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The *Drosophila* kinesin-like protein KLP3A is required for proper behavior of male and female pronuclei at fertilization

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SUMMARY

Drosophila melanogaster **females homozygous for mutations in the gene encoding the kinesin-like protein KLP3A are sterile (Williams et al., 1995). We have investigated the basis of this sterility. The eggs produced by *KLP3A* mutant mothers are fertilized by sperm, and female meiosis appears to occur normally. However, the large majority of these embryos arrest their development soon thereafter with a characteristic phenotype. The four nuclei produced by female meiosis associate together in a polar body-like structure, while a bipolar spindle is established around the metaphase-arrested male pronucleus. Thus, the major defect caused by depletion of the KLP3A protein is either in specification of the female pronucleus, or in migration of the male and female pronuclei toward each other. We have also found that the KLP3A protein is located throughout the metaphase spindle during meiosis and the early embryonic mitotic divisions, but later accumulates specifically at the midzone of these same spindles during telophase. The protein is also present on two other microtubule structures: the sperm aster; and the radial, monastral array of microtubules established between the two meiosis II spindles. We discuss these results in light of possible functions of the KLP3A protein in pronuclear specification and migration.**

Key words: kinesin-like protein, *Drosophila*, embryo, pronuclear migration

INTRODUCTION

The process of fertilization leading to formation of a zygote requires several unique behaviors of haploid nuclei. The maternal and paternal pronuclei must arrive in a common cell, recognize and migrate toward each other, fuse, and together enter the diploid mitotic cell cycle. Very little is currently known about the molecular basis of these initial events in development. Here, we describe our investigations revealing a critical role for a *Drosophila* microtubule motor protein of the kinesin superfamily in the proper conduct of the male and female pronuclei prior to their fusion.

In the ovaries of *Drosophila melanogaster* females, mature eggs are arrested at metaphase of the first meiotic division (Sonnenblick, 1950). Because unfertilized eggs can complete meiosis, the trigger for meiotic maturation appears not to be sperm penetration, but rather passage through the oviduct (Doane, 1960). The two meiotic divisions are completed very rapidly within 15 minutes of ovulation, producing four haploid nuclei that typically are arranged in a line perpendicular to the long axis of the egg. Normally, the three nuclei closest to the egg cortex become polar bodies. These polar body nuclei apparently undergo DNA replication (Rabinowitz, 1941), but then arrest in a metaphase-like state. During the first few mitotic divisions of the zygotic nuclei, the three polar bodies often fuse together, eventually disintegrating later in embryogenesis. The remaining, innermost meiotic product becomes the female pronucleus. The female pronucleus appears to migrate toward the male pronucleus along microtubules of the sperm aster, which is nucleated from the centrosomes associated with the male pronucleus. During the first mitosis, the maternal and paternal sets of chromosomes remain in separate 'gonomeric' groupings, so fusion of the parental genomes does not occur until telophase of this first division (Sonnenblick, 1950; Callaini and Riparbelli, 1996; for a detailed discussion of these earliest events in *Drosophila* development see Foe et al., 1993).

We have previously found that mutations in the *KLP3A* gene, which encodes the novel kinesin-like protein KLP3A, cause male and female sterility in *Drosophila*. In particular, we observed that *KLP3A* mutant females are sterile because they lay eggs that arrest development very soon after fertilization (Williams et al., 1995). We report in this paper our detailed analysis of the maternal effect phenotypic consequences associated with *KLP3A* mutations. The most obvious defect associated with mutations at this locus is in pronuclear migration: most embryos arrest after the completion of meiosis with separated paternal and maternal genetic complements. In the

arrested embryos, the four products of female meiosis fuse together into a single polar body, while a metaphase-like spindle is organized around the male pronucleus. We have also observed less common aberrations in meiotic and mitotic divisions that suggest the KLP3A protein may play a subsidiary role in spindle function. We discuss these results in the light of our findings on the cell cycle-dependent intracellular distribution of the KLP3A protein in early embryos, and with respect to our previous studies on the utilization of this molecule during meiosis in *Drosophila* males (Williams et al., 1995).

MATERIALS AND METHODS

Stocks

Construction of the *KLP3A^{e4}* stock has been described by Williams et al. (1995). The mutant *KLP3A* alleles *KLP3A^{521}*, *KLP3A^{835}*, *KLP3A^{1124}*, *KLP3A^{1161}* and *KLP3A^{2096}* were induced by ethylmethansulphonate (EMS) in a *y cv v f* background and had been originally named as alleles of the female-sterile complementation group *fs(1)M4* (Mohler, 1977; Mohler and Carroll, 1984). The *mei-218^{a4}* stock, which was employed to obtain post-metaphase I meiotic figures (McKim et al., 1993) was provided by Kim McKim (University of California, Davis, CA). *Df(1)54* is a deficiency from *zeste* to *zw13* and deletes *KLP3A* (Goldberg et al., 1989). 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* (for further explanation of chromosomes and genetic symbols employed see Lindsley and Zimm, 1992). The wild-type strain used was Oregon-R.

Fertility tests

Females to be tested were mated to wild-type males for several days prior to egg collections. Eggs were laid on agar-based media containing grape juice (Elgin and Miller, 1980); a drop of wet yeast was placed in the center of the plate to induce egg-laying. To determine the fraction of eggs hatched, flies were allowed to lay eggs overnight onto the grape plates. On the following day, the flies were removed and the eggs were left to develop for 24 hours. During this time the first instar larvae crawl to the wet yeast in the center of the plate, leaving the eggs (which are primarily laid at the periphery) undisturbed. Hatched eggs, as evidenced by their empty shells, and unhatched eggs were then counted to determine hatching rate.

Tests for meiotic nondisjunction

KLP3A mutant virgin females homozygous for *spa^{pol}* were crossed to *Y^{s}X·YL, In(1)EN; C(4)RM, ci ey^{R}* males (for further details see Baker and Carpenter, 1972). Although the vast majority of embryos laid by these females arrest very early in development (see Results), rare escaper adult flies emerge from these crosses. These progeny were scored for the presence of visible markers on the X and 4th chromosomes to detect meiotic nondisjunction, as described by Baker and Carpenter (1972).

Feulgen/Giemsa staining of eggs and early embryos

A Feulgen/Giemsa double staining technique (Puro and Nokkala, 1977) was used for visualizing the first meiotic metaphase in mature (stage 14) oocytes. All procedures were performed at room temperature unless otherwise noted. Female abdomens were dissected in Ringer's solution, transferred to 75 mM KCl for 10 minutes, and fixed for 2 hours in Carnoy fixative (99% ethanol, chloroform and glacial acetic acid, in a ratio of 6:3:1). Fixed abdomens were transferred to 99% ethanol for 2 hours, and then rehydrated via 70% ethanol (overnight at 4ºC), 50% ethanol (5 minutes), and 30% ethanol (3 minutes) to distilled water. After soaking in 1 N HCl, the abdomens were carried through the Feulgen procedure, including an 8 minute hydrolysis at 60°C and a 4 minute staining with modified Feulgen reagent containing 8 ml of 1 N HCl and 0.8 g $K_2S_2O_5$ for 200 ml of staining solution. The abdomens were then transferred to distilled water. Four to six eggs were removed from fixed and stained abdomens, and transferred to a drop of water on a microscope slide. Eggs were cut in half, bisecting the long axis. The posterior half, the chorion, and excess water were removed from the slide, and a drop of 45% acetic acid was added. Squashing was achieved by placing a coverslip over the drop without added pressure. After a quick preview in the phase contrast microscope, the slide was frozen on dry ice and the coverslip was removed. Slides were dehydrated in 99% ethanol, then immersed in glacial acetic acid for 20 seconds, and air dried. The final staining was carried out by a 30 minutes treatment with 4% Giemsa (Merck No. 9204) solution in phosphate buffer, pH 6.8. Slides were made permanent after a rinse in distilled water, drying and mounting in Entellan (Merck No. 7960).

To collect eggs in meiotic or early embryonic stages, well-fed, inseminated females were allowed to lay eggs in fresh culture bottles overnight before they were etherized. Eggs were squeezed out of the uteri of etherized females, and immediately fixed for 30-60 minutes in Carnoy fixative (see above) that was modified by replacing 99% ethanol with 80% ethanol. Roughly an equal amount of 99% ethanol was added, and fixing was allowed to continue overnight. Eggs were hydrated and carried through the Feulgen/Giemsa procedure described above, with the exception that only one egg with both halves was prepared per slide.

