The conserved kinase NHK-1 is essential for mitotic progression and unifying acentrosomal meiotic spindles in *Drosophila melanogaster*

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Conventional centrosomes are absent from the spindle in female meiosis in many species, but it is not clear how multiple chromosomes form one shared bipolar spindle without centrosomes. We identified a female sterile mutant in which each bivalent chromosome often forms a separate bipolar metaphase I spindle. Unlike wild type, prophase I chromosomes fail to form a single compact structure within the oocyte nucleus, although the integrity of metaphase I chromosomes appears to be normal. Molecular analysis indicates that the mutant is defective in the conserved kinase nucleosomal histone kinase-1 (NHK-1). Isolation of further alleles and RNA interference in S2 cells demonstrated that NHK-1 is also required for mitotic progression. NHK-1 itself is phosphorylated in mitosis and female meiosis, suggesting that this kinase is part of the regulatory system coordinating progression of mitosis and meiosis.

Introduction

Accurate partitioning of replicated DNA to daughter cells is fundamental for life. In eukaryotes, DNA is packaged into multiple chromosomes and separated by a microtubule-based machine called a spindle during mitosis and meiosis. Bipolarity of the spindle is essential for accurate segregation of chromosomes. It is equally crucial to limit the number of spindles so that all chromosomes share one unified spindle. Centrosomes and motor-based microtubule bridging are sufficient to establish and maintain a bipolar spindle (Nedelec, 2002). Therefore, strict control of centrosome duplication and separation in the cell cycle can ensure the bipolarity and the unity of the spindle.

Although centrosomes dictate spindle formation in mitosis, it is known that conventional centrosomes are lacking from the spindle in female meiosis of many species, such as mice, *Xenopus laevis*, and *Drosophila melanogaster* (McKim and Hawley, 1995; Waters and Salmon, 1997). The absence of centrosomes challenges us to readdress the questions of how a cell can define spindle bipolarity and limit the number of spindles to a single spindle without centrosomes. These questions have

important implications beyond female meiosis because the centrosome-independent pathway is also operating in mitosis (Khodjakov et al., 2000; Budde and Heald, 2003). We hope that the study of female meiosis will uncover underlying mechanisms usually masked by centrosomes in mitosis.

Cell-free systems using *X. laevis* extracts revealed how bipolar spindles can form without centrosomes and demonstrated critical roles of chromosomes and microtubule motors in the process (Heald et al., 1996; Walczak et al., 1998). Chromosomes drive spindle assembly around them by activating certain proteins required for spindle formation through the Ran-importin system (Gruss et al., 2001; Wiese et al., 2001; Blower et al., 2005). These Ran-activated spindle assembly factors include TPX2 (targeting protein for *X. laevis* kinesin-like protein 2), NuMA (nuclear mitotic apparatus protein), and the Rae1 ribonucleoprotein complex, but the full complement of factors regulated by this system remains to be determined.

D. melanogaster has proven to be a valuable model system for studying spindle formation in female meiosis in vivo (McKim and Hawley, 1995). Real-time analysis revealed a dynamic process of spindle assembly driven by chromosomes in oocytes (Matthies et al., 1996; Skold et al., 2005). As *D. melanogaster* female meiosis arrests in metaphase I until activation (Mahowald and Kambysellis, 1980), spindle formation can be studied independently of chromosome separation.

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Undoubtedly, the greatest advantage of *D. melanogaster* is its sophisticated genetics. So far, genetic analysis has identified a handful of genes involved in the meiotic spindle formation, including microtubule motors, a membrane-bound protein, and microtubule-associated proteins (Endow et al., 1990; McDonald et al., 1990; Tavosanis et al., 1997; Cullen and Ohkura, 2001; Giunta et al., 2002; Kramer and Hawley, 2003). Obviously, more genes need to be identified to reconstruct the whole molecular pathway of acentrosomal (also called anastral or acentriolar) spindle formation in female meiosis.

To understand how a spindle is formed in female meiosis, we searched for mutants that show abnormal morphology of the metaphase I spindle in *D. melanogaster*. In this paper, we report the analysis of a mutant (*triplet*) that is defective in the unification of the spindle. Molecular analysis indicates that *triplet* encodes a conserved protein kinase, nucleosomal histone kinase-1 (NHK-1). Isolation of new alleles and RNA interference (RNAi) in S2 cells demonstrate that NHK-1 is essential for mitotic progression as well. This study identified NHK-1 as a new regulator for mitosis and meiosis and revealed an intriguing role in the formation of an acentrosomal spindle in female meiosis.

Results

Individualized acentrosomal spindles in a *triplet* mutant

In contrast to centrosome-mediated spindle formation in mitosis, molecular information of centrosome-independent spindle formation in female meiosis is still limited. To tackle this problem using a genetic approach, we decided to cytologically search for mutants defective in acentrosomal spindle formation in female meiosis in *D. melanogaster*. In *D. melanogaster*, mature oocytes arrest at metaphase I and the rest of meiosis restarts when they are activated during ovulation, which coincides with fertilization (Mahowald and Kambysellis, 1980). Our screen focused on the morphology of the metaphase I–arrested spindle in nonactivated mature oocytes.

