos laid by females homozygous for *KLP3A* mutant alleles, but the signal is restored in the presence of a *KLP3A*+ transgene (Fig. 5). These results show that these antibody preparations are in fact specifically directed against the KLP3A gene product.

On the basis of sequence analysis, we had anticipated that animals carrying the *KLP3A^{e4}* allele would produce a truncated, but nonetheless near full-sized, variant of the KLP3A protein. The Western blots shown in Fig. 5 do not indicate large amounts of such a variant, suggesting that mutant KLP3A proteins might be rapidly degraded. We believe that small amounts of partially degraded KLP3A polypeptides are in fact produced in all of the mutants we have examined. Presumptive degradation products are weakly visible in extracts of animals with the *KLP3A^{1611}* allele (Fig. 5 *A*), and longer exposures of the same Western blot reveal immunoreactive bands of smaller size and much lower intensity that vary between alleles (data not shown). Differences in the accumulation of such breakdown products might account for some of the quantitative variation in phenotype severity associated with these alleles (Table I).

We have used indirect immunofluorescence with both crude and affinity-purified antisera to examine the distribution of KLP3A in *Drosophila* testes during the course of spermatogenesis. The two antibody preparations gave identical results. Furthermore, the localizations of KLP3A antigens observed in testes fixed according to several different standard protocols (including fixation in methanol-acetone [Pisano et al., 1993; Cenci et al., 1994], cold methanol alone [Neighbors et al., 1987], and formaldehyde [Kremer

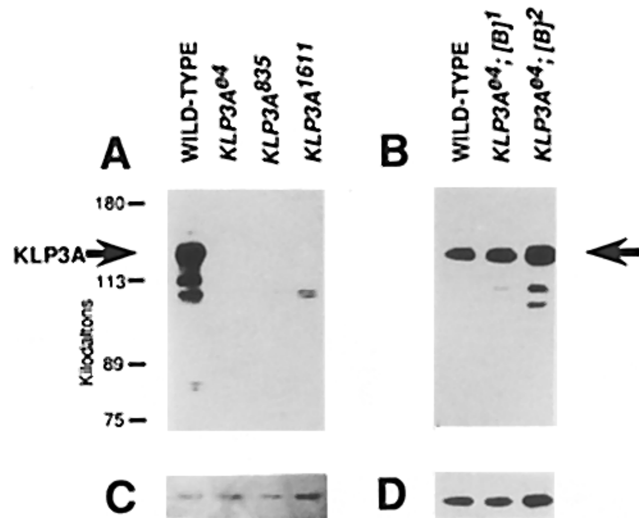

Figure 5. Identification of the KLP3A protein on Western blots. Immunoblots of total protein extracts from 0–12-h-old embryos laid by the females of the designated genotype, detected in *A* and *B* with an affinity-purified rabbit polyclonal antibody raised against KLP3A non-motor domain sequences. (*A*) The KLP3A protein is altered in *KLP3A* mutants. In wild-type embryos (lane *1*) the *KLP3A* protein appears primarily as a single band of about 140 kD (*arrow, left*). In embryos laid by females homozygous for *KLP3A^{e4}*, *KLP3A^{835}*, and *KLP3A^{1611}* mutations, no protein of this size is detectable (lanes *2–4*, respectively). Faint bands specific to these mutant strains are visible in overexposures of the same immunoblot (not shown); we presume these represent unstable forms of mutant KLP3A proteins. (*B*) Restoration of KLP3A protein levels by the fragment B transgene. Whereas no KLP3A protein is seen in *KLP3A^{e4}* embryos (*A*, lane 2), the addition of fragment B allows accumulation of the KLP3A protein (lanes *2* and *3*) to wild-type levels (lane *1*). Two independently derived insertions of the transgene ([B]^1 and [B]^2) were used. (*C* and *D*) Reaction of the filters depicted in *A* and *B*, respectively, with antibodies to a 100-kD *Drosophila* protein identified by an immune serum unrelated to KLP3A as a loading control.

