Author Correction


The name of the last author is S. Saighal not S. Shigali.

The authors apologise for this error.
Novel nuclear defects in KLP61F-deficient mutants in Drosophila are partially suppressed by loss of Ncd function

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Summary
KLP61F in Drosophila and other BimC kinesins are essential for spindle bipolarity across species; loss of BimC function generates high frequencies of monopolar spindles. Concomitant loss of Kar3 kinesin function increases the frequency of bipolar spindles although the underlying mechanism is not known. Recent studies raise the question of whether BimC kinesins interact with a non-microtubule spindle matrix rather than spindle microtubules. Here we present cytological evidence that loss of KLP61F function generates novel defects during M-phase in the organization and integrity of the nuclear lamina, an integral component of the nuclear matrix. Larval neuroblasts and spermatocytes of klp61F mutants showed deep involutions in the nuclear lamina extending toward the centrally located centrosomes. Repositioning of centrosomes to form monopolar spindles probably does not cause invaginations as similar invaginations formed in spermatocytes lacking centrosomes entirely. Immunofluorescence microscopy indicated that non-claret disjunctional (Ncd) is a component of the nuclear matrix in somatic cells and spermatocytes. Loss of Ncd function increases the frequency of bipolar spindles in klp61F mutants. Nuclear defects were incompletely suppressed; micronuclei formed near telophase at the poles of bipolar spindle in klp61F ncd spermatocytes. Our results are consistent with a model in which KLP61F prevents Ncd-mediated collapse of a non-microtubule matrix derived from the interphase nucleus.

Introduc}
Spindle bipolarity is essential for accurate segregation of chromosomes to daughter cells at cell division. Members of the BimC family of kinesin-related motor proteins are essential for spindle bipolarity across species; loss of BimC function results in monopolar rather than bipolar spindles (Hildebrandt and Hoyt, 2000; Kashina et al., 1997). BimC kinesins are bipolar (Kashina et al., 1996) homotetramers (Cole et al., 1994) and spindle-associated during mitosis (Sharp et al., 1999a). Despite the implications of spindle localization and spindle defects, the BimC kinesins Eg5 in Xenopus remains statically positioned in spindles as microtubules flux toward spindle poles (Kapoor and Mitchison, 2001) as if Eg5 is tethered to an immobile non-microtubule spindle matrix rather than to dynamic microtubules fluxing toward spindle poles. It is not yet clear whether BimC kinesins apply motive force to spindle microtubules to establish or to maintain spindle bipolarity or whether these kinesins prevent collapse of a non-microtubule spindle matrix that is connected in some way to spindle microtubules.

Non-microtubule components of spindles include constituents of the nuclear matrix. First defined as non-chromatin material in the interphase nucleus (Fawcett, 1966), the nuclear matrix is in part composed of a nuclear lamina and a ribonucleoprotein network involved in gene expression (Nickerson, 2001). The nuclear lamina is a supportive meshwork of nuclear-specific intermediate filaments called lamin and lamin-binding proteins (Holaska et al., 2002). The nuclear lamina is attached to the inner nuclear envelope and to heterochromatin at the nuclear periphery (Holaska et al., 2002). A number of nuclear matrix proteins have been implicated in spindle assembly, including NuMa in vertebrates (Becker et al., 2003; Compton and Cleveland, 1994; Dionne et al., 1996; Saredi et al., 1996; Tulu et al., 2003), TPX2 in Xenopus and humans (Bayliss et al., 2003; Eyers and Maller, 2004; Garrett et al., 2002; Gruss et al., 2002; Wittmann et al., 2000), the chromatin bound Skeletor protein in Drosophila (Silverman-Gavrila and Forer, 2003; Walker et al., 2000) and the recently identified nucleolar protein NuSAP in vertebrates (Raemaekers et al., 2003). Although inactivation generates defects in spindle and/or centrosome organization, the precise role of these nuclear proteins in spindle assembly is not clear. Although still controversial (Bloom, 2002; Scholey et al., 2001; Wells, 2001), some components of the nuclear matrix have been proposed to form a spindle matrix that assists in spindle assembly. However, spindle defects need not reflect an active role in spindle assembly, but rather collapse of a compressible non-microtubule matrix that is connected to spindle microtubules.

Key words: Spindle, Centrosome, BimC, Kar3, Mitosis, Meiosis, Nuclear matrix, Kinesin, Spindle matrix
Members of the Kar3 family of kinesins provide the strongest link between BimC kinesins and the nucleus. The Kar3 family of kinesins was first identified in Saccharomyces cerevisiae as a karyogamy (Kar) mutant that was defective in nuclear fusion (Meluh and Rose, 1990). BimC function in spindle assembly is antagonized by Kar3 kinesins; concomitant loss of BimC and Kar3 function increases the frequency of bipolar spindles in comparison with the frequency of monopolar spindles in bimC mutants (O’Connell et al., 1993; Saunders and Hoyt, 1992). The motor domains of BimC (Barton et al., 1995; Sawin et al., 1992) and Kar3 kinesins (Endow and Komma, 1998; Vale and Milligan, 2000) translocate toward the plus and minus ends of microtubules, respectively. Collectively, these results suggest that genetic interactions between BimC and Kar3 kinesins reflect the application of opposing motive forces to spindle microtubules to establish or maintain spindle bipolarity (Cottingham and Compton, 2000; Walczak et al., 1997). Kar3 kinesins are nuclear during interphase in C. The chambers at 25°C. The klp61F1, klp61F3 (Heck et al., 1991), klp61F4 (Wilson et al., 1997) and ncd9 mutants (Komma et al., 1991) have been described. The ncd point mutant described herein as ncd9 was isolated in a screen to uncouple ncd and ca (O’Tousa and Szauter, 1980) and obtained from Scott Hawley. Df3Lhab PG was obtained from James Posakony (Godt et al., 1993). Chromosomes bearing mutations in KLP61F and Ncd were constructed by meiotic recombination and standard genetic methods. The genotypes of klp61F ncd mutant stocks were confirmed by cytological and immunoblot analyses. Mutant animals were maintained as heterozygotes with TM6B or TM6C balancer chromosomes bearing dominant mutations in Tubby, Humoral and/or Stubble (Lindsley and Zimm, 1992).