We identified one female sterile mutation, which we tentatively called *triplet* after its spindle phenotype. This mutant was originally identified among lethal mutants through the analysis of oocytes produced from germline clones. Detailed genetic analysis indicated that a female sterile mutation could be separated from an unrelated lethal mutation on the same chromosome and was solely responsible for the phenotype in female meiosis. The female meiotic phenotypes were identical in homozygotes of the female sterile mutation and in germline clones from heterozygotes of the original chromosomes.

We studied the metaphase-arrested spindle in female meiosis I by immunostaining. In a wild-type oocyte, one bipolar spindle is formed that contains an entire set of meiotic chromosomes. Typically, three chiasmatic bivalent chromosomes are located at the equator and two small achiasmatic chromosomes are located closer to the poles (Fig. 1 A; Theurkauf and Hawley, 1992). In the *triplet* mutant, one bipolar spindle was formed in only half of the spindles observed. In the other half, two or three bipolar spindles were formed and each associated with one or two bivalent chromosomes (Fig. 1 B).
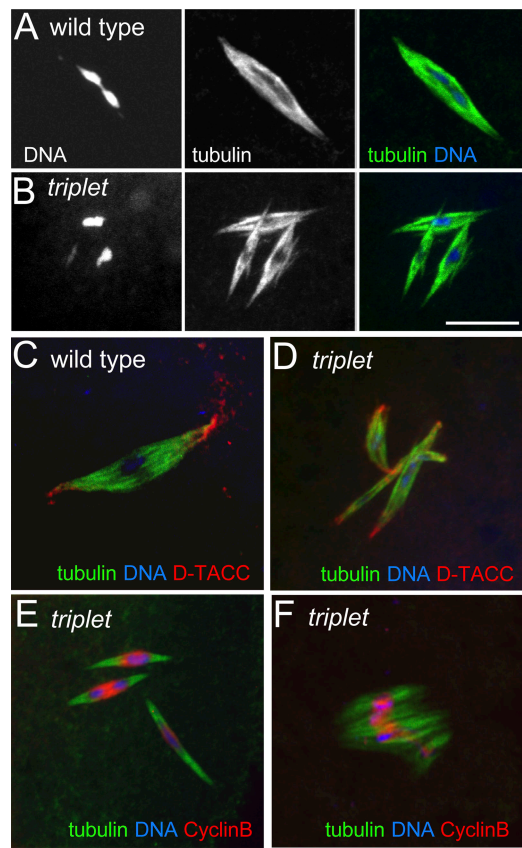

Figure 1.   ***triplet* mutant is defective in holding a spindle together in female meiosis.** Metaphase I spindles in nonactivated oocytes from wild type and the *triplet* mutant. (A) One single bipolar spindle formed in wild-type nonactivated oocyte. (B) Three bivalent chromosomes form three separate spindles in the *triplet* mutant. Note that bivalent chromosomes are stretched between poles as in wild type. (C) D-TACC localizes to the acentrosomal poles of a wild-type spindle. (D) D-TACC localizes to each acentrosomal spindle pole in the *triplet* mutant oocytes. (E) Cyclin B localizes to spindle equators in *triplet* as in wild type. (F) Rare unfocused poles in the *triplet* mutant. Bars, 10 μm.

Even when multiple spindles were formed, the morphology of each spindle appeared to be normal, namely, a symmetrical bipolar spindle with focused poles. To confirm the correct microtubule arrangement, we examined the localization of proteins that normally localize to spindle poles or equators. *D. melanogaster*–transforming acidic coiled coil (D-TACC) protein localizes to the acentrosomal spindle poles in wild-type flies (Fig. 1 C; Cullen and Ohkura, 2001). In the *triplet* mutant, D-TACC correctly localized to each pole of each individual spindle (Fig. 1 D), suggesting that individual spindles have proper pole structures. To visualize another spindle subdomain, we examined the localization of cyclin B, which we recently showed to be concentrated at the equator of a female meiotic spindle in wild type (Pearson et al., 2005). Similarly, in the *triplet* mutant, each spindle has cyclin B at the equator (Fig. 1 E). Correct localization of pole and equator proteins suggests that individual spindles in the *triplet* mutant have a normal microtubule configuration.

Abnormalities were restricted to the formation of multiple spindles, and other aspects were rarely affected in the *triplet*
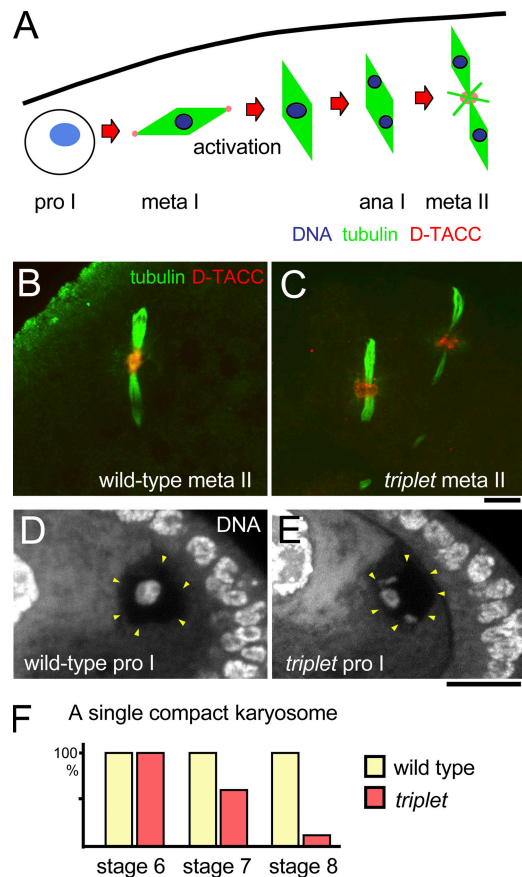
Figure 2. Meiotic progression in the *triplet* mutant. (A) A diagram of meiotic progression in wild type. In prophase I (pro I), chromosomes form a compact cluster called a karyosome. Fully matured oocytes arrest at metaphase I (meta I). After activation, the spindle rotates and undergoes anaphase (ana I). A pair of tandem spindles is formed with prominent central poles in meiosis II (meta II). (B) A pair of tandem spindles in wild-type meiosis II. (C) Two pairs of tandem spindles in meiosis II formed in the *triplet* mutant. (D) Prophase chromosomes forming a single compact karyosome in the wild-type oocyte nucleus at stage 7. The arrowheads indicate the outline of the oocyte nucleus. (E) Separate prophase I chromosomes in the oocyte nucleus of the *triplet* mutant at stage 7. (F) Frequencies of oocyte nuclei containing a single compact karyosomes in wild type and the *triplet* mutant at different stages. Differences between wild type and *triplet* at stages 7 and 8 and differences between stages in *triplet* are statistically significant (P < 0.01; χ^2 test). Bars, 10 μm.