et al., 1986; Williams and Goldberg, 1994]), were indistinguishable. In contrast to wild-type, testes mutant for several *KLP3A* alleles showed a complete or near-complete absence of antibody staining in the structures described below (Fig. 6). This staining reappeared to wild-type levels when mutant strains also carried the *KLP3A*+ transgene (data not shown). These results verify that the signal we observed indeed reflects the distribution of KLP3A protein and not any cross-reacting species. In our analysis, we have assigned developmental stages to particular cells based upon morphological criteria described by several previous studies of spermatogenesis in *Drosophila* (Fuller, 1993; Cenci et al., 1994).

The KLP3A protein is exclusively located within the nucleus throughout the growth of the primary spermatocyte to its maturity (stages S0 to S5 according to the stage terminology of Cenci et al., 1994; Figs. 7 *a* and 8, *A* and *B*). This intranuclear distribution during meiotic prophase appears mostly uniform, but in many cells, a higher level of staining is observed in the diffuse chromosomal bivalent clumps (Fig. 8, *A* and *B*). During late prophase and early prometaphase of the first meiotic division, concomitant with the migration of centrosomes and bivalent condensation (stage Mla and Mlb), KLP3A protein remains in the nucleus. However, the

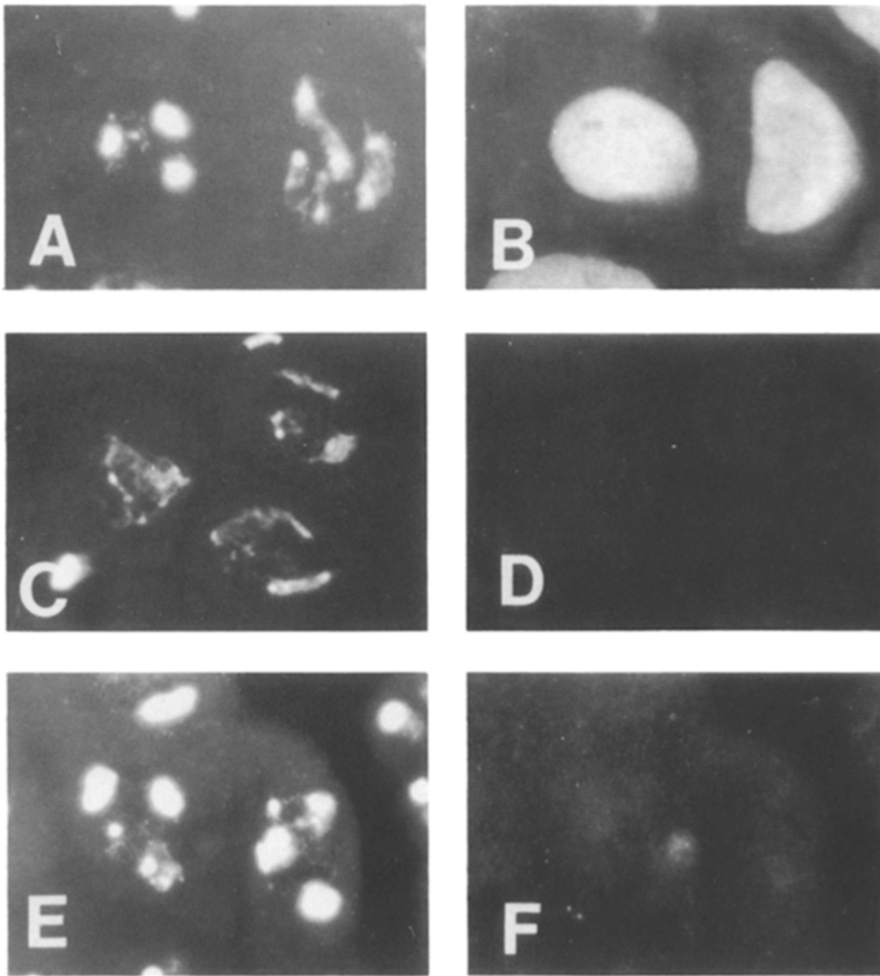

Figure 6. KLP3A mutants abolish staining by KLP3A antibodies. Testes were fixed and stained to visualize DNA (*A*, *C*, and *E*) and KLP3A antigen (*B*, *D*, and *F*). (*A* and *B*) Wild-type primary spermatocytes at stages S5 (*right*) and M1 (*left*) according to Cenci et al. (1994), exhibiting high levels of KLP3A antigen within their nuclei. (Also see Figs. 7 and 8 below.) (*C–F*) Mutant spermatocytes showing lack of nuclear staining with KLP3A antibodies. (*C* and *D*) $KLP3A^{e4}$ spermatocytes at approximately stage S5. (*E* and *F*) $KLP3A^{1611}$ spermatocytes at stage M1.

relative concentration on the bivalents observed earlier is no longer evident; in fact, KLP3A even appears to be excluded from the chromosomes (Figs. 7 *b* and 8, *C* and *D*).