DNA sequence analysis

DNA was extracted from larval brains of klp61F4 ncd1 and ncd9 mutants with DNeasy Tissue Kit (Quiagen). Mutant genes were amplified with Fail Safe PCR System (Epicare) and selected oligonucleotides (Integrated Device Technology). Oligonucleotide sequences and reaction conditions are available upon request. Amplification products were purified from agarose gels with QiAQuick Gel Extraction Kit (Quiagen), sequenced (DNA Core facility, Georgia State University) and analyzed with MacVector software (Accelrys). Sequence analysis of independent amplification products confirmed mutational changes. The molecular lesion in klp61F4 is probably the result of aberrant excision of the mutating transposon (Wilson et al., 1997). The ncd9 mutant contains a deletion of a single nucleotide in the motor domain. Sequences for klp61F4 and ncd9 have been deposited in GenBank as AY729989 and AY729990, respectively.

Antibodies

Antibodies against Ncd were directed against bacterially expressed fusion protein. The Ncd stalk domain was expressed in bacteria by cloning the 0.6 kb EcoRI/HindIII fragment of pBSncd (McDonald and Goldstein, 1990) into pQE32 (Quiagen). Fusion protein expression was induced with IPTG as directed by the manufacturer. Two New England white rabbits were immunized and boosted (Research Animal Care Facility, University of Wisconsin-Madison) with immunogen in slices of 10% SDS-PAGE gels. To affinity purify antibodies, fusion protein was fractionated on preparative 10% SDS-PAGE gels and transferred to nitrocellulose membranes with standard methods. Strips of membrane-bound bacterial fusion protein, revealed by Ponceau S (Sigma-Aldrich) staining, were incubated for 30 minutes in 10 ml PBS-TT, PBS with 0.2% Triton-X-100 (v/v) and 0.2% Tween 20 (v/v), and then with 1:10 dilution of crude antisera in 10 ml PBS-TT for 2 hours. Antibodies were eluted with 100 mM Glycine HCl (pH 2.3) and combined with an equal volume of 3 M Tris-HCl (pH 8.0). Antibodies were stabilized by addition of BSA to approximately 100 μg/ml, repeatedly dialyzed in a more than 1000× volume of PBS, brought to 50% glycerol (v/v), and frozen in aliquots at –86°C.

Immunoochemical methods

To perform immunoblot analysis, larval brains and imaginal discs were dissected out of third instar larvae in PBS and macerated in 1% SDS. Protein concentrations were established with BCA Protein Assay system (Pierce) and 50 μg of each extract was fractionated in a wide lane of a 10% SDS-PAGE gel and transferred to Protran 0.2 μm nitrocellulose (Fisher Scientific) by standard methods. Antibodies against Ncd and KLP61F were diluted 1:1000 in TBS-TT (TBS with 2% Triton-X 100, 2% Tween 20, pH 7.5) supplemented with 5% dry milk. Membranes were incubated in antibody solution with for 1-2 hours and washed three times for 15 minutes with 50 ml TBS-TT. Membranes were then incubated in a solution of anti-rabbit antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch

Materials and Methods

Fly husbandry and genetics

Drosophila flies were maintained on standard medium in humidified chambers at 25°C. The klp61F1, klp61F3 (Heck et al., 1993), klp61F4 (Wilson et al., 1997) and ncd9 mutants (Komma et al., 1991) have been maintained as heterozygotes with TM6B or TM6C balancer chromosomes bearing dominant mutations in Tubby, Humoral and/or Stubble (Lindsley and Zimm, 1992).
Laboratories) diluted 1:2000 in TBS-TT supplemented with 5% dry milk for 1 hour and washed three times for 15 minutes with 50 ml TBS-TT. Signal was detected with SuperSignal West Dura Chemiluminescent System (Pierce) as directed by the manufacturer. To perform immunofluorescence, tissues were spread under a coverslip in PEMS (PIPEs 80 mM, EGTA 10 mM, MgCl2 10 mM, 100 mM sucrose, pH 6.8), placed in a 50 µl PEMS with 1% (w/v) glycerol. Tissue was gently spread under a silanized coverslip by wicking media from beneath the coverslip. Slides were frozen in liquid nitrogen, the coverslip was flicked off with a razor blade and the slide was placed in 100% methanol cooled in dry ice for 6 minutes. Slides were moved to a postfixative of 4% paraformaldehyde in PBS for 6-12 minutes and washed in several changes of PBS-TT at room temperature. Following application of PBS-TT supplemented with 3% BSA, primary antibodies diluted in PBS-TT with 3% BSA were applied for 1-2 hours. Following several washes in PBS-TT, tissues were incubated with conjugated secondary antibodies for 1-8 hours. Samples were washed in PBS-TT and mounted in Vectashield (Vector) or Prolong (Pierce). Antibodies against Ncd, KLP61F, and γ-tubulin were diluted 1:2000 and mouse monoclonal antibodies against lamin (Harold Saumweber), E7 antibodies against tubulin (Developmental Studies Hybridoma Bank) and mAb1A1 antibodies against Skeletor (Kristen Johansen) were used at 1:5-10. When necessary, secondary antibodies were directed against the FCγ fragment of mouse IgG or the μ chain of IgM (Jackson ImmunoResearch Laboratories). Chromatin was routinely stained with a combination of DAPI and SYTO 43 (Molecular Probes). Immunofluorescence images were single z-stacks of deconvolved sections obtained with a 1.4 oil emersion objective at 1.2 dry objective mounted on an IX70 Olympus microscope. Images were captured with a Soft Imaging Systems CCD camera and imported into Photoshop for processing.