mutant. However, we noticed that a small minority (6%) of *triplet* spindles shows loose microtubule bundling and/or unfocused spindle poles (Fig. 1 F). As this morphology is not seen in wild type, we considered this to be a significant defect in spite of the low frequency.

Meiotic progression in the *triplet* mutant

The aforementioned cytological studies indicate that the structure of individual spindles in the *triplet* mutant is generally normal. In addition, to examine whether these spindles are functional, we looked at the interaction between a spindle and chromosomes. The *triplet* mutant, like wild type, contained bivalent chromosomes with condensed morphology that were stretched along the spindle axis and usually consisted of two domains. This chromosome morphology is consistent with chiasmatic chromosomes pulled toward both poles by kineto-

chore microtubules. Moreover, achiasmatic fourth chromosomes were often located closer to poles within individualized spindles as seen in wild type. These results suggest that spindle–chromosome interactions are not impaired in the *triplet* mutant.

To confirm that each *triplet* spindle is functional, we examined meiotic progression after the activation of oocytes. In wild type, after the activation of oocytes, a metaphase I spindle is first rotated 90 degrees, followed by chromosome separation. It is then reorganized to form a pair of meiosis II tandem spindles with prominent poles in the middle (Fig. 2, A and B; Endow and Komma, 1997). To test whether *triplet* spindles can undergo chromosome separation and spindle reorganization, we examined spindles in activated oocytes from the *triplet* mutant. In these oocytes, we sometimes observed more than one pair of meiosis II spindles (Fig. 2 C), indicating that the individual spindles can undergo meiotic progression. Spindle orientation relative to the cortex was generally unaffected in the mutant.

Wild-type prophase I chromosomes in *D. melanogaster* oocytes form a single compact cluster called the karyosome within the nucleus (Fig. 2 D; King, 1970). A failure to form this compact karyosome may result in condensed chromosomes too distant from each other to assemble a unified spindle after nuclear envelope breakdown. To test this possibility, we examined prophase I chromosomes in earlier stages of the oocyte in the *triplet* mutant. DNA staining revealed that early oocytes (at stage 6) in the mutant had a compact karyosome structure within the nucleus (Fig. 2 F). At later stages (7 and 8), however, chromosomal DNA in the oocyte nucleus was often seen as fragmented and/or filamentous masses (Fig. 2 E) and the abnormality increased through the progression of stages (Fig. 2 F). Therefore, the *triplet* gene activity is required for the maintenance but not the formation of the karyosome structure. Nevertheless, because bivalent chromosomes in metaphase I appear to be normal, the integrity of individual chromosomes is unlikely to be disrupted. Unlike known mutants, which affect karyosome structure (Morris et al., 2003), the *triplet* mutation does not significantly affect other aspects of oogenesis, including specification of oocytes, polytenization in nurse nuclei, and the egg shape. In summary, the *triplet* mutation is specifically defective in holding chromosomes together in prophase I and forming a single unified spindle in metaphase I.

The *triplet* mutation is epistatic to *mini spindles (msps)* and *d-tacc* mutations

Although the *triplet* phenotype is unique among reported mutants, the *triplet* gene may function in concert with other genes required for female meiosis. To examine genetic interactions, we constructed double mutants with *msps*, *d-tacc*, and *ncd* mutations.

Msps and its binding partner D-TACC localize to acentrosomal spindle poles in female meiosis (Cullen and Ohkura, 2001). A female sterile mutation of either gene leads to the formation of one tripolar spindle in a third to a half of oocytes, whereas in the rest of the oocytes, the spindles maintain their bipolarity.