A modification of this technique was used specifically for collecting eggs that were in meiotic or early postmeiotic stages. After fixation and rehydration, the eggs were transferred to 2 N HCl for 2 hours, and then subsequently removed to distilled water. Eggs that were at early stages became transparent, while older eggs turned opaque. Transparent eggs were cut and squashed on slides without a preceeding Feulgen treatment. After removal of the coverslip and dehydration in 99% ethanol, the slides were air dried and stored at 37°C. Slides were stained by a procedure including hydrolysis at 60°C for 8 minutes, treatment with the modified Feulgen reagent for 10-15 minutes, 3 washes in distilled water, a 15 minute postfixation in a 3:1 mixture of ethanol and glacial acetic acid, air drying, and a 30 minute treatment with 4% Giemsa.

Immunostaining

For examination of embryonic phenotypes, embryos were collected at timed intervals and fixed in methanol-EGTA (Warn and Warn, 1986; Hatsumi and Endow, 1992). Embryos were collected on yeasted plates, rinsed in egg wash buffer (0.4% NaCl, 0.2% Triton X-100), and dechorionated in 50% Chlorox bleach for 2 minutes. They were then transferred to heptane and swirled for 15 seconds, followed immediately by the addition of an equal volume of methanol-EGTA (15 mM, pH 6.0) and shaken vigorously for an additional 20 seconds. Fixed, devitellinized embryos sank to the bottom of the lower methanol layer. Embryos were then washed three times (5 minutes each) with methanol/EGTA, followed by three 10 minutes washes in Tris-buffered saline + Triton (TBST; 50 mM Tris-HCl [pH 7.4], 50 mM NaCl, 0.02% sodium azide, 0.1% Triton X-100). RNAse (DNAse-free; Boehringer Mannheim, Indianapolis, IL) was added to antibody incubations at a concentration of 1 μg/ml if embryos were to be later stained with YOYO-1 (see below). A monoclonal anti-β-tubulin antibody (Calbiochem, La Jolla, CA) or monoclonal anti-sperm tail antibody (Karr, 1991) were used at a 1:10 dilution in TBST overnight at 4°C, followed by three 5 minute washes in TBST, and incubation for 2 hours at room temperature with TRITC-conjugated anti-mouse IgG antibodies (7.5 μg/ml in TBST; Jackson Immunoresearch, West Grove, PA). Nuclei were stained with Hoechst 33258 (Sigma Chemical Co, St. Louis, MO) at a concentration of 0.05 μg/ml for 5 minutes, and with YOYO-1 (benzoxazolium-4-quinolinium dimer) iodide (Molecular Probes, Eugene,

OR) at 0.2 μM for 5 minutes, after which embryos were extensively washed with TBST to reduce background.

To study female meiosis I, oocytes were isolated and fixed in formaldehyde according to protocols of Theurkauf and Hawley (1992), and stained to visualize tubulin and DNA as described above.

For immunolocalization of KLP3A protein in early embryos, wild-type embryos were fixed in formaldehyde and methanol according to the method of Karr and Alberts (1986). To examine KLP3A localization in female meiosis I, unactivated wild-type oocytes and oocytes isolated from *mei-218*^{a4} females (McKim et al., 1993) were fixed according to the method of Theurkauf and Hawley (1992). To examine KLP3A in meiosis II and with respect to the sperm aster, embryos were collected at 15 minute intervals and fixed with methanol-EGTA as described above. KLP3A antibodies were used exactly as detailed by Williams et al. (1995).

Confocal microscopy was carried out using a Zeiss Axiovert 10 attached to a Bio-Rad MRC600 confocal imaging system equipped with a Krypton/Argon laser (Bio-Rad Laboratories, Cambridge, MA) at the Cornell Biotechnology Flow Cytometry and Imaging Facility. Image collection was performed utilizing low laser settings to attenuate photobleaching, and assisted by Kalman averaging to improve the signal/noise ratio. Double fluorescence images were collected simultaneously as two full screen images and merged in pseudocolor using COMOS software (Bio-Rad). 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.

Fluorescence in situ hybridization (FISH)

To determine the chromosome constitution of nuclei within arrested embryos, fluorescence in situ hybridization (FISH) was performed using probes specific for the *Drosophila* sex chromosomes. Embryos were fixed as above with methanol/EGTA and carried through the FISH procedure described in detail by Dernburg et al. (1996), and briefly outlined here. The X-chromosome probe was the 359-bp satellite, which was amplified from genomic DNA using primers designed from the published sequence (Hsieh and Brutlag, 1979). This PCR product was digested with *Alu*I or Tsp509I (New England Biolabs, Beverly, MA) and 3′-end labeled with biotin-14-dCTP (Gibco-BRL, Grand Island, NY) as described previously (Dernburg et al., 1996). As a probe for the Y chromosome, an oligonucleotide comprising the sequence $(AATAC)_7$ was synthesized on a MilliGen Cyclone DNA synthesizer and end-labeled with digoxigenin-11-dUTP (Boehringer Mannheim).

Following hybridization, the embryos were washed in 2× SSCT (0.3 M NaCl, 0.03 M sodium citrate, 0.1% Tween-20), blocked with 0.5% (w/v) bovine serum albumin, and stained with rat monoclonal anti-tubulin antibodies (Sera-Lab, Sussex, UK). Secondary detection of the hybridization probes and anti-tubulin antibody was performed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-rat antibodies (Jackson Immunoresearch), rhodamine-conjugated anti-digoxigenin F(ab) fragments (Boehringer Mannheim), and Cy5-conjugated streptavidin (Jackson Immunoresearch). The embryos were then counterstained with diamidinophenylindole (DAPI), mounted in VectaShield mounting medium (Vector Laboratories, Burlingame, CA) and imaged using wide-field optical sectioning microscopy (Dernburg et al., 1996). Images were processed using an iterative, constrained deconvolution algorithm and are presented here as volume-rendered projections of three-dimensional data stacks.

RESULTS

Mutations in *KLP3A* result in non-rescuable maternal effect lethality

Our appreciation for a role of the *KLP3A* gene in female fertility first came from examination of the synthetic allele *KLP3A*^{e4} (Williams et al., 1995). This lesion in *KLP3A* was made by combining a deletion that removes sequences encoding both the C-terminal 82 amino acids of KLP3A and part of the adjacent gene *l(1)zw4*, with a transgene that rescues mutations in *l(1)zw4*. We subsequently found that five EMS-induced mutations in the formerly described locus *fs(1)M4* (Mohler, 1977; Mohler and Carroll, 1984) were alleleic to *KLP3A*^{e4}, and we have renamed these *KLP3A*^{521}, *KLP3A*^{835}, *KLP3A*^{1124}, *KLP3A*^{1611} and *KLP3A*^{2096} (for details see Williams et al., 1995).

As shown in Table 1, the large majority (>99%) of eggs laid by mothers homozygous for *KLP3A*^{e4}, *KLP3A*^{1611} and *KLP3A*^{2096} that are mated to wild-type males fail to hatch into larvae. The effects of *KLP3A*^{835} are much less pronounced. We presume that this latter mutant is weakly hypomorphic, though we cannot exclude the possibility that this stock contains extragenic phenotypic modifiers of the sterility. Females homozygous for the remaining two alleles (*KLP3A*^{521}and *KLP3A*^{1124}) produced insufficient eggs for analysis, probably due to background mutations affecting oogenesis or oviposition. We have established that maternal effect sterility is the direct consequence of mutations in *KLP3A* by two criteria. (1) More than 97% of the eggs laid by all of the transheterozygotes analyzed (here, females one of whose X chromosomes carries *KLP3A*^{e4} while the other X chromosome contains one of the EMS-induced *KLP3A* mutations) also fail to hatch (Table 1). (2) The addition of a transgenic copy of *KLP3A*$^{+}$ (Williams et al., 1995) to either homozygous or transheterozygous mothers restores the viability of the embryos they produce to near wild-type levels (Table 1).

By classical genetic tests, *KLP3A*^{e4}, *KLP3A*^{1611} and *KLP3A*^{2096} appear to represent the null or near-null state of the gene. The proportions of non-hatching embryos laid by homozygotes are identical to those laid by females transheterozygous for these alleles over a deficiency for the chromosomal region including the *KLP3A* gene (Table 1). In the case of *KLP3A*^{e4}, this supposition is supported by molecular evidence. We see no KLP3A-reactive bands on western blots of embryos laid by *KLP3A*^{e4} homozygous mutant mothers (Williams et al., 1995). Because the *KLP3A*^{e4} allele would be expected to encode a truncated form of the KLP3A protein, it appears that these aberrant KLP3A molecules are highly unstable.