In late prometaphase (stage M2), KLP3A protein becomes dispersed into the cytoplasm so that the exclusive nuclear localization is lost (Figs. 7 *b* and 8, *E* and *F*) The dispersion of KLP3A protein coincides with the disappearance of the nuclear–cytoplasmic demarcation, as visualized by phase contrast microscopy (Cenci et al., 1994). The distribution of KLP3A between metaphase I and mid-anaphase I (stages M3 through M4b) remains diffuse throughout the cell; no colocalization with either the spindle or chromosomes is observed (Figs. 7 *b* and 8, *E* and *F*).

In late anaphase (stage M4c), the protein distribution again becomes distinct as a wide, striated band extending across the midzone of the central spindle (Fig. 7 *c*). The appearance of KLP3A protein in this region coincides precisely with the first visible constriction of the midzone at late anaphase (Fig. 7, *c* and *d*). As late anaphase progresses into telophase (stages M4c to M5), the meiotic spindle becomes "pinched" at the midbody by the contractile ring during cytokinesis (Cenci et al., 1994). During this time, KLP3A becomes even more sharply concentrated at the midbody region (Fig. 7 *e*).

After completion of the first meiotic division, KLP3A becomes dispersed so that interphase secondary spermatocytes (stages M6a-M6b) again show a diffuse pattern of KLP3A staining throughout the cell. In contrast to interphase primary spermatocytes, KLP3A antigen does not appear to be constrained to the nuclear domain (Fig. 7 *f*). In the second meiotic division, like the first meiotic division, KLP3A is not localized to discrete structures from prometaphase (M7) through mid-anaphase (stage M10a; Fig. 7 *g*). Again, KLP3A staining becomes distinct during late anaphase II (M10b-c) and telophase II (M11), when the concentration of KLP3A at the midbody is once more clearly apparent (Fig. 7 *g*). The antibody produces a weak signal within spermatid nuclei during the onion stage that occurs immediately after completion of meiosis II. Staining persists through spermatid elongation, but disappears in mature sperm (not shown).

We have also examined the distribution of KLP3A protein during mitotic divisions in the male germline (data not shown). Four successive spermatogonial mitotic divisions originating from a germline stem cell take place before primary spermatocyte growth (Fuller, 1993). During these divisions, KLP3A protein appears to follow broadly the same dynamic localization as in meiosis. Interphase spermatogonia contain KLP3A exclusively in the nucleus but not as-