### Results

This study was initiated with a genetic test to determine whether loss of Ncd suppressed the lethal effects of mutations in KLP61F (Heck et al., 1993; Wilson et al., 1997). Animals bearing mutant alleles of both KLP61F and Ncd were constructed by meiotic recombination. Alleles of KLP61F included klp61F1, klp61F2 and klp61F4. The hypomorphic klp61F1 and klp61F3 mutants (Wilson et al., 1997) contain transposon insertions in the upstream non-coding region of KLP61F (Heck et al., 1993) and express a low level of wild-type KLP61F protein (Wilson, 1999). The klp61F4 mutant is a severe loss-of-function allele (Wilson et al., 1997). Alleles of Ncd included the protein null ncd1 allele (Komma et al., 1991) and ncd0 that contains a nucleotide deletion in the motor domain (Materials and Methods). General references here to double mutants are designated as klp61F ncd for simplicity.

Mutant klp61F ncd adults were recovered in almost all combinations tested (Table 1). In most combinations, fully formed adults often died within pupal cases and viable adults died within a few days of hatching, possibly because of residual klp61Fmutant effects and/or additive effects of klp61F and ncd on animal viability. Although viable adults were not recovered, klp61F4ncd1 mutant animals survived longer as third instar larvae and showed increased cell proliferation in comparison to klp61F4 mutants, as indicated by the larger size of larval brains and imaginal discs. These results indicated that Ncd function limits cell proliferation and contributes to lethality of klp61F mutants.

### Immunoblot analysis of Ncd and KLP61F expression

Improved viability and increased cell proliferation in klp61F ncd mutants suggested that Ncd is expressed in the soma of wild-type animals. To examine Ncd expression directly, antibodies were directed against the stalk domain of Ncd and used to probe crude extracts of larval brains of wild-type animals by immunoblot analysis. Affinity purified antibodies against Ncd recognized 90 kDa and 150 kDa proteins that were not recognized by pre-immune antibodies (Fig. 1A). The 90 kDa protein was designated Ncd since it was close to the size

### Table 1. Viability of klp61F ncd double mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Heterozygotesa</th>
<th>Expected no. of homozygotesb</th>
<th>Observed no. of homozygotesb</th>
<th>Observed/expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>klp61F1 ncd0</td>
<td>170</td>
<td>85</td>
<td>24</td>
<td>0.28</td>
</tr>
<tr>
<td>klp61F1 ncd+</td>
<td>201</td>
<td>101</td>
<td>46</td>
<td>0.46</td>
</tr>
<tr>
<td>klp61F3 ncd0/klp61F1 ncd0</td>
<td>309</td>
<td>155</td>
<td>192</td>
<td>1.24</td>
</tr>
<tr>
<td>klp61F1 ncd0/Df(3) bab PG ncd0</td>
<td>200</td>
<td>100</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

*aHeterozygous progeny bearing a mutant and wild-type copy of KLP61F and ncd.

bThe expected number of fully viable homozygous mutant adults is ≈0.5 of the number of viable heterozygous adults.

*cScored within 48 hours of eclosion from pupal case.
expected and was not detected in ncd1 or klp61F4 ncd1 mutants (Fig. 1B). These results show that Ncd is expressed in somatic tissue as well as in the female germ line. At the onset of this study, neither the level of protein expression nor the mutational change in klp61F4 was known. Membranes here were probed with antibodies against the stalk domain of KLP61F (Wilson, 1999). The ~130 kDa KLP61F protein was detected in ncd1 mutant brains, but not in klp61F4 ncd1 mutants (Fig. 1B). DNA sequence analysis of the klp61F4 gene revealed an insertion of four nucleotides near the start codon that shifts the translational reading frame. Conceptual translation of the mutant gene predicted expression of a truncated 6.3 kDa polypeptide of 66 amino acids that includes the amino terminal 40 amino acids of KLP61F. These data indicate that klp61F4 is effectively a protein null allele and that klp61F4 ncd1 mutants lack KLP61F as well as Ncd.

Spindle organization in klp61F ncd mutants

We next compared spindle organization in wild type, klp61F and klp61F ncd1 mutants. Spindles in wild-type animals are bipolar and spindle poles are organized by centrosomes, designated here as biastral spindles (Fig. 2A). Spindles in klp61F mutants are predominantly monopolar (Fig. 2B) or monastral bipolar structures (Wilson et al., 1997) in which one pole lacks detectable immunostaining of γ-tubulin or shows a very tiny mass (Fig. 2C). The frequency of biastral spindles was increased in klp61F ncd1 mutants in comparison with the corresponding klp61F mutant. Biastral spindles comprised less than 30% (Wilson et al., 1997) and 15% of the spindles in klp61F1 and klp61F2 mutant brains, respectively, but more than 96% of ≥100 spindles scored in klp61F1 ncd1 (n≥3) and klp61F2 ncd1 (n≥3) mutant brains. Biastral spindles were not observed in klp61F4 mutants (Wilson et al., 1997), but comprised more than 66% of ≥150 spindles klp61F4 ncd1 mutants (n≥3). Biastral spindles in klp61F ncd mutants sometimes showed multiple masses of γ-tubulin at spindle poles and an apparently polyploid complement of chromatin, suggesting that mitosis and/or cytokinesis failed in a previous cell cycle. The frequency of monastral bipolar spindles was also decreased in all mutant combinations, but most dramatically in klp61F1 mutants in which monastral bipolar spindles comprised ~65% of the spindles scored (Wilson et al., 1997). Some spindles in klp61F ncd mutants could not be unambiguously assigned as monopolar or monastral bipolar, showing multiple centrosomal asters and condensed chromosomes, but without recognizable spindle organization. Tabulation of spindle defects in klp61F and klp61F ncd mutants is available online (Table S1, see supplementary material). Collectively, these results indicate that neither KLP61F nor Ncd are essential for crosslinking and/or bundling microtubules into bipolar spindles. Furthermore, Ncd contributes to assembly of monastral bipolar spindles as well as monopolar spindles in klp61F mutants.