To determine the stage at which the embryos laid by strong *KLP3A* mutants arrest development, we stained collections of these eggs with Hoechst 33258 to visualize the nuclei (Table 1). The large majority of the embryos appeared to undergo very few if any nuclear divisions. However, a significant minority of embryos (roughly 10%) could develop past this point. In such embryos at late syncytial blastoderm stages, mitotic defects were commonly seen, such as disorganized spindles, unequal spacing of nuclei, and free centrosomes (data not shown). At gastrulation, embryos typically displayed a range of apparently nonspecific defects accompanied by cellular degradation (not shown). Examination of the cuticles of fully developed but unhatched embryos revealed variable defects in cuticle formation and incomplete segmentation. However, some rare embryos (about 1% of total eggs) were ‘escapers’ that hatched into larvae, almost all of which eventually became normal adults.

The emergence of these adult escapers is independent of any paternally supplied *KLP3A*$^{+}$ gene, since male and female

Table 1. Development of embryos laid by klp3A mutant females

	Eggs hatched (%)		Developmental stage (%)					
Genotype	%	n	E	EC	SB	CB	L	n
Wild-type	96.6	1630	5.9	4.1	6.0	2.4	81.6	414
e4/e4;[B]	0.2	1008	91.8	0.0	1.1	0.1	7.0	823
1611/1611	0.9	1020	87.9	1.3	1.7	0.0	9.1	203
2096/2096	0.0	410	93.8	0.6	1.2	0.2	4.2	162
835/835	53.3	450	8.7	1.1	13.0	3.6	73.6	277
e4/521	1.2	1934	93.2	0.0	0.0	0.0	6.8	133
e4/835	2.9	463	90.3	0.8	0.8	0.3	7.8	382
e4/1124	2.3	645	80.0	0.0	0.6	1.7	17.7	179
e4/1611	1.7	415	96.2	0.0	0.4	0.9	2.5	235
e4/2096	0.2	430	97.5	0.4	0.0	0.0	2.1	236
e4/e4;[B];[D]	81.4	620	13.3	1.0	2.1	0.7	82.9	286
e4/521;[D]	80.5	145	15.5	1.9	2.9	4.9	74.8	103
e4/835;[D]	84.4	450	3.4	1.2	1.2	0.0	94.2	86
e4/1124;[D]	84.8	105	19.4	0.0	1.5	1.5	77.6	67
e4/1611;[D]	84.5	110	7.9	1.6	0.0	0.0	90.5	63
e4/2096;[D]	80.1	555	5.7	0.0	1.4	0.0	92.9	70
1611/1611;[D]	89.0	262	13.5	3.4	5.6	2.2	75.3	89
2096/2096;[D]	87.7	285	17.4	1.4	2.9	5.8	72.5	69
e4/Df;[B]	0.2	1520	79.9	0.7	0.7	0.0	18.7	134
521/Df	1.2	510	83.3	0.4	1.3	0.0	15.0	226
835/Df	0.7	144	69.1	1.7	3.4	1.7	24.1	58
1124/Df	0.5	195	57.1	9.5	2.3	2.3	28.8	42
1611/Df	1.1	370	57.8	1.9	1.9	1.9	36.5	52
2096/Df	0.1	1585	48.7	2.1	6.4	0.7	42.1	140
e4/FM7a;[B]	85.0	453	14.4	0.5	5.1	1.8	78.2	1707
521/FM0	81.5	314	22.4	4.1	4.1	4.1	65.3	49
835/FM0	91.0	275	2.8	3.5	7.0	1.4	85.3	142
1124/FM0	71.4	70	18.5	3.7	7.4	1.9	68.5	54
1611/FM0	72.8	725	17.1	1.2	7.3	2.4	72.0	164
2096/FM0	70.6	340	11.6	0.0	6.7	1.7	80.0	120

All females were mated to wild-type males for several days before egg collections. For 'Eggs hatched (%)' females of the appropriate genotype were allowed to lay eggs for 14 hours onto well-yeasted grape medium. The flies were removed and the number of hatched eggs was scored after another 24 hours (see Materials and Methods). For 'Developmental stage (%)', 0- to 14-hour collections of embryos were fixed and stained with Hoechst to examine the extent of their development: E, very early embryo (1-2 nuclei); EC, early cleavage divisions (cycles 2-9); SB, syncytial blastoderm; CB, cellular blastoderm; L, later embryos (gastrulation). [B] and [D] are transgenes that supply zw4+ and klp3A+ activity, respectively (Williams et al., 1995). Df= Df(1)54, a deficiency for the KLP3A region (Goldberg et al., 1989). *n*, total number of eggs examined for each experiment.

escapers were recovered in equal proportion from *KLP3A* mutant homozygous mothers (data not shown). Male escapers had only a single, mutant copy of the X-linked *KLP3A* gene that they obtained from their mothers, while their sisters had a *KLP3A*$^+$ allele from their fathers. It is also remarkable that genetic tests (see Materials and Methods) revealed that none of these adult escapers are aneuploid for the X or 4th chromosome. This suggests that chromosome segregation during meiosis in eggs derived from *KLP3A* mutant females is generally regular.

We note here the curious finding that females heterozygous for a *KLP3A* mutant allele and a deletion for the *KLP3A* region produced embryos that generally continued further through embryogenesis than those laid by homozygotes or transheterozygotes (Table 1). This effect was relatively modest, and was not reflected by any obvious differences in the proportions of eggs that hatched into larvae (Table 1). It is conceivable that these *KLP3A* mutations produce small amounts of unstable but deleterious KLP3A protein fragments. Thus, embryo viability would be prolonged when the mutant alleles are reduced in dose. Alternatively, the deletion-bearing stock might include a weak dominant genetic suppressor of the *KLP3A* phenotype.

Mutations in *KLP3A* cause developmental arrest subsequent to fertilization and the completion of meiosis, but prior to fusion of the male and female pronuclei

We have performed several experiments to determine the nature of the early arrest encountered by roughly 90% of the embryos produced by *KLP3A* homozygotes or transheterozygotes. First, to establish if these embryos were in fact fertilized, we stained embryo collections with Hoechst 33258 and (by indirect immunofluorescence) with an antibody directed against a component of the sperm tail (Karr, 1991). In *Drosophila melanogaster*, the sperm tail enters completely into the egg and remains relatively intact for several hours after fertilization (Karr, 1991). The large majority (>80%) of these early-arrested *KLP3A* embryos did in fact contain a detectable sperm tail, indicating that the primary block in development must occur subsequent to fertilization.

Next, we examined the embryonic progeny of mutant mothers for DNA and microtubules by immunofluorescence. The most common type of aberrant embryos displayed two discrete DNA-containing regions (Fig. 1). The region containing the most DNA was that located closest the embryonic cortex. The chromosomes within this region often exhibited a 'starburst' configuration, and the microtubules were radially arranged around the chromosomes. These observations all indicated that this region corresponds to the polar bodies (Foe et al., 1993). The second DNA-positive area was more centrally located within the egg, and was surrounded by microtubules organized into a bipolar spindle. The chromosomes were organized into a tight mass at the center of the spindle, suggesting that this spindle was arrested in a metaphase-like state. In contrast with the polar bodies, the latter nucleus was not observed in unfertilized eggs from mutant virgin females, indicating that it involved male pronuclear DNA.

This single metaphase spindle could represent either an arrested first (gonomeric) division that includes chromosomes contributed by both the male and female pronucleus, or it could be formed solely around the genome of the male pronucleus. To distinguish between these possibilities, we simultaneously examined the embryos by immunofluorescence for DNA and tubulin and by in situ hybridization with probes specific for the X and Y chromosomes (Fig. 2; see Materials and Methods for procedural details). The interiorly located, metaphase-like spindle always hybridized either with an X chomosome probe or with a Y chromosome probe, but never with both. This indicates that the spindle contained only those chromosomes contributed by the male pronucleus. In accordance with this idea, the most favorable preparations showed four discrete X chromosome signals in the polar body mass (Fig. 2), suggesting that the other nucleus was formed from the fusion of all four female meiotic products.