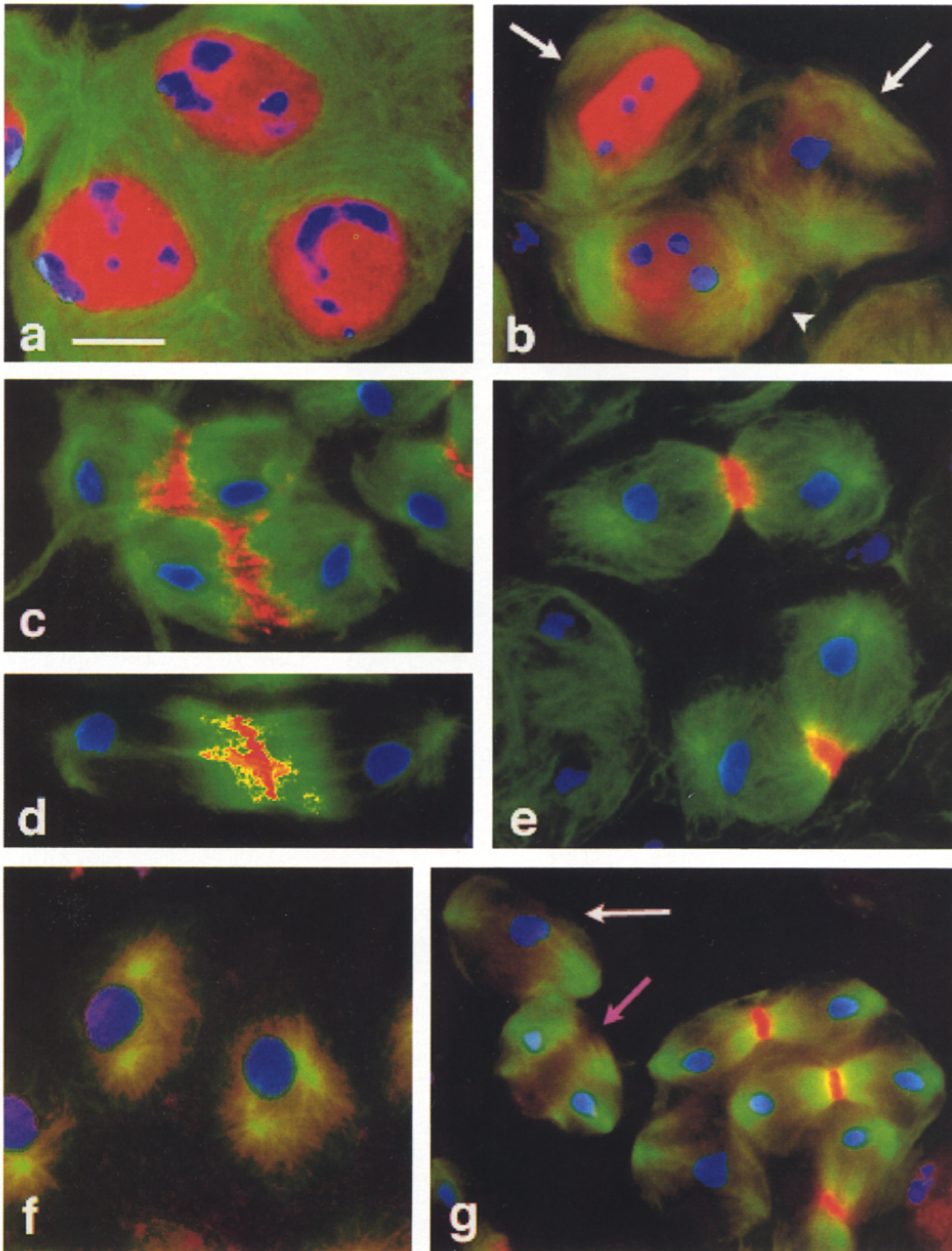

Figure 7. Localization of KLP3A protein during male meiosis. Wild-type testes were fixed and stained for indirect immunofluorescence to visualize KLP3A protein (*red*), microtubules (*green*), and chromosomes (*blue*) during the two meiotic divisions. Yellow- and orange colors indicate overlap of KLP3A and microtubule signals. (*a*) Three mature primary spermatocytes at the S5 stage where chromatin has not fully condensed and the spindle has not formed: KLP3A protein appears at high levels confined to the nuclear domain. (*b*) Prometaphase-metaphase transition. At early prometaphase I (stage M1b; *arrow, top left*) chromosomes have condensed into bivalents but have not congressed. KLP3A remains in the nucleus as in *a*. Later in prometaphase (stage M2; *arrowhead, bottom*) when the bipolar spindle is formed and the nuclear–cytoplasmic demarcation has disappeared (Cenci et al., 1994), some KLP3A protein moves into the cytoplasm. At metaphase (stage M3; *arrow, top right*), when bivalents have fully congressed, the KLP3A protein is dispersed evenly throughout the

sociated with chromosomes. Furthermore, KLP3A protein moves to the midbody during telophase of these gonial mitotic divisions.