Double mutants between female sterile *triplet* and *msps^{MJ208}* alleles did not affect viability and remained female sterile. Immunostaining of nonactivated oocytes showed that the *msps^{MJ208} triplet* double mutant has a spindle phenotype
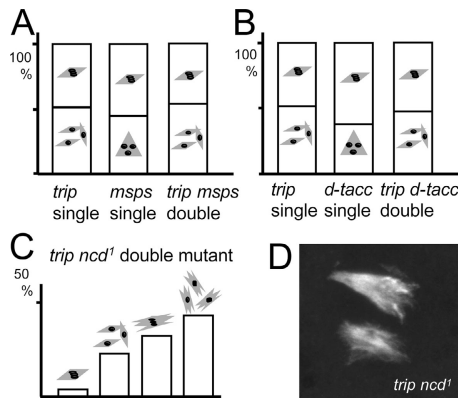
Figure 3. **triplet is epistatic to msps and d-tacc.** (A) *trip* is epistatic to *msps*. *trip* single, *msps*[MJ208] single, and *trip msps*[MJ208] double mutants were examined for the morphology of metaphase I spindles. Single bipolar spindles, multispindles, and single tripolar spindles were observed and counted. (B) *trip* is epistatic to *d-tacc*. The morphology of metaphase I spindles from *trip* single, *d-tacc*[stella] single, and *trip d-tacc*[stella] double mutants. (C) The phenotypes of *trip* and *ncd* are additive. The morphology of metaphase I spindles from *trip* single, *ncd*[1] single, and *trip ncd*[1] double mutants. A combination of *ncd* (unfocused poles) and *triplet* (multiple spindles) phenotypes was observed. (D) Multiple metaphase I spindles with unfocused poles were formed in the *trip ncd*[1] double mutant.

identical to that of the *triplet* single mutant (Fig. 3 A). About half of the oocytes contain two or three separate spindles, each of which is associated with bivalent chromosomes. Although the remaining oocytes contain one bipolar spindle shared by an entire set of chromosomes, we did not observe any tripolar spindles as seen in the *msps* single mutant. We also examined oocytes from a *triplet d-tacc*[stella] double mutant, which showed a phenotype identical to that of the *triplet* single mutant and no *d-tacc* phenotype (Fig. 3 B). These results indicated that the *triplet* mutation is epistatic to the *msps* and *d-tacc* mutations.

Ncd is a minus end–directed microtubule motor that is uniformly localized along spindle microtubules (McDonald et al., 1990; Walker et al., 1990; Hatsumi and Endow, 1992a). *ncd* mutants show defects in focusing acentrosomal poles in female meiosis (Hatsumi and Endow, 1992b; Matthies et al., 1996). In addition, Ncd plays a role in efficient localization of Msps (Cullen and Ohkura, 2001). A double mutant between *ncd*[1] and *triplet* showed a mixture of the *ncd* and *triplet* phenotypes (Fig. 3, C and D), namely, multiple spindles and unfocused poles. Therefore, unlike *msps* or *d-tacc*, the *ncd* mutation is additive to the *triplet* mutation for the phenotype of metaphase I spindles.

triplet encodes NHK-1

To determine the molecular identity of the gene, we first mapped the mutation finely by using visible genetic markers, single nucleotide polymorphisms (SNPs), and chromosomal deficiencies (Fig. 4, A and B). We narrowed the region down to within 50 kb of the third chromosome (cytological region 97D). All coding regions and their surroundings within the region were sequenced, and we found one nonsense mutation in the gene (CG6386) encoding NHK-1 (Fig. 4 C). The identity of the gene was further verified by using a *P* element insertion
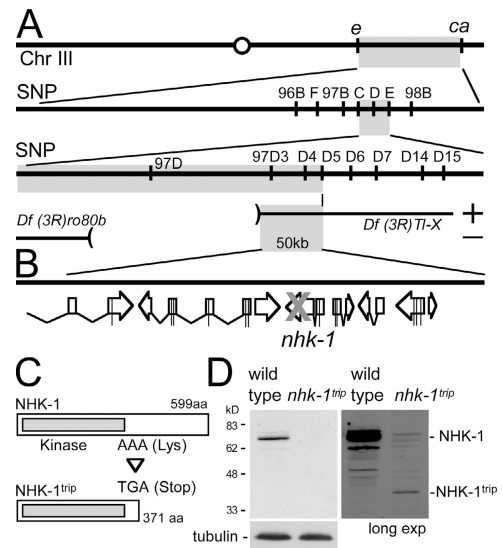

Figure 4. **triplet encodes NHK-1.** (A) Mapping of the *triplet* mutation using visible markers, SNPs, and deficiencies. (B) Molecular map around *trip*. Transcribed regions are shown in arrows. (C) Predicted protein structure of NHK-1 in wild type and the *trip* mutant (*nhk-1*[trip]). *trip* has a nonsense mutation in NHK-1, which results in truncation of the noncatalytic region. (D) Truncated NHK-1 protein in the *trip* mutant. Protein samples from adult females of wild type and *nhk-1*[trip] were prepared for immunoblots using an antibody against the full-length NHK-1 (Aihara et al., 2004).

line and its derivatives, which were isolated independently from the original *triplet* mutant (see below).

NHK-1 was originally identified as a protein kinase that phosphorylates nucleosome-bound histone H2A at threonine 119 in vitro (Aihara et al., 2004). It was shown to have an affinity for nucleosomes and indeed localizes to chromosomes in syncytial embryos. The kinase is conserved from *Caenorhabditis elegans* to humans. Previously, NHK-1 and its homologues had not been shown to have functions in spindle formation or cell division in either mitosis or meiosis.

To confirm the molecular nature of the *triplet* mutation, immunoblots against *triplet* mutant adults were performed using an NHK-1 antibody (Aihara et al., 2004). Consistent with a nonsense mutation in NHK-1, the 65-kD band corresponding to the full-length NHK-1 disappeared and instead a band of ∼40 kD appeared in the *triplet* mutant (Fig. 4 D). This result confirmed that the band recognized by the antibody is indeed the NHK-1 protein and that the *triplet* mutation results in a truncated protein.