The spindle established around the male pronucleus in embryos from *KLP3A* mutant mothers often displayed striking abnormalities (Fig. 4). Occasionally, a few chromosomes were not found at the metaphase plate, and remained either near the poles or at a slight distance from the spindle (Fig. 4B). The centrosomes also sometimes behaved in an aberrant fashion. Most commonly, centrosomes appeared to detach from the spindle apparatus and wander a considerable distance from it (Fig. 4A; see also Fig 1B,F). Both these 'free' centrosomes as well as those still found at the spindle termini often appeared to be replicated (Fig. 4B,C). It should be noted that centrosome replication normally occurs at the end of telophase while here, the spindle seemed to be arrested in metaphase. Similar defects in centrosome behavior are associated with other *Drosophila* maternal-effect mutations that cause difficulty in mitotic progression (Freeman et al., 1986; González et al., 1990; Girdham and Glover, 1991; Hatsumi and Endow, 1992; Gomes et al., 1993; Williams and Goldberg, 1994), and appear to result from the uncoupling of the centrosome and nuclear

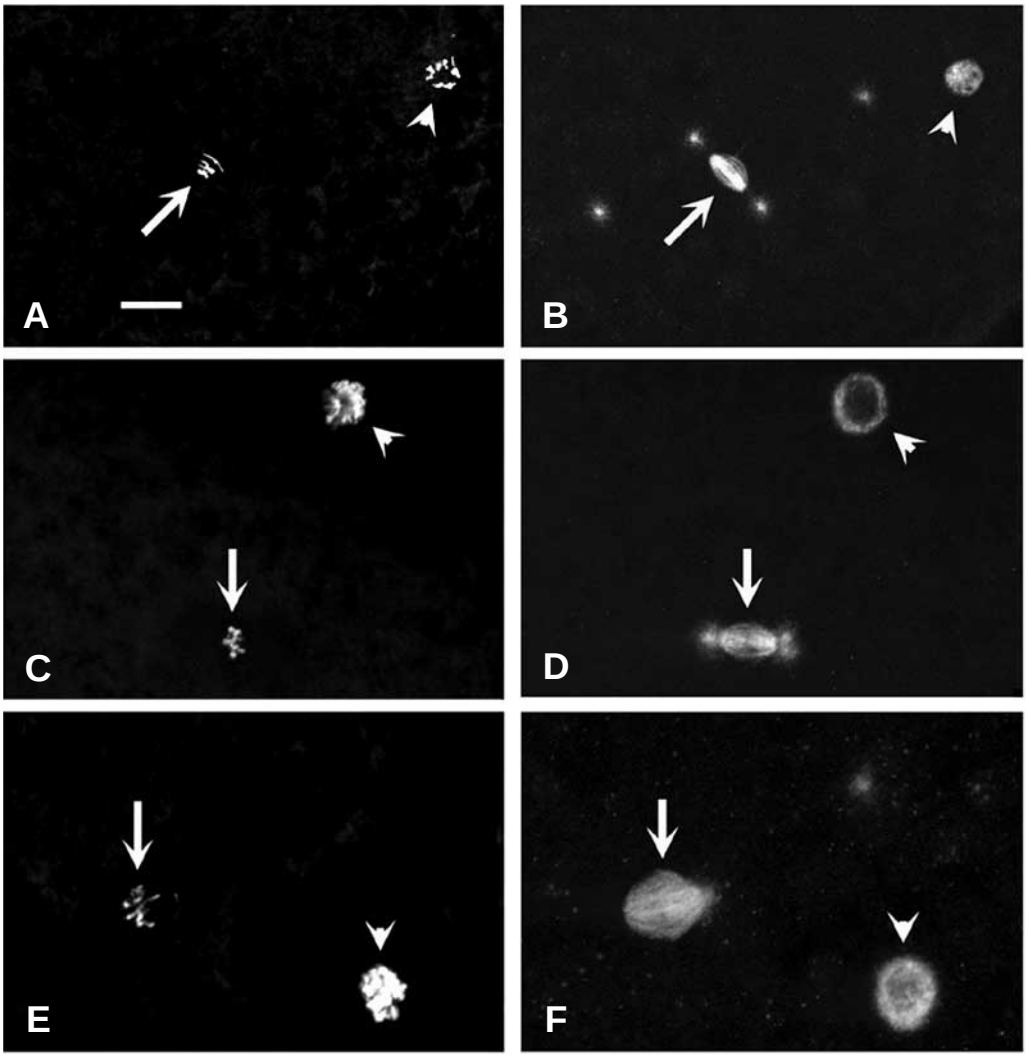

Fig. 1. Early arrest phenotype of embryos obtained from *KLP3A* mutant females. Embryos from *KLP3A*^{1611} (A-D) and *KLP3A*^{e4} (E,F) females were fixed and stained to visualize DNA (A,C,E) and tubulin (B,D,F) by confocal microscopy (see Materials and Methods). Only two DNA-containing foci are generally present in each mutant embryo: (1) a small haploid nucleus with chromosomes aligned on a metaphase plate in the center of a bipolar spindle (arrows), and (2) a larger, polyploid nucleus associated with a circular array of microtubules (arrowheads). Note the abnormal presence of asters (centrosomes) detached from the bipolar spindles in B and F. Bar, 20 μm.

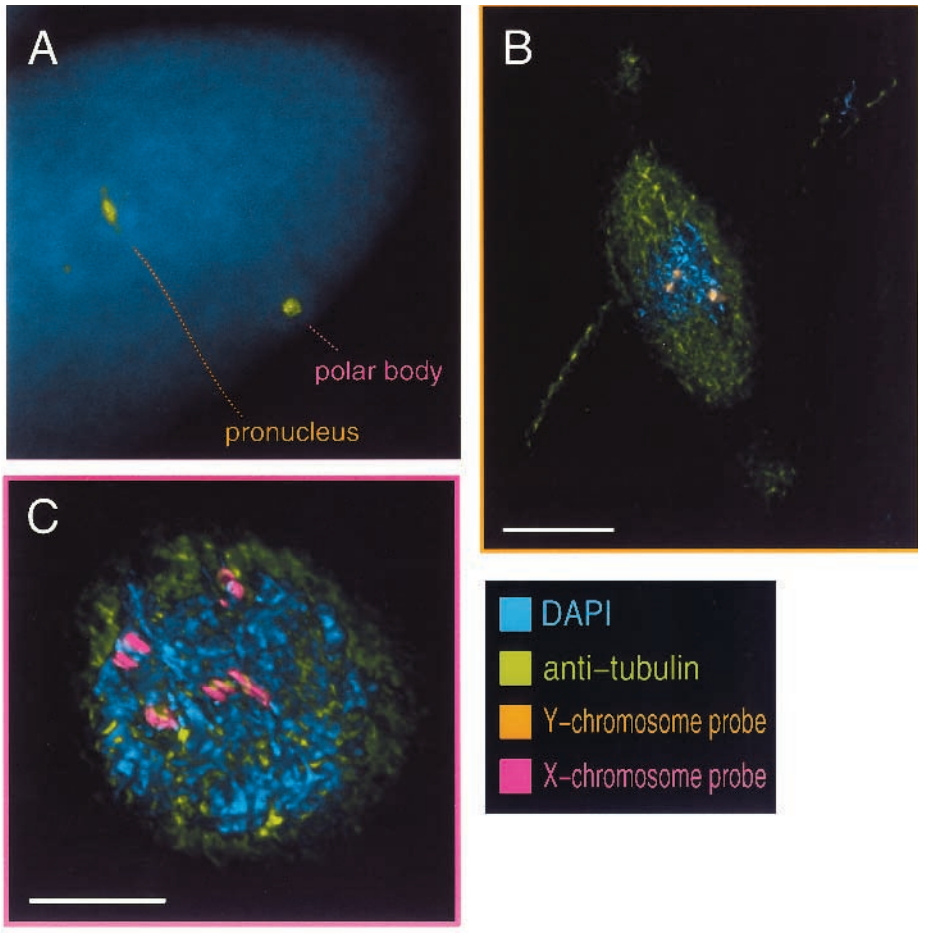

Fig. 2. Fate of the maternal and paternal genomes in *KLP3A* mutant embryos analyzed by in situ hybridization with chromosome-specific probes. Embryos arrested with the phenotype depicted in Fig. 1 were simultaneously stained for DNA (DAPI; blue), tubulin (green), and the hybridization of probes specific for the *Drosophila* X (red) and Y (orange) chromosomes as described by Dernburg et al. (1996). (A) The polar body, with the radial (starburst) arrangement of microtubules, never hybridizes with the Y chromosome probe, but reveals four X chromosome signals (C). This suggests that the polar body in the mutant embryos is made by the fusion of all four female meiotic products. The other focus of DNA, which is found in the context of a bipolar spindle, hybridizes with either an X or a Y chromosome probe, but never both, indicating that it corresponds to the male pronucleus (B). Bars, 5 μm.

division cycles in the rapidly developing early embryo (Glover, 1992).