Discussion

Requirements for KLP3A+ Function Vary in Development

The only phenotypic consequences of mutations in the *KLP3A* gene that we have detected are seen in the embryonic progeny of homozygous mutant mothers, and in the testes of mutant males, as detailed in this report. Even though expression of KLP3A protein is not restricted to the germline (for example, KLP3A staining is seen in larval brains; data not shown), animals can develop to adulthood without synthesizing wild-type KLP3A protein. These findings suggest that KLP3A product serves as a member of a network of related KLPs that perform overlapping functions. Most somatic cells in *Drosophila* would be relatively insensitive to loss of wild-type KLP3A, as redundant proteins could fulfill the same role. The requirement for *KLP3A*+ activity in embryos and spermatocytes could reflect either tissue-specific variations in the amounts of the various members of the network, or the occurrence in these cell types of processes more dependent upon KLP3A protein. There is ample precedent for the idea that various KLPs can in fact provide overlapping functions (Hoyt et al., 1992).

The Intracellular Distribution of KLP3A Protein

The KLP3A gene product undergoes rapid redistribution during many phases of the cell cycle. We do not yet understand how these movements are related to the function of this protein. The intranuclear location of KLP3A in interphase spermatogonia, in primary spermatocytes during the growth phase, and very weakly in onion stage spermatids probably reflects the existence of consensus nuclear localization signals in the middle, stalk-like part of this protein. Two other KLPs, MKLP-1/CHO1 (Nislow et al., 1992), and XKLP1 (Vernos, I., and E. Karsenti, personal communication), which are also found in the nucleus of interphase cells, contain such motifs. It is of interest that this intranuclear interphase localization is developmentally regulated: KLP3A protein appears to be excluded from the nuclei of interphase secondary spermatocytes (Fig. 7 *f*) and from the nuclei of syncytial blastoderm embryos (our unpublished results). The cell cycles in these latter cell types are unusual, as there is no S phase (DNA synthesis) in secondary spermatocytes, and the regulation of embryonic cell cycles is generally less stringent than in other cells. Moreover, interphase in secondary spermatocytes and blastoderm nuclei is much shorter than in primary spermatocytes, which might not allow sufficient time for KLP3A to move back to the nucleus. Both the physiological rationale for differences in KLP3A localization, and the mechanisms that alternatively prevent or allow nuclear targeting of this protein, remain obscure at present.

Our immunofluorescence results indicate that the KLP3A product diffuses throughout the cell at metaphase and early anaphase. Later in anaphase and during telophase, the KLP3A protein relocalizes exclusively to the spindle midbody. We speculate that KLP3A first attaches to microtubules throughout the spindle and then rapidly migrates to the midbody. The structure of the KLP3A product, with an NH_2-terminal motor domain, suggests that this protein is likely to be a mechanochemical motor moving toward the plus ends of microtubules (Endow and Titus, 1992; Goldstein, 1993). However, this assumption about the biochemical properties of KLP3A remains to be verified by in vitro experiments.

The KLP3A gene product can be added to a growing list of proteins that localize to the spindle midbody. Most of the known midbody components have been proposed to fall into a class of "chromosomal passenger proteins" that associate with chromosomes (particularly at the centromere/kinetochore) early in mitosis, then move to the spindle midbody by telophase (reviewed by Earnshaw and Bernat, 1991). At least two other KLPs, MKLP-1/CHO1 (Nislow et al., 1992), and CENP-E (Yen et al., 1992), have been classified as such chromosomal passengers, and at least some proportion of the intracellular complement of the related XKLP1 also follows this pattern of distribution (Vernos, I., and E. Karsenti, personal communication). However, in contrast with these proteins, the KLP3A gene product is excluded from the chromosomes just prior to its movement to the midzone or midbody (see above). It thus appears unlikely that the KLP3A product could directly transport material from the chromosomes to the midbody.