Nuclear defects in the soma of KLP61F-deficient mutants

Spindle pole bodies in yeast move through the nuclear envelope to side-by-side positions when temperature sensitive bimC mutants are shifted to non-permissive temperatures (Saunders and Hoyt, 1992). To examine the relationship between centrosomes and the nuclear envelope here, larval brains of wild-type and mutant animals were stained with antibodies against lamin and γ-tubulin as well as a chromatin dye and examined by deconvolution immunofluorescence microscopy (Fig. 3). The nuclear envelope in Drosophila becomes fenestrated at spindle poles (Hiraoka et al., 1990), appearing in larval brains of wild-type animals as a fusiform

Fig. 1. Immunoblot analysis of Ncd and KLP61F expression. (A) Crude extracts of ovaries of wild-type females probed with antibodies from immune (I) and preimmune (P) antibodies against Ncd. (B) Crude extracts of larval brains of wild type (wt), ncd1 and klp61F4 ncd1 mutants fractionated in three wide lanes (underscores) of a 10% SDS-PAGE gel. Parallel channels of an Immunetics manifold were probed with antibodies against Ncd (Ab Ncd) and KLP61F (Ab KLP61F). Positions of Ncd (⁎) and KLP61F (⁎⁎) proteins are indicated.

Fig. 2. Spindle organization in klp61F and klp61F ncd mutants. Larval brains of wild type (A), klp61F2 (B,C) and klp61F3 ncd1 (D) animals were stained with antibodies against α-tubulin (green), γ-tubulin (red) and a fluorescent chromatin dye (blue). (C) Arrow indicates a tiny dot of γ-tubulin at a nearly anastral spindle pole and asterisk indicates chromosomes at opposing pole. Bar, 2 μm.
structure with diffuse lamin staining extending toward the plasma membrane (Fig. 3A). The nuclear lamina in klp61F3 and klp61F4 mutant cells with monopolar spindles (n≥50), inferred by monopolar organization of chromosomes and centrosomes, showed involutions that extended toward centrally located centrosomes as well as blebbing and diffuse staining throughout cells (Fig. 3B, C). Similar staining was detected in cells (n≥20) of klp61F4 ncd1, klp61F3 ncd1 and klp61F3 ncd4/klp61F4 ncd4 mutants with monopolar structures. Invaginations in the nuclear lamina extending toward centrally located centrosomes suggested that the nuclear lamina is, directly or indirectly, attached to centrosomal material and/or centrosomal asters.

Ultrastructural analysis of the nuclear lamina in the soma of klp61F ncd mutants

Unexpectedly, cells in klp61F ncd mutants with bipolar spindles, inferred by metaphase alignment of chromosomes and bipolar positioning of centrosomes, showed extensive blebbing and regions of disorganization (Fig. 3D). The nuclear lamina in ncd1 mutants was similar to the nuclear lamina in wild-type animals (data not shown), indicating that the defects could not be attributed to loss of Ncd. However, diffuse staining of lamins in klp61F ncd mutant animals made evaluation of nuclear lamina integrity difficult to evaluate by immunofluorescence. To examine the nuclear lamina at a higher resolution, larval brains in the wild type and klp61F4 ncd4/klp61F3 ncd1 mutants were examined by transmission electron microscopy (Fig. 4). Transheterozygotes klp61F4 ncd4/klp61F3 ncd1 mutant animals were chosen for these experiments because mutant effects were representative. The nuclear lamina in interphase cells of klp61F ncd mutants was indistinguishable from the nuclear lamina in wild-type animals. Mitotic cells (n=8) in wild-type brains showed a nuclear lamina lying between condensed chromatin and mitochondria in the cytoplasm (Fig. 4A). Although 1 of 20 mitotic cells in mutant brains showed a nuclear lamina similar to the nuclear lamina in wild-type brains, a nuclear lamina could not be detected or appeared to be very disorganized (Fig. 4B) in 16 of the remaining mitotic cells in klp61F ncd mutant brains (n=5). The positioning of condensed

![Fig. 3. Nuclear lamina organization in klp61F and klp61F ncd mutants. Larval brains of wild type (A) and klp61F3 (B), klp61F3 ncd1/Df(3L)bab PG (C) and klp61F3 ncd1/klp61F4 (D) mutants stained with antibodies against lamins (grayscale), γ-tubulin (red) and a chromatin dye (blue). Arrows in (B, C) indicate involutions of the nuclear lamina that extend toward centrosomes. (D) Cell with bipolar positioning of centrosomes and metaphase alignment of chromosomes, showing regions of (arrow) disorganization and blebbing of nuclear lamina. Images of γ-tubulin and chromatin are single projected images of a stack of deconvolved sections. Images of lamin staining are single deconvolved sections chosen to show invasions in nuclear lamina. Bar, 5 μm.](image1)