The failure in pronuclear fusion seen in *KLP3A* mutants could be the indirect result of defects in female meiosis. To check this possibility, meiotic figures from fertilized eggs were examined by Feulgen/Giemsa double staining (Fig. 5). With very few exceptions, *KLP3A* mutant eggs appeared to behave normally at all stages of meiosis. Metaphase I chromosome configurations and spindle morphology were similar to wild-type in mature oocytes (Fig. 5A). Chromosome segregation through meiosis I and II was regular (Fig. 5B-F). Four meiotic products were produced, and were separated from each other at the conclusion of the second meiotic division (Fig. 5G-H). However, the 'middle pole material' (Puro, 1991) separating the two meiosis II spindles appeared reduced in mutants relative to wild-type (Fig. 5D-F; compare with wild-type in Fig. 5C). Although this diminution of middle pole material did not obviously affect meiotic progression, it may still be of significance in eliciting the terminal phenotype (see Discussion). It is also of interest that the sperm aster established in the vicinity of the male pronucleus during meiosis II in the fertilized egg (Callaini and Riparbelli, 1996) appeared unaffected by *KLP3A* mutations (Fig. 6).

Eggs from *KLP3A* mutant mothers occasionally display defects at meiotic stages prior to that represented by the terminal phenotype. Thus, approximately 10% of the meiotic or immediately postmeiotic figures observed displayed abnormalities (not shown). These included metaphase I spindles from oocytes with missing chromosomes, distorted anaphase I spindles, meiotic figures with chromosomes scattered over a large area, and the integration of postmeiotic chromosomes into mininuclei.

In summary, the large majority of *KLP3A* mutant embryos arrested development after the completion of meiosis. In the terminal state, the presumptive female pronucleus remained associated with the other three products of female meiosis in a single polar body. The male pronucleus remained separated a considerable distance from this polar body. Although the gonomeric spindle could not be formed in these conditions, a bipolar spindle was still established around the haploid paternal genome in *KLP3A* mutants.

The KLP3A protein is associated with the spindle at metaphase and with the spindle midzone at ana-telophase

In order to better understand the basis for the phenotypic effects of *KLP3A* mutations on early development, we have employed an affinity-purified anti-KLP3A serum (Williams et al., 1995) to determine the location of the KLP3A protein during wild-type female meiosis and during the cleavage divisions of normal *Drosophila* embryogenesis (Fig. 7). The distribution of KLP3A during ana/telophase of the female meiotic divisions and of embryonic cleavage divisions was analogous to what we previously observed during the male meiotic divisions (Williams et al., 1995): KLP3A protein accumulated in the vicinity of the spindle equator (Fig. 7B,C,F,H,I).

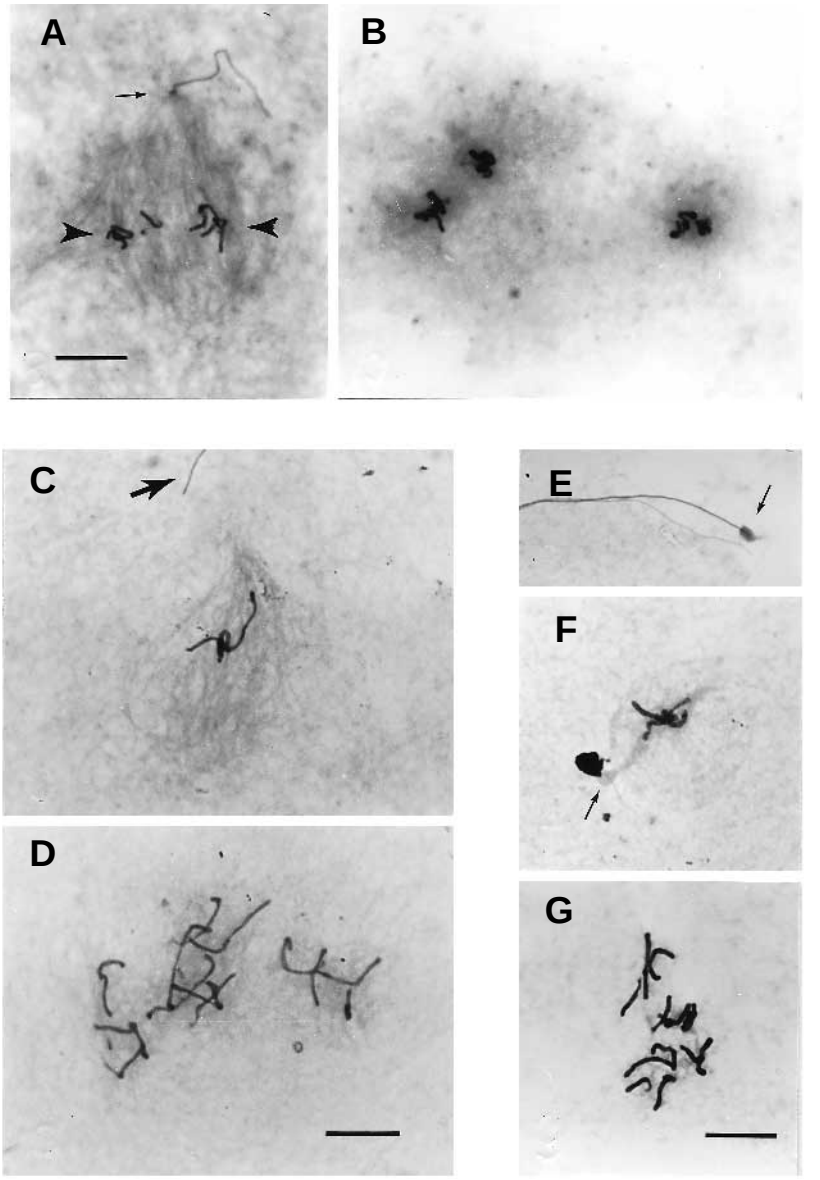

Fig. 3. Defective pronuclear migration in *KLP3A* mutant embryos. Chromosomes and spindles in fertilized eggs were examined by a modification of the Feulgen/Giemsa staining technique (Puro and Nokkala, 1977; see Materials and Methods). (A,B) First mitotic (gonomeric) division in wild-type embryos. Male and female pronuclei become closely apposed, and their condensed chromosomes remain two adjacent but separate groups on the spindle (arrowheads; A). The sperm tail often remains associated with one of the spindle poles (arrow). In another region of the same embryo (B), the chromosomes of the three polar bodies become condensed in a metaphase-like arrested state. (C-G) Pronuclei in fertilized eggs produced by $KLP3A^{1611}$ (C,D) and $KLP3A^{e4}$ (E-G) mothers at the corresponding stage. Here, the bipolar spindle is instead organized around a haploid set of metaphase-arrested paternal chromosomes (C,F), with the sperm tail present nearby (arrows in C,E). In contrast to the three sets of chromosomes in wild-type polar bodies (B), there are instead four sets of maternal chromosomes (D,G). (The arrow in (F) points to a spindle pole; the prominent dark mass above the arrow is a contaminating piece of dirt.) Bars, 10 μm.

The zone of distribution in the mitotic telophase divisions was very narrow, clearly in the midbody of the central spindle's interdigitating microtubules (Fig. 7F,I). On the ana/telophase spindles of the female meiotic divisions, the zone of KLP3A staining in the equatorial region was wider, reflecting the relatively elongated shape of these spindles (Fig. 7B,C). Previous

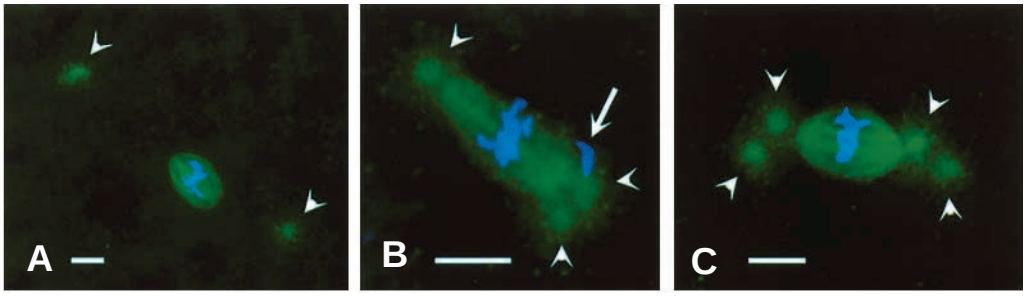

Fig. 4. Abnormal centrosome and chromosome behavior in spindles organized around the male pronucleus in *KLP3A* mutant eggs. Embryos from $KLP3A^{1611}$ (A,B) and $KLP3A^{e4}$ (c) females were fixed and stained to visualize DNA (blue) and tubulin (green). Shown here are examples of spindles associated with the male pronucleus. The positions of the centrosomes are inferred by the position of the asters (arrowheads). (A) Abnormal detachment of centrosomes from the ends of the spindle. (B) Premature duplication of the aster at one pole (lower right pole) during metaphase. Also, one chromosome is not aligned at the metaphase plate (arrow). (C) Improper duplication of centrosomes and their detachment from the ends of the spindle. These defects in centrosome attachment indicate the separation of centrosome and nuclear division cycles in these mutant embryos. Bars, 10 μm.

results from other laboratories have shown that anti-tubulin staining is excluded from most of the broad region between the separating chromosomes (McKim et al., 1993), so we believe that KLP3A antibodies recognize the equivalent midbody structure in all spindles at this late stage of the cell cycle.