The KLP3A Protein, the Central Spindle, and Anaphase B

Although the KLP3A protein is required for the integrity of the central spindle as a whole, it is localized during late anaphase and telophase exclusively to the midbody, which comprises only a small part of the midzone. At the present time, we can only guess at the function of KLP3A in central spindle assembly. It is possible that the protein migrates along polar microtubules toward their plus ends, bundling antiparallel microtubules as it moves. However, in regions of the central spindle exclusive of the midbody, these microtubules must subsequently remain bundled along their lengths

nucleus and cytoplasm. (*c* and *d*) Cells in late anaphase I showing the localization of KLP3A at the equator of the prominent central spindle formed by interdigitated microtubules. The anaphases in *c* are in the M4c/M4d stage, while the anaphase in *d* is in the late M4c stage. (*e*) Two telophase I divisions (M5 stage) exhibiting the concentration of KLP3A at the spindle midbodies. The centrosomes have already duplicated in preparation for the second meiotic division. (*f*) Two secondary spermatocytes at interphase between the first and second meiotic divisions (stage M6b). The duplicated asters have begun to migrate towards opposite ends of the nuclei. In contrast to *a*, KLP3A protein appears dispersed in the cytoplasm. (*g*) Metaphase to telophase during meiosis II. KLP3A protein is dispersed throughout the cell during metaphase II (stage M9; *white arrow, top*) and early anaphase II (stage M10a; *purple arrow*). At telophase II (stage M11), KLP3A is sharply localized to the midbody region (cells on right). Bar, 10 μm.