![Fig. 4. TEM of nuclear lamina defects in klp61F ncd mutants. Somatic cells in larval brains of wild-type (A) and klp61F4 ncd4/klp61F3 ncd1 mutant (B-D) animals with nuclear lamina indicated with red arrows, condensed chromatin indicated by blue arrows and mitochondria indicated by green arrows. (A) Nuclear lamina in a wild-type cell lies between condensed chromatin and cytoplasmic mitochondria, shown in the inset at 2× magnification. (B) Somatic cell in klp61F4 ncd4/klp61F3 ncd1 larval brain lacks detectable nuclear lamina lying between mitochondrion and condensed chromatin. A region between a mitochondrion and chromatin is shown in the inset at 2× magnification. (C) Nuclear lamina surrounding separate masses of condensed chromatin. (D) Region lying between marked lamina is shown at 3× magnification. Fenestration of the nuclear lamina in prometaphase or metaphase cells was inferred from uniform staining throughout mitotic cells whereas interphase cells showed lighter staining of nucleoplasm in comparison to darker staining of the cytoplasm and by position of condensed chromatin near the cell center rather than the nuclear periphery. Interphase cells were more than tenfold more frequent than cells in mitosis. Bars, 2 μm.](image2)
We first examined KLP61F localization in wild-type testes with confocal microscopy. Immunostaining of KLP61F in wild-type primary spermatocytes in G2 showed diffuse cytoplasmic distribution that was similar to immunostaining of somatic cells in interphase (Wilson, 1999). Although KLP61F localizes to centrosomal asters and spindles in somatic cells of wild-type animals (Wilson, 1999) and cultured clone 8 cells (Fig. S1, see supplementary material), KLP61F remained diffusely distributed and did not show detectable localization to centrosomal asters at the G2/M transition (Fig. 5A) or to meiotic spindles at metaphase (Fig. 5B). KLP61F showed septin-like localization in midbodies in late telophase of meiosis (Fig. 5C,D) that was similar to its localization during telophase of mitosis in germ cells (Wilson, 1999). Male germ cells within a cyst proceed through spermatogenesis in near synchrony (Fuller, 1993) and an exceptional cyst caught in the transition from metaphase to anaphase (Fig. 5E) revealed the transition in KLP61F localization; KLP61F showed slight enrichment in the polar regions of spermatocytes at metaphase (Fig. 5F). KLP61F repositioned to the interpolar region of spindles near anaphase (Fig. 5G) whereas spermatocytes in late anaphase and early telophase showed KLP61F nearly removed from the cellular pool and localized in a plane that bisected the entire spermatocyte (Fig. 5H). We found similar localization (data not shown) with antibodies against the phosphorylated BimC Box of Eg5 (Sharp et al., 1999a) and with antibodies against KLP61F tagged with the myc epitope (Barton et al., 1995). Taken together, these results show the germ-cell-specific localization of KLP61F to cleavage furrows and forming ring canals near telophase. However, in contrast to mitosis in somatic cells and germ cells, meiotic spindles assemble in wild-type spermatocytes without detectable spindle enrichment of KLP61F.

Failure to detect enriched centrosomal and spindle localization of KLP61F suggested either that KLP61F function is not required for male meiosis or that enriched spindle localization is not required for KLP61F function. To address this question, we examined male meiosis in larval testes of klp61F3 homozygotes, klp61F/Df(2L)Df(3L)1 and klp61F/Df(3L)bab PG animals that express one copy of klp61F3. Testes are not generated in klp61F4 mutants, precluding analysis here of spermatocytes lacking KLP61F entirely. Immunofluorescence analysis indicated that centrosomes separated the G2/M transition in those klp61F mutant spermatocytes with two or more centrosomes (Fig. 6A). Acentrosomal meiotic spermatocytes showed microtubules, but did not show microtubule asters or detectable spindle structures (Fig. 6B). Although spermatocytes containing centrosomes showed a range of spindle defects, classic monopolar spindles (Fig. 6C) comprised less than 10% of spindle structures.
substantially lower than the 50-75% frequency found in somatic cells of these mutants (Wilson et al., 1997). The lower frequency of monopolar spindles in mutant spermatocytes most likely reflects progression through meiosis in spermatocytes with spindle defects (Kemphues et al., 1980; Rebollo and Gonzalez, 2000; White-Cooper et al., 1993) whereas somatic cells with monopolar spindles are severely delayed in mitosis (Gatti and Baker, 1989). Most meiotic spindles had characteristics of both monopolar and monastral bipolar spindles, showing microtubules splayed into broad spindle poles that lacked detectable γ-tubulin or showing splintered spindle poles with foci of γ-tubulin at the ends of microtubule bundles (Fig. 6C,D). Bivalents were closely apposed to spindle poles, positioned symmetrically or asymmetrically within microtubule bundles. Despite the abnormal organization of microtubules, spermatocytes in telophase showed a cytokinetic furrow that divided the spermatocyte unequally (Fig. 6E), with one daughter receiving most or all of the bivalents and centrosomes. Telophase was also abnormal in that bivalents typically remained paired, as indicated by the presence of ~3-4 chromatin masses in spermatocytes at telophase that were similar in size to the chromatin masses near metaphase. Nonetheless, these data indicate that, despite its dispersed distribution, loss of KLP61F function in spermatocytes produces the same range of spindle defects that are found in somatic cells.

**Nuclear matrix defects in spermatocytes with and without centrosomes**

Confocal microscopy was used to evaluate the relationship between centrosomes and the nuclear lamina in klp61F mutant spermatocytes. Similar to wild-type spermatocytes (Fig. 7A), centrosomes separated at the G2/M transition in klp61F3 and klp61F3/klp61F4 mutant spermatocytes with an intact nuclear envelope (Fig. 7D). Fusiform structures, indicative of a bipolar spindle in wild-type spermatocytes (Fig. 7B), were not detected in klp61F mutant spermatocytes. Rather, the nuclear lamina showed deep involutions that were occupied by closely apposed centrosomes (Fig. 7E). Remarkably, acentrosomal spermatocytes in the same cyst showed similar involutions (Fig. 7E). The nuclear lamina appeared to collapse around bivalents in spermatocytes and form micronuclei, irrespective of the presence or absence of centrosomes (Fig. 7E). Several
inferences can be drawn from these observations. One inference is that formation of involutions and collapse of the nuclear lamina in klp61F mutant spermatocytes are probably not caused by microtubule-dependent repositioning of centrosomes. Another inference is that failure of most homolog pairs to disjoin in klp61F mutant spermatocytes probably reflects collapse of the nuclear lamina around bivalents. A final inference is that KLP61F function is required, directly or indirectly, to prevent collapse of the nuclear matrix during M-phase.