Based on these results, we concluded that the *triplet* gene encodes NHK-1, and we renamed the original *triplet* mutation *nhk-1*[trip].

NHK-1 is an essential gene for viability

The *D. melanogaster* genome project has identified a line (EP[3]863) in which a *P* element (EP; Rørth, 1996) is inserted in the noncoding region of the NHK-1 gene (which we call *nhk-1*[EP]). This line is viable but poorly female fertile. The poor fertility was not complemented by *nhk-1*[trip].

To determine whether NHK-1 is required for somatic mitosis, we created a variety of new alleles by remobilization of the EP in the allele *nhk-1*[EP]. Molecular analysis indicated that

part of the EP was left in some alleles, whereas various genomic regions flanking the EP were deleted in the others (Fig. 5 A). Only the alleles in which the molecular aberration is confined within the NHK-1 gene were studied further.

We found that one of the alleles, E107, has a 1.1-kb deletion of the genomic region corresponding to the kinase domain and is therefore considered a kinase-null mutation. E107 is lethal as a homozygote or hemizygote over a small deficiency, indicating that NHK-1 is essential for viability. We also found that another lethal allele, E60, has a part of the original EP left. We also isolated a female sterile allele, E24, which has a 50–base pair insertion in the 5′ nontranslated region of the NHK-1. All alleles were female sterile over the *triplet* allele.

To determine the level of the NHK-1 protein in these alleles, we performed immunoblots of total protein samples using anti–NHK-1 antibody (Fig. 5 B). In the lethal hypomorphic E60 allele, the level of protein in a third instar larva is reduced compared with wild type. In viable alleles, the amount of NHK-1 in adult females was correlated with the level of fertility. The female sterile E24 allele, which reduces the expression level of NHK-1 but does not affect the integrity of the coding sequence, showed the same spindle defects as the original allele in female meiosis (unpublished data), confirming that the defect is not attributable to any special nature of the original allele.

NHK-1 is required for spindle organization and chromosome segregation in mitosis

To uncover the essential role of NHK-1 for viability, we examined the lethal alleles of NHK-1 in detail. Homozygotes of the kinase-null E107 allele died as early pupae. The mutant larvae grew to the same size as wild type with no obvious defects in behavior. Most larval cells are polytenized cells that undergo rounds of DNA replication and cell growth without cell divisions. Cell division is limited to adult progenitor cells, such as cells in the central nervous system (CNS) and imaginal discs (Gatti and Baker, 1989). Dissection of mutant larvae revealed that the imaginal discs were missing or degenerated and that the CNS was very small. These results suggest that *nhk-1* is essential for dividing cells but largely dispensable for nondividing cells.

To elucidate the role of NHK-1 in dividing cells, we examined the kinase-null allele E107 by chromosome staining of squashed larval CNSs (a so-called brain squash). Consistent with the striking reduction in the size of the CNS, the number of cells was greatly reduced. Furthermore, we did not find any dividing cells in most of the CNSs from late third instar larvae. However, in some CNSs that contained mitotic cells, mitotic chromosomes appeared highly overcondensed (Fig. 6 A).

To analyze the mitotic defects in detail, we examined another lethal allele (E60) that produces a low level of NHK-1. Brain squash revealed a higher mitotic index and frequent overcondensation of chromosomes (Fig. 6, A and B), results that are indicative of mitotic arrest. The frequency of anaphase was also decreased (Fig. 6 B), and we observed some anaphase cells with lagging chromosomes. Except for overcondensation, which is associated with mitotic arrest, the morphology of
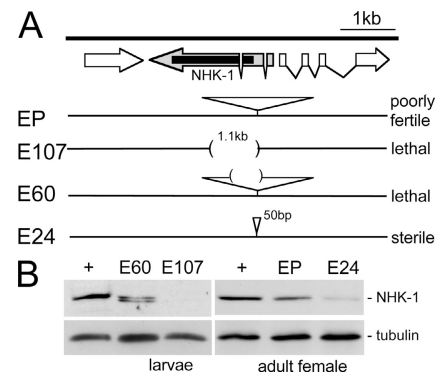

Figure 5. **Molecular analysis of new NHK-1 alleles.** (A) Molecular lesions of representative NHK-1 alleles resulted from excision of the *P* element in the *nhk-1^EP^* mutant. EP is viable and poorly female fertile, E107 and E60 are lethal, and E24 is viable but female sterile. Only alleles in which the aberrations are confined within the NHK-1 gene were studied in detail. (B) The NHK-1 protein in new NHK-1 alleles. Protein samples were prepared from third instar larvae for E60 and E107 or from adult females for other alleles, together with equivalent wild type (+).

mitotic chromosomes was generally unaffected. These observations suggest that NHK-1 is required for chromosome segregation in mitosis.

To further study the role of NHK-1 in mitosis, we visualized α-tubulin and the centrosomal protein CP190 by immunostaining of whole mount preparations of larval CNSs. Wild-type mitosis has a bipolar spindle in which most of the microtubules emanate from two foci where centrosomes (CP190) are located (Fig. 6 C). In the *nhk-1^E60^*, about one third of the spindles (11/33) exhibited defects in bipolarity. The defects included monopolar spindles, multipolar spindles, and formation of ectopic microtubule foci that lacked CP190 (Fig. 6 C). Therefore, NHK-1 is required for proper spindle organization in somatic mitosis.