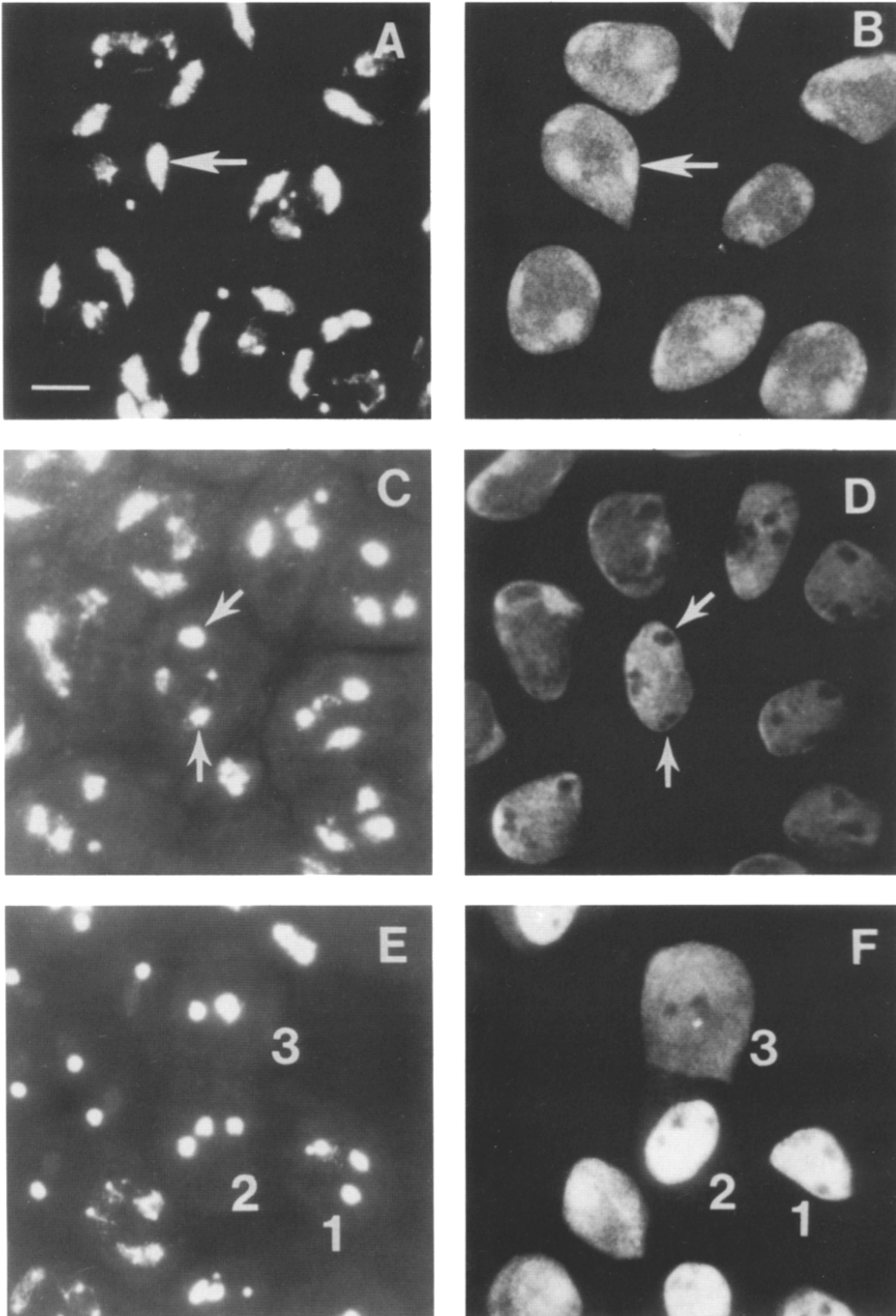

Figure 8. Localization of KLP3A protein from meiotic prophase I to metaphase I. Fixed cells were simultaneously stained with Hoechst 33258 for DNA (*A*, *C*, and *E*) and with anti-KLP3A (*B*, *D*, and *F*). (*A* and *B*) KLP3A occupies the nuclear domain in primary spermatocytes during early meiotic prophase I (stage S5). Additional accumulation of KLP3A antigen can often be visualized on the uncondensed chromosomal bivalents (*arrows*). (*C* and *D*) KLP3A protein, though still within the nucleus, is excluded from chromosomes during late prophase

in the absence of KLP3A protein. Another possibility is that KLP3A at the midbody in some fashion recruits microtubules to the central spindle, and other molecules would be responsible for their bundling. Finally, KLP3A may transport other molecules to the midzone or midbody that could function in either of these roles.

The absence of a dense central spindle during meiosis in *KLP3A* mutant spermatocytes has little obvious effect on anaphase B spindle elongation. We believe this finding provides strong support for the hypothesis that spindle elongation in higher cells can be powered by pulling forces exerted upon the asters through the astral microtubules (Aist et al., 1993; Waters et al., 1993). In this view, pushing forces associated with sliding of the central spindle are not essential for anaphase B, but might instead act as a ratchet that regulates the process. This interpretation of our results must be tempered by the caveat that there appears to be a small amount of interaction between microtubules emanating from the two hemispindles in mutant ana/telophases (Fig. 4). It remains possible that molecular motors associated with this minor component of overlapping microtubules might still create pushing forces through the central spindle, but we would anticipate these forces would be much diminished relative to normal.

Our results appear to be at variance with previous experiments that convincingly show that poles are pushed apart during anaphase B by the sliding of interdigitated central spindle microtubules in diatoms (Cande and McDonald, 1985) and *Saccharomyces cerevisiae* (Sullivan and Huffaker, 1992). However, recent experiments with cells derived from higher eukaryotes support our finding that the central spindle is expendable for anaphase B spindle elongation. (*a*) Physical severing of the central spindle appears to actually increase the rate at which the poles move apart (Kronebusch and Borisy, 1982; Aist et al., 1991). (*b*) Newt cell hemispindles can move independently (Bajer and Mole-Bajer, 1981; Waters et al., 1993). (*c*) Irradiation or removal of asters blocks anaphase B movements at the damaged pole, but speeds up motion at the other pole (Hiramoto et al., 1986; Aist et al., 1993). (*d*) Reconstructions of serial sections through spindles indicate that most interpolar microtubules dissociate from the poles during anaphase, so force could not be directly transmitted from the central spindle to the poles via these microtubules (Mastronarde et al., 1993). Along with the findings reported here, these investigations suggest at a minimum that pulling forces on the spindle poles are sufficient for anaphase B spindle elongation. The central spindle may contribute to this process, but it is non-essential.