Nuclear lamina defects at poles of bipolar spindles in klp61F ncd spermatocytes

We next asked whether loss of Ncd function both restored spindle bipolarity and prevented formation of micronuclei in klp61F ncd mutant spermatocytes. For these experiments, we examined homozygous klp61F ncd1 mutants and mutants homozygous for ncd1, but transheterozygous for klp61F3, klp61F4 or the deficiency Df(3L)bab PG in which the wild-type KLP61F gene has been deleted. Immunostaining of α- and γ-tubulin in mutant spermatocytes indicated that spindles at metaphase of both meiotic divisions were typically bipolar with a single centrosome at each pole (Fig. 8A). Homolog pairs and sister chromatids disjoined and segregated to opposing spindle poles at anaphase of meiosis I and meiosis II, respectively (Fig. 8B). However, many spermatocytes (~50%) showed several small nuclei at the poles of telophase spindles (n≥50) and some post-meiotic spermatids also contained micronuclei (Fig. 8B,C). These observations suggested the presence of residual nuclear matrix defects in klp61F ncd mutants, despite assembly of bipolar spindles.

Immunostaining of lamins in klp61F ncd mutant testes showed that virtually all primary spermatocytes contained a single nucleus, indicating that formation of micronuclei did not reflect progression through meiosis with multiple nuclei. Although a single nuclear lamina was present in spermatocytes at metaphase and anaphase of meiosis I and meiosis II (Fig. 8D), 50-75% of spermatocytes at telophase of meiosis I and meiosis II (n≥50) showed a nuclear lamina about individual homolog pairs or chromosomes clustered at centrosomes (Fig. 8E). Cysts of post-meiotic spermatids showed multiple nuclei (Fig. 8F), but the frequency was typically less than 10%, suggesting that fusion of individual nuclear envelopes in mutant spermatocytes is delayed or blocked. The nuclear laminas in the wild type (Fig. 8G) and ncd1 mutant spermatocytes were indistinguishable (Fig. 8H,I), indicating that the nuclear lamina defects in klp61F ncd mutants are probably not a result of loss of Ncd alone. Given the apparent collapse of the nuclear lamina around bivalents in klp61F mutants, the simplest explanation for these results is that micronuclei in klp61F ncd mutant spermatocytes reflects residual defects caused by loss of KLP61F function.

Ncd localization in wild-type and KLP61F-deficient spermatocytes

Ncd-mediated suppression of klp61F mutants raised the question of Ncd function in spindle and nuclear matrix organization in spermatocytes. In contrast to embryos (Hatsumi and Endow, 1992; Sharp et al., 2000a; Sharp et al., 1999b), Ncd was distributed throughout the interphase nucleus and enriched near heterochromatin attached to the nuclear lamina in wild-type spermatocytes (Fig. 9A). Similar localization was detected in somatic cells of larval brains and in cultured clone 8 cells (Fig. S2, see supplementary material). Near the G2/M transition, Ncd showed diffuse distribution throughout the nucleus (Fig. 9B), as if Ncd is released from chromatin-associated material as bivalents become condensed. Spermatocytes at metaphase of both meiotic divisions showed immunostaining of Ncd near centrosomes, but immunostaining in spindles was weak (Fig. 9C) or undetectable. Ncd localized to midbodies near bivalents in klp61F mutants, the simplest explanation for these results is that micronuclei in klp61F ncd mutant spermatocytes reflects residual defects caused by loss of KLP61F function.

**Fig. 8.** Micronuclei in klp61F ncd spermatocytes at telophase. Testes in klp61F ncd mutants (A-F), wild-type animals (G) and ncd1 mutants (H,I) were stained with antibodies against γ-tubulin (green) and a fluorescent chromatin dye (blue) and α-tubulin (green, A-C) or lamins (green, D-I). (A,D) Multiple nuclei were not detected in metaphase of meiosis, but were detected at the poles of telophase spindles (B, arrows) and in spermatids (C,F arrows). Multiple nuclei were not detected in wild-type (G) or in ncd1 (H) spermatocytes in telophase of meiosis or in post-mitotic spermatocytes (I). Bar, 2 μm.
(Fig. 1) as staining was not detected in spermatocytes (Fig. 9E) or in somatic cells (Fig. S2, see supplementary material) of ncd mutants. In contrast to spermatocytes, immunostaining of Ncd in larval brains and cultured clone 8 cells showed immunostaining of pole-to-pole fibers in mitotic cells (Fig. S2, see supplementary material) that were similar to the pole-to-pole fibers observed in embryos (Endow and Komma, 1996b). These observations show that Ncd localization in somatic cells and spermatocytes is similar during interphase and telophase, but the precise pattern in prometaphase and metaphase varies. Ncd localization in primary spermatocytes and in spermatocytes near the G2/M transition in klp61F and other klp61F mutants was similar to localization in wild-type spermatocytes. However, Ncd was diffusely distributed in meiotic spermatocytes (Fig. 9F) and immunostaining of spindles, centrosomes or micronuclei was not detected. Taken together, these observations indicate that Ncd is a component of the nuclear matrix and is mislocalized in klp61F mutant spermatocytes. One implication of these findings is that KLP61F is required for disposition of nuclear matrix constituents during M-phase.