In contrast to the male meiotic divisions, where we previously observed a diffuse distribution of KLP3A throughout the metaphase cell (Williams et al., 1995), this protein was clearly associated with the metaphase spindle both in female meiotic cells and in syncytial blastoderm nuclei (Fig. 7A,G). This finding of KLP3A localization to the metaphase spindle was particularly clear-cut in the case of stage 14 eggs (Fig. 7A). Here, the assignment of the cell cycle stage to metaphase of meiosis I is unambiguous, because of the meiotic arrest that occurs prior to passage of the egg through the oviduct.

KLP3A protein was also associated with the radial, monastral array of microtubules that emanates from between the twin meiosis II spindles (Riparbelli and Callaini, 1996) and that presumably corresponds to the 'middle pole' material seen in orcein-stained preparations (see Fig. 5 and Puro, 1991). The KLP3A protein was also localized at the sperm aster which forms near the male pronucleus shortly after fertilization (Fig. 7E).

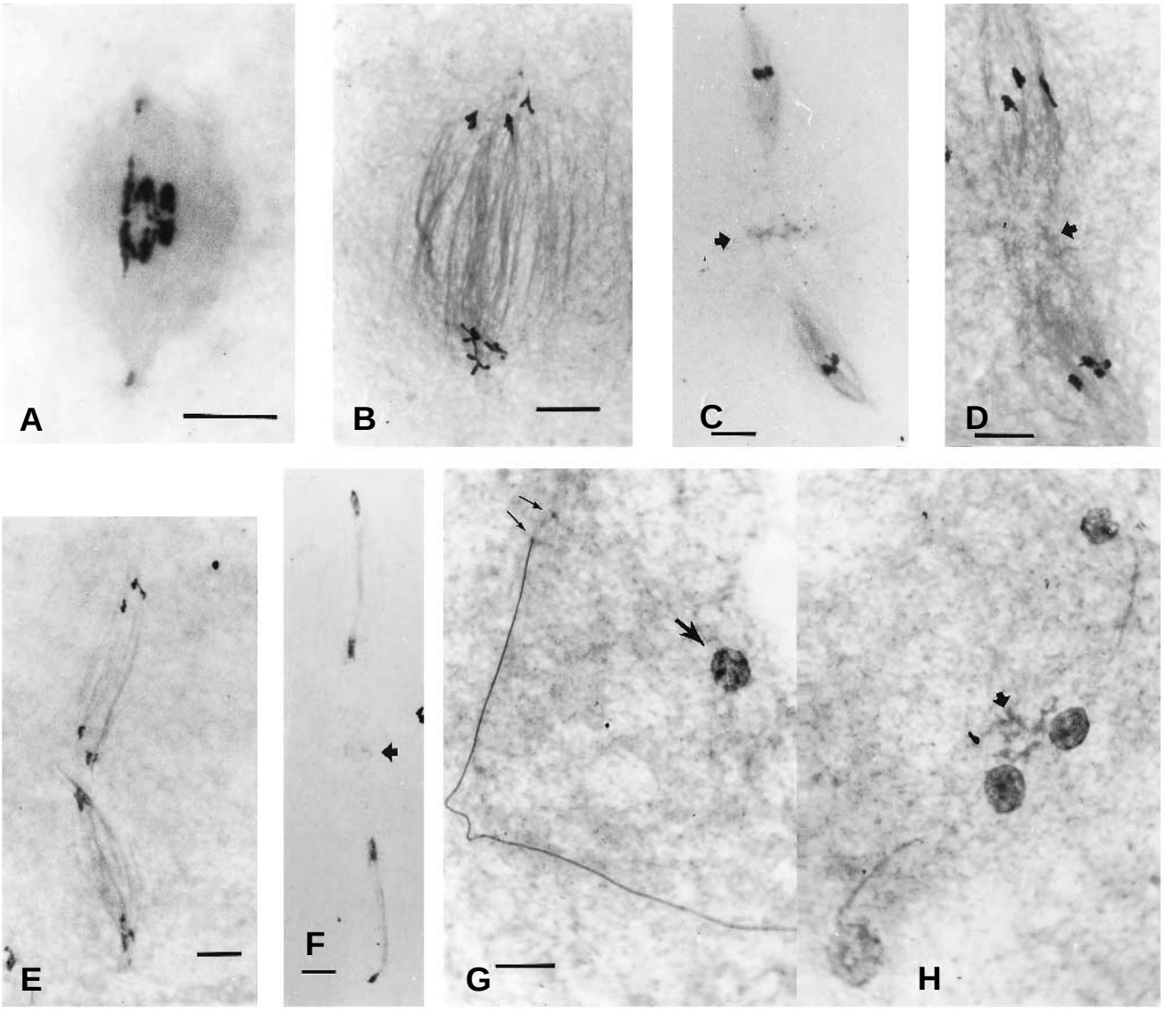

Fig. 5. Female meiosis in *KLP3A* mutants. Eggs from *KLP3A*^{e4} (a,b,d-h) and wild-type (c) mothers were fixed and stained by the Feulgen/Giemsa technique (Puro and Nokkala, 1977; see Materials and Methods). (A) Typical metaphase I of normal appearance from a mutant stage 14 oocyte. As in wild-type (Puro and Nokkala, 1977; Theurkauf and Hawley, 1992), the three major bivalents are located at the center of the spindle, while the 4th chromosomes are near the poles. (B) The anaphase I spindle also appears normal with no obvious defects in chromosome segregation. (C) Metaphase II in wild-type showing the tandem orientation of the two spindles, which are separated by conspicuous 'middle-pole material' derived from the midbody of the first division spindle (arrow). (D) Metaphase II, (E) anaphase II and (F) telophase II figures from *KLP3A* mutant eggs showing diminution (arrows in D,F) or absence (E) of the middle-pole material. (G,H) Two fields from one egg after completion of meiosis. This egg was fertilized, as seen by the sperm tail associated with duplicated centrosomes (G; smaller arrows.) The male pronucleus has decondensed (G; larger arrow). In h, the four products of female meiosis have separated from each other, though they remain associated with remnants of the meiotic spindle and some middle pole material (arrow). Bars, 10 μm.

DISCUSSION

Developmental requirements for the KLP3A protein

The fate of the large majority of fertilized eggs produced by *KLP3A* mutant females is quite consistent. Both meiotic divisions occur relatively normally, and the embryos arrest with a terminal phenotype consisting of a polar body containing the four female meiotic products and a metaphase-arrested spindle containing the paternal chromosome complement. It should be stressed that these consequences of *KLP3A* mutations result from the lack of the KLP3A protein in the egg itself, rather than its absence from other, somatic cells (like follicle cells) that contribute to female fertility. By mitotic recombination techniques (Perrimon et al., 1989), we generated homozygous $KLP3A^{e4}$ mutant germline clones in $KLP3A^{e4}/KLP3A^{+}$ heterozygous females. The mutant clones did not display oogenic defects. Embryos obtained from these mutant clones arrested development with the same phenotypic syndromes described above, establishing that the effects of *KLP3A* mutations are germline-dependent (data not shown). This result is anticipated because we have demonstrated that maternally supplied KLP3A protein is in fact associated with the spindles within the developing embryo (Fig. 7).

Not all embryos arrest with this same terminal phenotype. In embryos from *KLP3A* mutant mothers, we observed rare defects in earlier meiotic stages. The frequency of any particular kind of aberration was very low, so it is possible that these apparent meiotic defects represent either extremely sporadic cases or artefacts of preparation. However, roughly 10% of all embryos derived from *KLP3A* mutant mothers develop past the block in pronuclear migration encountered by most of their siblings. Of these later-developing embryos, the large majority fail to proceed beyond gastrulation and do not hatch. Remarkably, between 1-10% of all escaper embryos (i.e., 0.1-1% of the total eggs) nevertheless develop into normal adults.