Depletion of NHK-1 in S2 cells results in chromosome misalignment during mitosis

To analyze the mitotic role of NHK-1 in detail, we turned to the *D. melanogaster*–cultured S2 cell line. A genome-wide survey of protein kinases required for cell cycle progression was previously performed using RNAi in S2 cells (Bettencourt-Dias et al., 2004). Although NHK-1 was included in the survey, significant defects in cell cycle progression were not observed. We wondered whether RNAi in the survey had depleted NHK-1 sufficiently. To clarify this, we exposed S2 cells to double-stranded RNA for a longer period (5–7 d instead of 3 d) to achieve better depletion. Quantitative immunoblots indicated that >98% of NHK-1 protein was depleted (Fig. S1, available at www.jcb.org/cgi/content/full/jcb.200508127/DC1). Indeed, immunostaining revealed that this depletion resulted in a significant increase in mitotic abnormalities compared with control RNAi cells.

NHK-1 depletion resulted in a significantly higher proportion of mitotic cells with unaligned chromosomes (Fig. 6, D and F), compared with wild type. In some cases, irregular chromosome condensation was observed. In addition, more anaphases or telophases had lagging chromosomes or chro-
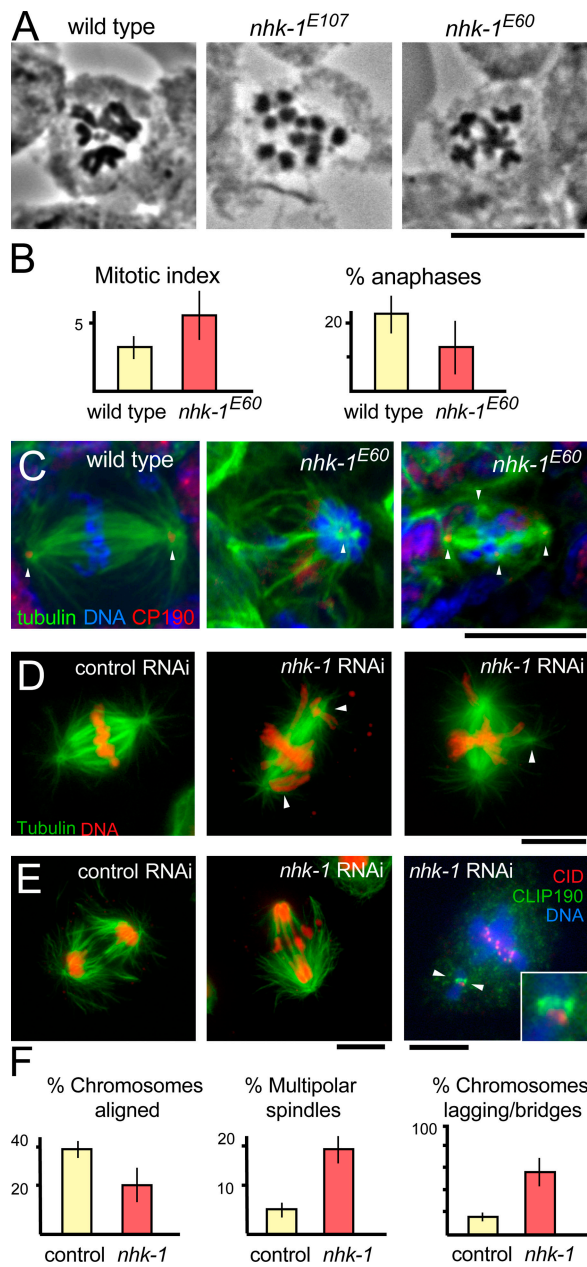
Figure 6. NHK-1 is required for spindle organization and chromosome segregation in mitosis. (A) Orcein staining of mitotic chromosomes in cells of squashed larval CNSs in wild type, the kinase-null *nhk-1^{E107}*, and the lethal allele *nhk-1^{E60}*. (B) Mitotic parameters of larval CNS cells from wild type and *nhk-1^{E60}*. Mitotic index represents the number of mitotic cells per microscopic field, which typically contains 200–400 cells (Cullen et al., 1999). Percentage of anaphase represents the frequencies of anaphases among mitotic cells. Both parameters are significantly different between wild type and the *nhk-1^{E60}* mutant (P < 0.05). The mean values and standard deviations from five CNS samples are shown as bars and lines. (C) Mitotic spindles from larval CNS cells in wild-type and *nhk-1^{E60}*. Arrowheads indicate foci of the centrosomal protein CP190. The polarity of spindles is disrupted in the *nhk-1* mutants. (D) A mitotic spindle in S2 cells after RNAi of control and *nhk-1* genes. The arrowheads in the middle panel indicate misaligned chromosomes, and the arrowhead in the right panel indicates an ectopic pole. (E) Mitotic S2 cells after RNAi of control (left) and *nhk-1* (middle and right) genes. Depletion of NHK-1 results in lagging chromosomes in anaphase (middle). D-CLIP-190 localizes to kinetochores of misaligned chromosomes (arrowheads and inset), indicating that microtubules are not attached to kinetochores. (F) Frequencies of mitotic phenotype after RNAi of control and *nhk-1* genes. The difference is significant in each category (P < 0.05). The mean values and standard deviations from three independent experiments were shown as bars and lines. Bars, 10 μm.

mosome bridges in NHK-1–depleted cells (Fig. 6, E and F). Spindle morphology was sometimes disrupted, typically leading to a multipolar spindle (Fig. 6 D, right).