KLP3A Function and Cytokinesis

Our results clearly show that mutations in the *KLP3A* gene lead to the appearance of spermatids containing multiple nuclei associated with Nebenkern (mitochondrial derivatives) of greater than normal size. In accordance with other investigators (Fuller et al., 1988; Casal et al., 1990; Castrillon et al., 1993), we have interpreted these aberrations as resulting from failures in cytokinesis during the two meiotic divisions. In support of this interpretation, it appears that cleavage furrows fail to form subsequent to either meiotic division in the strongest mutant lines. This suggests in particular that mutant spermatocytes are deficient in the signal that initiates furrow formation.

How might the lack of the central spindle in *KLP3A* mutant spermatocytes disrupt cytokinesis? It is of course possible that such defects in cytokinesis are a nonspecific problem of spindle assembly. However, it must be remembered that many aspects of spindle function, including chromosome disjunction and anaphase B elongation (see above) appear unaffected in *KLP3A* mutant spermatocytes. We instead believe that the midzone or midbody may normally play an essential role in providing the stimulus for cleavage furrow formation.

There are several precedents for this concept. It has long been clear that the mitotic apparatus is the source of the stimulus dictating assembly of the contractile ring equidistant between the spindle poles. Once this signal has been given, the mitotic apparatus is no longer required for furrowing to occur. In the large cells of amphibian embryos, the asters at the poles determine the position of the cleavage furrow (reviewed by Rappaport, 1986). However, certain micromanipulation experiments indicate that in mammalian cells, this signal instead emanates from the central spindle (Rappaport and Rappaport, 1974; Kawamura, 1977). Our results provide strong support for this view. One interesting mechanistic possibility is that the midzone or midbody elaborates a "telophase disk" that extends perpendicularly to the axis of division (Andreassen et al., 1991). When this disk reaches the cell cortex, it would signal (or become a part of) the cleavage machinery. Proteins whose intracellular distribution during anaphase and telophase are consistent with their localization in such a telophase disk have recently been described (Andreassen et al., 1991; Jiménez and Goday, 1993).

The KLP3A Protein May Have Additional Functions

Regardless of the precise mechanisms by which *KLP3A* mutations disrupt the central spindle and cytokinesis during meiosis in males, it is likely that these cannot be the only processes in which the KLP3A protein participates. Homozygous *KLP3A* mutant mothers yield embryos that arrest very early in their development, at some stage after metaphase of the first meiotic division but prior to anaphase of the first mitotic division. This arrest is unlikely to involve the central spindle, since meiotic spindles in the female have no clearly discernable central spindle, and the mutant embryos clearly arrest before a mitotic central spindle is formed. Moreover, it should be remembered that no cytokinesis takes place in the syncytium of the early embryo. Thus, the developmental arrest cannot result from an effect of *KLP3A* mutations on cytokinesis. To understand the gamut

(stage M1a; *arrows*). (*E* and *F*) As cells progress from early prometaphase I (stage M1b; cells *1* and *2*) to late prometaphase I (stage M2; cell *3*), the nuclear location of KLP3A is replaced by a uniform distribution of KLP3A protein in both nuclear and cytoplasmic compartments (also see Fig. 7). Again, KLP3A is excluded from chromosomal regions during these stages. Bar, 10 μm.

of roles played by the KLP3A protein, we are currently attempting to characterize this embryonic phenotype in greater detail.

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