The view of the requirement for KLP3A function during the *Drosophila* life cycle that emerges is as follows. Several developmental processes are substantially unaffected by the absence of KLP3A protein. (1) It appears that oogenesis occurs normally in *KLP3A*-deficient females. (2) The KLP3A protein plays, at most, a minor role in female meiosis, because most embryos from mutant mothers complete an apparently normal meiosis and arrest their development at a later time. However, we have noted a diminution of 'middle pole' material between the two meiosis II spindles in mutant oocytes (Fig. 5) that could affect some subtle aspect of meiosis II. (3) Subsequent to larval hatching, we see no evidence of a requirement for KLP3A function. Animals that survive to hatching can develop normally independently of KLP3A protein. This is consistent with the results of developmental northern analysis showing that larvae and pupae contain much less *KLP3A* mRNA than do embryos (Williams et al., 1995).

In contrast, our results clearly demonstrate a strong requirement for the KLP3A protein at three developmental stages. (1) Absence of KLP3A causes severe cytokinesis defects during male meiotic divisions, leading to subsequent problems in spermatogenesis (Williams et al., 1995). (2) KLP3A protein is needed in very early embryos for some process subsequent to the completion of meiosis but prior to pronuclear fusion. (3) A requirement for the KLP3A protein to complete gastrulation is indicated by the fact that almost all embryos that proceed past the usual terminal phenotype arrest their development in gastrulation. The KLP3A-dependent processes that occur at the time of the pronuclear migration or gastrulation arrests must be partially redundant, allowing a few KLP3A-deficient embryos to continue their development past these blocks.

The KLP3A protein is involved in pronuclear specification or pronuclear migration

The clear-cut phenotypic effect of mutations in *KLP3A* is the failure of the female pronucleus to separate from the three other haploid products of meiosis and subsequently to migrate toward the male pronucleus. As a result, the four nuclei generated by female meiosis fuse together into a single polar body (as they do in unfertilized wild-type eggs), and a metaphase-arrested spindle forms around the paternal genome. This syndrome appears to be the fate of approximately 90% of the embryos derived from *KLP3A* mutant mothers. Our observations demonstrate that several events occur normally prior to this arrested state. The eggs are fertilized, and the chromosomes in the male pronucleus soon decondense (Fig. 5G and data not shown), suggesting that the male-specific basic chromatin proteins are replaced by histones (Das et al., 1964; Yasuda et al., 1995). Cytologically normal female meiosis occurs, producing four meiotic products that generally have euploid chromosome complements. It further appears that the four female meiotic products as well as the male pronucleus undergo DNA replication prior to the arrest. This is suggested by the facts that the chromosomes in these nuclei are recondensed in the terminal phenotype (Figs 3C-G, 4A-C), and that a bipolar spindle is assembled around the paternal chromosome complement. However, it should be cautioned that our cytological resolution of chromosomes has been insufficient to verify whether the chromosomes in the arrested state have indeed replicated into sister chromatids.

The major defect associated with the lack of KLP3A protein occurs in the very short period (less than 6 minutes; Foe et al., 1993) between the end of meiosis and pronuclear fusion. Mutations in *KLP3A* could theoretically disrupt either of two processes that occur within this narrow time frame. First, it is possible that absence of KLP3A disrupts migration of the pronuclei towards each other, thereby preventing syngamy. Alternatively, KLP3A may participate in the events that specify one of the four female meiotic products as the female pronucleus. If individualization of the female pronucleus is not accomplished, an indirect consequence could be a subsequent failure in pronuclear migration. We note that the distinction between these two processes may well be artificial: for example, the female pronucleus may have no special properties other than being the first meiotic product to migrate toward the male pronucleus. However, we believe it is helpful to consider these two hypotheses separately, each in light of our current understanding of KLP3A function.

How might KLP3A protein be involved in pronuclear migration? At least at first glance, the simplest explanation of the phenotype is that the KLP3A kinesin-like protein serves as a molecular motor to transport the female pronucleus as a 'cargo' toward the male pronucleus along the sperm aster. Such a role has previously been proposed for the yeast Kar3 and for the *Drosophila* NCD kinesin-like proteins (Endow et al., 1994; Komma and Endow, 1995). However, we believe this hypothesis is probably incorrect with regard to KLP3A, because it demands that this protein be a minus-end directed microtubule

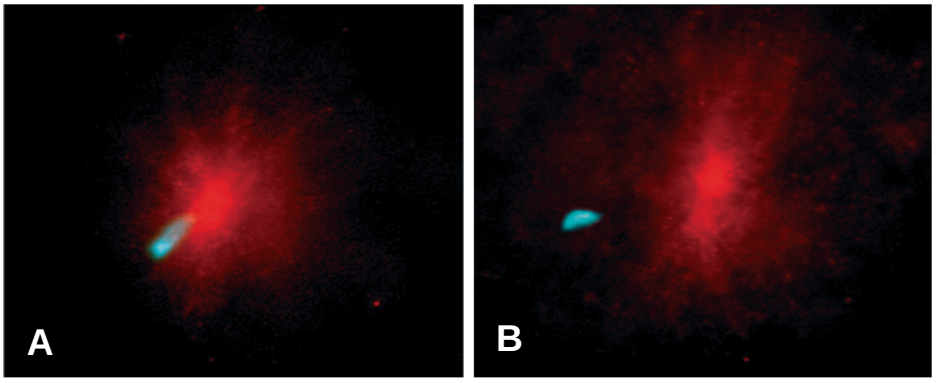

Fig. 6. The sperm aster in wild-type and mutant eggs. Fertilized eggs during telophase of meiosis II from wild-type (A) or from *KLP3A* mutant (*b*) mothers were fixed, stained, and then squashed to visualize DNA (blue) and microtubules (red; see Materials and Methods.) A sperm aster is found in the vicinity of the male pronucleus in both cases; the distance between the aster and the DNA is variable due to the preparation. Bar, 10 μm.

motor (as are Kar3 and NCD; Walker et al., 1990; McDonald et al., 1990; Endow et al., 1994). Several considerations instead imply that KLP3A is likely to have plus-end directed microtubule motor activity. The structure of the KLP3A protein, with the conserved motor domain near the N terminus, is similar to that of other plus end-directed kinesins. KLP3A is evolutionarily related to the XKLP1/KIF4 family of plus end-directed kinesin-like proteins (Goldstein, 1993; Goodson et al., 1994; Sekine et al., 1994; Moore and Endow, 1996). Finally, KLP3A's localization at the equator of the ana/telophase spindle suggests movements toward microtubule plus ends (Williams et al., 1995; this paper).

In spite of these arguments, it remains possible that KLP3A could be directly involved in pronuclear migration. The biochemical activity of this molecule has not been determined, so KLP3A could be a minus end-directed motor. Recent evidence suggests that CENP-E, another kinesin-like protein with an N-terminal motor domain, may be minus end-directed (Thrower et al., 1995). Even if it were a plus end-directed motor, the KLP3A protein might still be necessary for pronuclear migration, for example by helping to establish or stabilize the sperm aster. We do know that an apparently normal sperm aster forms in at least some fertilized *KLP3A* mutant eggs (Fig. 6B), but its function may be compromised by the absence of KLP3A protein that is normally associated with it (Fig. 7E). Finally, it should be remembered that although pronuclear migration is usually described as the movement of the female pronucleus toward the male pronucleus (Foe et al., 1993; Callaini and Riparbelli, 1996), this supposition is based upon images of fixed embryos. It therefore cannot be discounted that pronuclear migration may instead require movements of the male pronucleus along the sperm aster.

An alternative hypothesis is that KLP3A protein is needed for the selection of the female pronucleus or for maintaining its separation from the three other products of meiosis, rather than for pronuclear movements per se. Other than the fact that the post-meiotic nucleus furthest from the egg cortex almost invariably becomes the female pronucleus (Sonnenblick, 1950), very little is known about these processes. Is there something unique about the interior-most nucleus that predicts its selection as the pronucleus, or is it instead simply the nucleus which resides closest to (and is most likely to be influenced by) the male pronucleus? Why in wild-type do the other meiotic products fail to migrate toward the male pronucleus, and why do these nuclei usually fuse with each other to make a polar body subsequent to syngamy?

We cannot at present answer these questions, but we can exclude at least one simple way that KLP3A could be imagined to affect female pronuclear selection. Riparbelli and Callaini (1996) have recently reported that the female meiotic spindle rotates after fertilization. During normal metaphase I arrest, the meiotic spindle is oriented parallel to the egg cortex, but it then becomes perpendicular to the cortex by anaphase I and remains in this same orientation throughout the remainder of meiosis. These authors suggest that this rotation is required to bring the meiotic product destined to be the female pronucleus into sufficient propinquity with the male pronucleus. In the case of *KLP3A* mutant eggs, we have observed that meiosis II spindles are perpendicular to the egg cortex, so the lack of KLP3A does not disrupt meiotic spindle rotation.