Chromosome misalignment in NHK-1–depleted cells may be caused by defects in the attachment of microtubules to kinetochores or the movement of chromosomes to the equator after the attachment. To identify the basis of the chromosome misalignment, we examined the centromere/kinetochore proteins Cid and D-CLIP-190. D-CLIP-190 (*D. melanogaster* cytoplasmic linker protein 190) localizes to kinetochores that are unattached to microtubules (Dzhindzhev et al., 2005), whereas Cid (*D. melanogaster* centromere protein A) localizes to all centromeres (Henikoff et al., 2000; Blower and Karpen, 2001). Double staining of these two proteins revealed that most misaligned chromosomes in NHK-1–depleted cells had D-CLIP-190 signals at one or both of the kinetochores (Fig. 6 E). This suggests that NHK-1 is required for efficient attachment of kinetochores to microtubules. Although it is difficult to distinguish primary from secondary defects, these results confirm essential roles for NHK-1 in mitosis.

NHK-1 is phosphorylated during female meiosis and mitosis

Our results indicated that NHK-1 is a protein kinase essential for mitosis and female meiosis. Protein kinases that control mitosis or meiosis are tightly regulated (Barr et al., 2004; Ducat and Zheng, 2004; Glover, 2005). Immunoblots using an NHK-1 antibody originally detected a single 65-kD protein in the extract (Aihara et al., 2004), and we have confirmed its identity by mutations or RNAi. We realized that when we carefully prepared the extract from adult females for immunoblotting, one or two slower migrating bands were detected by the NHK-1 antibody (Fig. 7 A). To test whether these bands were phosphorylated forms, we treated the extract with λ protein phosphatase before electrophoresis. This phosphatase treatment converted slower migrating forms into the fast migrating form (Fig. 7 A), indicating that slow migrating forms are phosphorylated protein.

To examine the NHK-1 expression pattern during development, we performed immunoblots against protein samples prepared from various stages of the *D. melanogaster* life cycle. The NHK-1 protein was ubiquitously expressed throughout development but detected at a very high level in embryos and decreased in second instar larvae (Fig. 7 B). The level of the protein showed a good correlation with the occurrence of cell divisions. Interestingly, slow migrating phosphorylated forms were detected in a stage- or sex-specific manner. They were detected at a high level in adult females but were absent in males. They were also detected at a low level in embryos but were virtually absent from other stages of the life cycle.

To examine whether NHK-1 is phosphorylated during female meiosis, we examined the phosphorylated forms from different stages of oogenesis. We prepared the protein samples from mature nonactivated oocytes (metaphase I) and earlier stages of oogenesis. Immunoblots indicated that only the slower migrating phosphorylated forms of NHK-1 were detected in metaphase I–arrested oocytes, whereas only the faster migrating form was detected in earlier stages of oogenesis (Fig. 7 C).
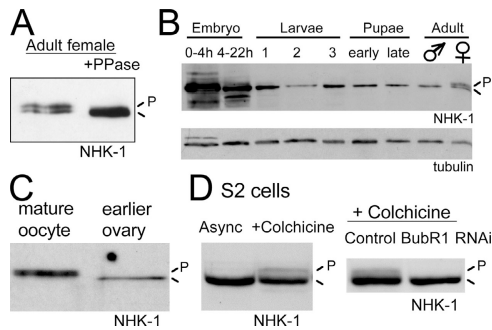
Figure 7. Phosphorylation of NHK-1 in female meiosis and mitosis. (A) NHK-1 is phosphorylated in adult females. Protein extracts from adult females were incubated with or without λ protein phosphatase before immunoblots for NHK-1. Slow migrating forms, which converted to the fast migrating form after phosphatase treatment, are phosphorylated forms. (B) The level and the phosphorylation of NHK-1 during development. Protein samples were prepared from various stages of development for immunoblotting for NHK-1 (top) and α-tubulin. (C) NHK-1 is fully phosphorylated in metaphase I in female meiosis. Adult females were dissected in methanol, and protein samples were prepared from mature nonactivated oocytes (stage 14; King, 1970) and ovarioles at earlier stages (stages 1–10) for immunoblotting to detect NHK-1. (D) NHK-1 is phosphorylated during mitosis in S2 cells. (left) Protein samples were prepared from an asynchronous cell culture (with a mitotic index of 3%) and from cells treated with colchicine for 16 h (with a mitotic index of 26%). (right) Cells were treated with colchicine after RNAi of control and *bubR1* genes for 3 d. Mitotic arrest is required for the accumulation of the phosphorylated form.

We then studied whether the phosphorylation is regulated in the mitotic cell cycle using the *D. melanogaster* S2 cell line. Although no effective synchronization methods have been reported for *D. melanogaster* cell lines, mitotic cells can be enriched by treating with a microtubule depolymerizing drug, colchicine (26% compared with 3% in an untreated population). Immunoblots showed that a slow migrating phosphorylated form appeared after colchicine treatment (Fig. 7 D). To confirm that this was attributable to the accumulation of mitotic cells, we inactivated the spindle checkpoint by RNAi of *bubR1* before treating with colchicine. We did not see an increase of the phosphorylated form in checkpoint-defective cells (Fig. 7 D), suggesting that mitotic arrest, not exposure to colchicine itself, was required for the increase in phosphorylation. These results indicated that NHK-1 is phosphorylated in mitosis as well as in female meiosis.