Skeletor localization in klp61F mutant spermatocytes

Immunostaining of Skeletor provided another marker for the nuclear matrix in klp61F and klp61F ncd mutant spermatocytes. In contrast to the rapid mitotic cycles in early embryos (Walker et al., 2000), Skeletor was below the level of detection in primary spermatocytes until chromatin condensation was detectable near the G2/M transition (Fig. 10A). Skeletor showed similar mitosis-specific expression in proliferating germ cells and in cultured clone 8 cells (data not shown), suggesting that Skeletor expression is upregulated near the G2/M transition in somatic cells and spermatocytes, as is
the nucleolar protein NuSAP (Raemaekers et al., 2003). Skeletor was diffusely distributed throughout the nucleus prior to prometaphase, but fusiform structures were not observed until metaphase (Fig. 10B,D). Skeletor usually appeared to be enriched near segregating chromatin during anaphase and chromatin-associated during telophase (Fig. 10C). Immunostaining of Skeletor in klp61F3 mutant spermatocytes resembled immunostaining in wild-type spermatocytes near the G2/M transition (Fig. 10G). However, meiotic spermatocytes showed immunostaining of aggregates (Fig. 10F) that were typically associated with chromatin within micronuclei (Fig. S3, see supplementary material). In contrast to klp61F mutant spermatocytes, Skeletor localization in klp61F3 ncdI and ncdI mutant spermatocytes was similar to that in wild-type spermatocytes (Fig. 10E,G,H). The simplest interpretation of these results is that neither KLP61F nor Ncd are essential for Skeletor localization and that Skeletor collapses with the nuclear matrix in KLP61F-deficient mutants.

**Discussion**

Here we present cytological evidence that loss of KLP61F function generates spindle defects as well as novel defects in organization of the nuclear matrix during M-phase in somatic cells and spermatocytes. Our results also show that Ncd is nuclear during interphase and spindle-associated in M-phase in the soma and male germ line. Loss of Ncd function increases the frequency of biastral spindles in klp61F mutants, but fails or incompletely restores nuclear matrix defects. These findings raise new questions about the molecular basis of genetic interactions between KLP61F and Ncd.

**Novel nuclear defects in klp61F and klp61F ncd mutants**

Somatic cells in klp61F and klp61F ncd mutants with monopolar spindles showed deep invaginations in the nuclear lamina that extended toward centrally located centrosomes (Fig. 3B,C). Similar invasions were found in klp61F mutant spermatocytes judged to be near prometaphase (Fig. 7E), irrespective of the presence or absence of centrosomes (Fig. 7E). These observations suggest that the driving force in forming invaginations in the nuclear lamina is associated with nuclear and/or cytoplasmic material rather than with centrosomes or centrosome organized microtubules. A contribution of nuclear forces to repositioning of centrosomes has precedence in yeast; spindle-pole bodies in preassembled spindles move through the nuclear envelope to side-by-side positions when temperature-dependent BimC function is inactivated at non-permissive temperatures (Saunders and Hoyt, 1992). Because spindle pole bodies assume face-to-face positions when microtubules are depolymerized (Jacobs et al., 1988), side-by-side positions suggest that nuclear forces contribute to spindle defects in BimC-deficient yeast as well.

Nuclear defects in somatic cells differed from those in spermatocytes, raising the question of whether KLP61F function in somatic cells and spermatocytes is mediated by a common mechanism or two different mechanisms. Arguments can be made for and against a common mechanism. The strongest argument for a common mechanism is the striking similarity of spindle defects in somatic cells (Wilson et al., 1997) and spermatocytes (Fig. 6). Another argument is the ability of ncd mutants to suppress the klp61F mutant phenotype in both cell types (Fig. 2, Fig. 8). At first glance, other aspects of the mutant phenotype are not consistent with a common function. Somatic cells in KLP61F-deficient animals showed extensive disorganization of the nuclear lamina (Fig. 3), including cells showing bipolar positioning of centrosomes and metaphase alignment of chromosomes. In contrast to somatic cells, the nuclear lamina appeared to collapse around bivalents near prometaphase and form micronuclei in klp61F mutant spermatocytes (Fig. 7). These differences could reflect different functions in somatic cells and spermatocytes. Alternatively, the difference may reflect cell cycle regulation; the spindle assembly checkpoint is active in somatic cells (Gatti and Baker, 1989), but inactive (Kemphues et al., 1982) or severely abrogated in spermatocytes (Rebollo and Gonzalez, 2000). This view is consistent with the disorganized state of the nuclear lamina in cultured clone 8 cells that were delayed in mitosis with an inhibitor of APC (Fig. S2, see supplementary material). Thus, disorganization of the nuclear lamina in somatic cells and formation of micronuclei in spermatocytes in KLP61F-deficient mutants could reflect a common underlying defect in different cell types.

KLP61F shows overlapping, but differential localization during mitosis and male meiosis. In somatic cells, KLP61F is highly enriched near centrosomal asters during prophase, spindle-associated in metaphase and located in midbodies in telophase (Wilson, 1999). In meiotic spermatocytes, KLP61F fails to show centrosomal enrichment or spindle association, but in late anaphase/early telophase KLP61F localizes to a sphere that bisects the entire spermatocyte and then follows the ingressing cleavage furrow (Fig. 5). Similar localization in proliferating germ cells in telophase was found to reflect interactions, directly or indirectly, with components of fusomes (Wilson, 1999). We cannot draw firm conclusions from the failure to detect KLP61F localization to centrosomal asters or to spindles as a small pool could escape our detection methods. However, given static positioning of Eg5 in spindles assembled in Xenopus egg extracts (Kaporov and Mitchison, 2001), the failure to detect KLP61F localization to male meiotic spindles may indicate that KLP61F is not associated with spindle microtubules, but with non-microtubule binding partners. In most cell types, BimC kinesins are diffusely distributed throughout the cytoplasm during interphase. Localization of BimC kinesins to spindles in vertebrate cells has been linked to phosphorylation of a Cdk1 target site in the conserved BimC Box near the carboxyl tail region of these kinesins (Blangy et al., 1995; Sawin and Mitchison, 1995; Sharp et al., 1999a), postulated to elicit or strengthen intrinsic microtubule binding activity and spindle localization. However, phosphorylation of the BimC Box of Cut7 in Saccharomyces pombe is not required for spindle association or for Cut7 function in assembly of a bipolar spindle (Drummond and Hagan, 1998). It is possible that KLP61F and other BimC kinesins crosslink microtubules and non-microtubule binding partners during interphase. BimC Box phosphorylation may downregulate microtubule binding activity and allow interactions with non-microtubule binding partners to direct KLP61F localization during M-phase. Identification of non-microtubule binding partners and genetic analysis of BimC Box function in KLP61F localization could test these possibilities.
Ncd and the nuclear matrix