Although the mechanism by which the lack of KLP3A protein prevents fusion of the male and female pronuclei remains mysterious, our knowledge of KLP3A function in other tissues provides an intriguing hint. In spermatocytes, mutations in *KLP3A* disrupt the central spindle between the separating chromosome complements during ana/telophase. This central spindle defect correlates with the localization of KLP3A protein to the midzone of male meiotic spindles (Williams et al., 1995). We have shown here that KLP3A similarly accumulates in the equatorial region of the wild-type meiosis I ana/telophase spindle (Fig. 7B,C). Moreover, we usually observe in *KLP3A* mutants a diminution in the amount of 'middle pole' material that forms at this equatorial position and that normally separates the two meiosis II spindles (Fig. 5; see also Puro, 1991). This is consistent with the localization of KLP3A to the microtubule aster nucleated by centrosomal material that is found between the two meiosis II spindles (Fig. 7D), which probably corresponds to the 'middle pole' material (Riparbelli and Callaini, 1996). This novel aster might be a source of forces that 'push' the female pronucleus away from the other meiotic products and toward the male pronucleus. Alternatively, interactions between this interstitial aster and the sperm aster could be critical for proper behavior of the pronuclei.

Why does the male pronuclear spindle arrest at metaphase?

One curious aspect of the usual terminal phenotype associated with *KLP3A* mutations is the fact that the spindle established around the paternal genome is arrested at a metaphase-like state. The male pronucleus nonetheless undergoes several events in the absence of a female pronucleus. The centrosome

associated with the male pronucleus can duplicate (Figs 3 and 4); this is unsurprising given that centrosome duplication normally precedes migration of the female pronucleus. The two resultant centrosomes can separate from each other, and migrate around the male pronucleus. Finally, as mentioned previously, it appears that the male pronuclear genome replicates in these defective eggs, even in the absence of pronuclear migration. These events eventually allow the establishment of a bipolar spindle around the haploid paternal chromosome complement (Figs 1-4). It is interesting that in rare cases of dispermy in wild type, a bipolar spindle also forms around the supernumerary male nucleus (Callaini and Riparbelli, 1996).

If all of these processes occur, it remains unclear why this haploid spindle in *KLP3A* mutant embryos is subsequently unable to enter anaphase. The hypothesis that the female pronucleus provides factors that cue onset of anaphase of the first, gonomeric mitotic division, appears unlikely. Haploid embryos containing the paternal but not maternal genome are generated by females heterozygous for a particular mutation (*aTub67C*^{3}) in an α-tubulin isoform-encoding gene (Komma and Endow, 1995). These embryos demonstrate that mitotic divisions can occur in the absence of any contribution from the female pronucleus. The metaphase arrest of the male haploid spindle in KLP3A-deficient eggs cannot therefore simply result from failure to achieve either pronuclear fusion or the diploid state per se. A second hypothesis to explain the metaphase arrest is that the lack of KLP3A protein on the haploid spindle compromises spindle function, thus preventing mitotic progression. This model is difficult to reconcile with our finding that the absence of this kinesin-like protein does not prevent anaphase entry during the meiotic divisions or during the early syncytial mitotic divisions (in escapers). However, females with certain other mutations in the *aTub67C* gene yield embryos that arrest with a phenotype that has not been exhaustively characterized but that nonetheless appears to be similar to the terminal phenotype described here (Matthews et al., 1993). This implies that cell cycle progression of the first mitotic spindle in the

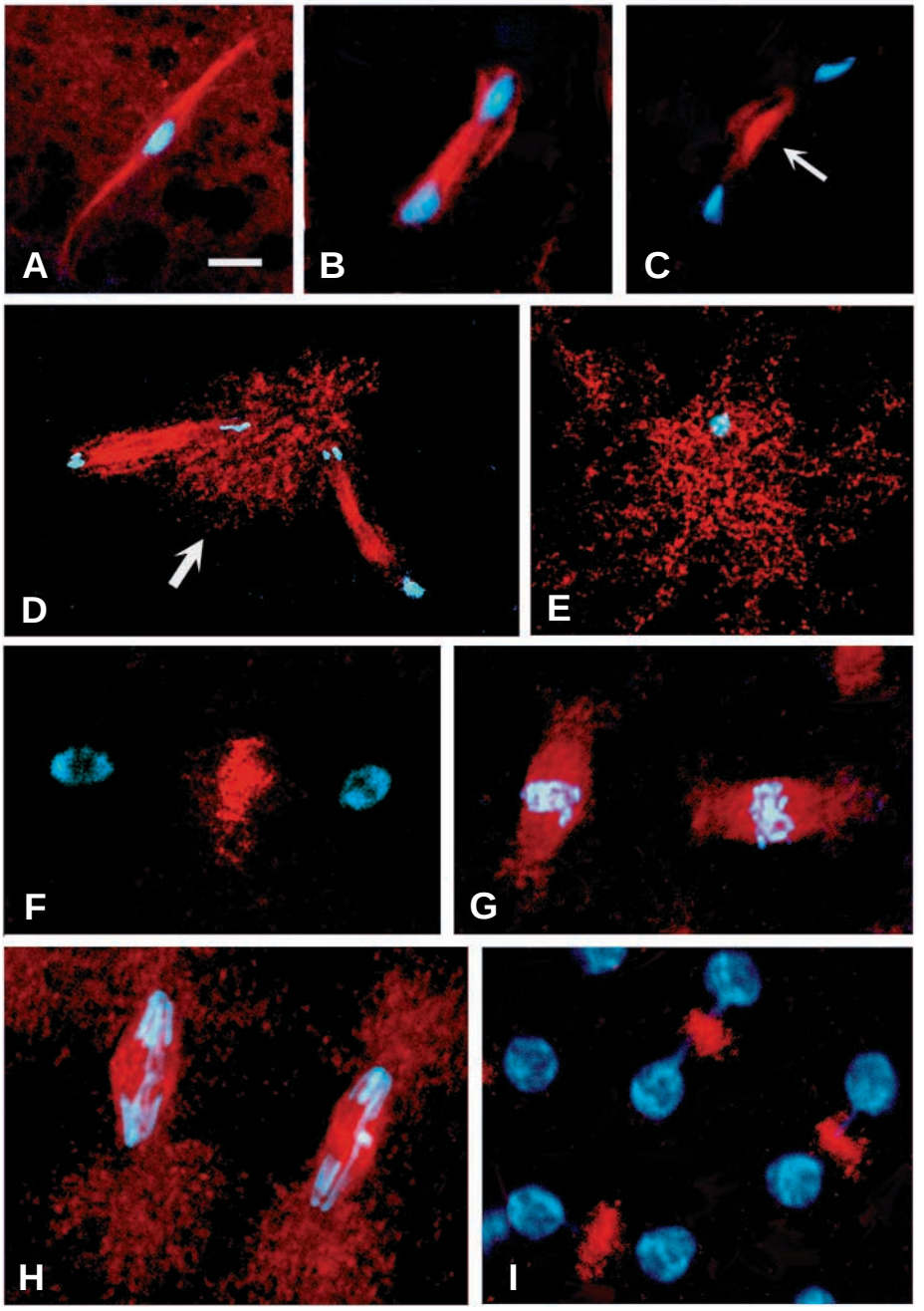

Fig. 7. Localization of KLP3A protein during female meiosis and embryonic mitosis. Oocytes from wild-type (A,D) or from *mei-218*^{a4} (B,C) mothers, and wild-type embryos (F-I) were fixed and stained to visualize DNA (blue) and KLP3A protein (red; see Materials and Methods.) (A) Metaphase I with KLP3A present along the length of the meiotic spindle (compare with meiotic spindles in Theurkauf and Hawley, 1992). At anaphase I (B) and telophase I (C), KLP3A moves progressively to the spindle midzone (compare with meiotic spindles in McKim et al., 1993). During anaphase of meiosis II (D), KLP3A is again associated with the midzones of the tandemly arranged spindles. KLP3A is also localized to the monastral array situated between the two meiosis II figures (arrow). (In D, the innermost meiotic product is at lower right.) During this time, KLP3A also associates with the growing sperm aster near the male pronucleus (E). KLP3A localizes to the spindle midbody during telophase of the first, gonomeric (mitotic) division (F). In the syncytial blastoderm embryo, at metaphase (G), KLP3A is present along the spindle, becoming enriched in the spindle midzone during anaphase (H), and later becomes sharply demarcated to the spindle midbody during telophase (I). Localization of KLP3A to astral microtubules during metaphase (G) and anaphase (H) can also be observed. Bar, 10 μm.

Drosophila life cycle may be particularly sensitive to perturbations in microtubule components.

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