Similar to Kar3 kinesins in vertebrates (Kuriyama et al., 1995; Mountain et al., 1999; Walczak et al., 1996), Ncd is nuclear during interphase in spermatocytes (Fig. 9A) and in somatic cells (Fig. S1A, see supplementary material) as well as in somatically derived clone 8 cells (Fig. S1C, see supplementary material). It is not clear why Ncd fails to show nuclear localization in early embryos, but the rapidity of the cell cycle in early embryos may preclude complete reorganization of the nuclear envelope and nuclear entry of Ncd through nuclear pores. Two lines of cytological evidence suggest that Ncd may be associated with the nuclear matrix during interphase in somatic and male germ line cells. First, Ncd shows subnuclear enrichment near heterochromatin attached to the nuclear envelope (Fig. 9 and Fig. S1, see supplementary material). Given that fibrillar components of the nuclear matrix connect chromatin to the inner nuclear membrane (Nickerson, 2001), subnuclear enrichment could reflect localization of Ncd to fibrillar components of the nuclear matrix and/or localization to chromatin-associated material at these sites. Second, Ncd localizes to fibers extending between the poles of metaphase spindles in somatic cells and cultured clone 8 cells (Fig. S2, see supplementary material), reminiscent of Ncd localization in embryos (Endow and Komma, 1997). The functional significance of these fibers is not clear since pole to pole fibers have not been reported in female meiotic spindles (Endow and Komma, 1997; Endow and Komma, 1998; Hatsumi and Endow, 1992) and we did not detect strong immunostaining of similar fibers in meiotic spermatocytes (Fig. 9). However, there is a precedence for localization of other nuclear matrix proteins to spindle fibers. The nuclear matrix protein NuSAP is localized to spindle-associated fibers in cultured vertebrate cells and loss of its function generates defects in spindle organization and chromosome segregation (Raemaekers et al., 2003). The nuclear matrix protein Skeleton also localizes to fibers in embryonic spindles although the fibers do not extend the full distance between spindle poles (Walker et al., 2000). Loss of Ncd function did not appreciably alter Skeleton distribution in spermatocytes (Fig. 10), indicating that Ncd is not necessary or plays only a very limited role in Skeleton localization. Nonetheless, our findings are consistent with the view that Ncd is a component of the nuclear matrix in somatic cells and spermatocytes.

Cooperation between KLP61F and Ncd in assembly of bipolar spindles

At this point, we can only speculate on the relationship between spindle and nuclear defects in klp61F mutants and the functional significance of Ncd-mediated suppression. With few exceptions, cooperation between BimC and Kar3 kinesins in spindle assembly has been ascribed to application of antagonistic motive forces to spindle microtubules to establish or maintain centrosome separation (Kashina et al., 1997; Sharp et al., 2000b). According to this view, nuclear defects in KLP61F-deficient animals could be secondary to primary defects in spindle organization; increasing the frequency of bipolar spindles in klp61F ncd mutants results in a decreased frequency of nuclear defects. However, this explanation does not easily explain formation of micronuclei at the poles of bipolar spindles in klp61F ncd spermatocytes (Fig. 8).

Moreover, collapse of the nuclear lamina about bivalents cannot be ascribed to spindle defects since similar defects are not found in meiotic spermatocytes of β2t n mutants (P.G.W., unpublished) that lack microtubules and spindle structures owing to loss of an essential testis specific β-tubulin (Kempfues et al., 1982; Kempfues et al., 1983; Kempfues et al., 1980). An alternative interpretation of our findings is that spindle defects are secondary; spindle defects reflect collapse of a nonmicrotubule spindle matrix that is derived from the nuclear matrix and attached to centrosomes and/or spindle microtubules. According to this view, interactions between KLP61F and nonmicrotubule binding partners prevent collapse of a compressible spindle matrix when nuclear and cytoplasmic contents mix at prometaphase, whether or not KLP61F is spindle associated.

Our results are in part unexpected because they question the assumed relationship between localization and function of a microtubule-dependent motor protein. KLP61F is required for spindle bipolarity, but its function in male meiosis does not require spindle association. Conversely, KLP61F localizes to cleavage furrows, but it is not required for cytokinesis. With these contradictions in mind, further work must address the mechanism of KLP61F function in spindle organization and the functional significance of nuclear localization of Ncd.

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References


Cut7 indicate divergence of regulation within the bimC family of kinesin-related proteins.


Table S1. Spindle organization in somatic cells of *klp61F* and *klp61F ncd* mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>no. brains</th>
<th>no. spindles</th>
<th>% biastral</th>
<th>% monastral bipolar</th>
<th>% monopolar</th>
</tr>
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<tr>
<td>Canton S</td>
<td>13</td>
<td>422</td>
<td>99.1</td>
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<td>0.0</td>
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<td>65.2</td>
<td>28.4</td>
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<tr>
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<tr>
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<td>66.5</td>
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<td>32.0</td>
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*a* Spindle organization in Canton S and *klp61F* mutants as previously established (Wilson et al., 1997)

*b* Spindle organization as assayed by immunofluorescence of α-tubulin, γ-tubulin and a chromatin dye.