Discussion

A bipolar spindle in female meiosis is formed without centrosomes in many species, but the molecular mechanism by which it is formed in vivo remains to be elucidated. In this study, we identified a novel *D. melanogaster* female sterile mutation that is defective in holding the chromosomes and the spindle together during female meiosis. Molecular study indicated that the mutation lies in the gene encoding NHK-1. Isolation of new alleles and RNAi in S2 cells demonstrated that this kinase is also essential for mitotic progression. We show that NHK-1 itself is phosphorylated during mitosis and female meiosis, suggesting that it is controlled by other mitotic and meiotic kinases.

Female meiosis and mitosis

Female meiosis is arguably the most important cell division in the animal life cycle. It is estimated that about a third of spontaneous abortions in humans are aneuploid, and the incidence of aneuploidy dramatically increases with maternal age (Hassold and Hunt, 2001). It is rather remarkable that a bipolar spindle is formed without conventional centrosomes in female meiosis in many species (McKim and Hawley, 1995; Waters and Salmon, 1997). Studies indicate that mitosis also has a chromosome-driven spindle assembly pathway, although it is not clear how important such a mechanism is in the presence of centrosomes (Khodjakov et al., 2000; Budde and Heald, 2003; Maiato et al., 2004). Therefore, the study of female meiosis should provide unique information applicable to mitosis, as well as advance our understanding of this equally important cell division itself.

Chromosome and spindle defects of the *nhk-1* mutant in female meiosis

We identified a female sterile mutant of *nhk-1* based on abnormal morphology of the metaphase I spindle in female meiosis. In the *nhk-1* mutant, each bivalent chromosome forms separate metaphase I spindles in female meiosis. We consider these individualized metaphase I spindles and bivalent chromosomes themselves to be structurally and functionally normal, based on the following observations. First, spindle pole proteins and an equator protein localize to the right place. Second, the chromosome number and morphology appears to be unaffected. Third, interactions between chromosomes and spindle microtubules appear to be normal. Finally, they can undergo meiotic progression normally upon activation.

We found that the *nhk-1* mutant is also defective in the maintenance of a single compact karyosome during prophase I. In wild type, all prophase I chromosomes together form and maintain a compact structure called the karyosome within the oocyte nucleus (King, 1970). In the *nhk-1* mutant, prophase I chromosomes form a single compact karyosome structure, but as oogenesis progresses, the chromosomes change their morphology to a filamentous form and occupy separate regions within the nucleus. However, this defect does not seem to have disrupted the integrity of individual meiotic chromosomes or the formation of each bivalent, as chiasma formation and the integrity of metaphase I chromosomes are not significantly affected. This failure to keep nonhomologous chromosomes together during prophase I is likely the primary cause of the separate metaphase I spindles formed after nuclear envelope breakdown.

In *D. melanogaster* male meiosis, each bivalent chromosome is separated in prophase I, but after nuclear envelope breakdown, all chromosomes are captured by microtubules emanating from a pair of centrosomes and integrated into one spindle (Cenci et al., 1994). Therefore, the formation of a single karyosome during prophase I seems particularly critical for centrosome-independent spindle formation in female meiosis.

Roles of NHK-1 in female meiosis

Molecular studies indicate that the mutant is defective in NHK-1. NHK-1 was originally purified from *D. melanogaster* embryonic extract as a major kinase that phosphorylates H2A

within nucleosomes in vitro (Aihara et al., 2004). The phosphorylation site was located at threonine 119 in the COOH-terminal tail of H2A and conserved among eukaryotes. The residue was shown to be phosphorylated in *D. melanogaster* embryos and human cultured cells. However, Aihara et al. (2004) did not establish the cellular roles of NHK-1 and the H2A phosphorylation.

NHK-1 was shown to be localized to mitotic chromosomes in embryos and S2 cells (Aihara et al., 2004; unpublished data). NHK-1 may phosphorylate H2A or other chromosomal proteins to promote the maintenance of a single karyosome. Although the molecular mechanism of karyosome formation and maintenance is not well understood, the substrates activated by NHK-1 may act as a "glue" protein, which holds non-homologous chromosomes together. This glue protein seems to be independent from proteins that hold homologous chromosomes or sister chromatids together, as bivalent chromosomes appear to be normal in metaphase I and can progress through meiosis upon activation.

Roles of NHK-1 in spindle formation in female meiosis

Our study suggests that the primary role of NHK-1 in female meiosis is to maintain a single compact karyosome in prophase I and, consequently, form a unified metaphase I spindle. However, two observations suggest that NHK-1 may play a more direct role in spindle formation.

First, metaphase I spindles in the *nhk-1* mutant show infrequent but significant microtubule bundling defects. Second, a female sterile *nhk-1* mutation is epistatic over *msps* or *d-tacc*. Msps and its binding partner D-TACC localize to acentrosomal spindle poles and play a critical role for spindle bipolarity (Cullen and Ohkura, 2001). Msps belongs to a conserved family of microtubule-associated proteins (Ohkura et al., 2001). Despite half of *nhk-1 msps* or *nhk-1 d-tacc* meiotic spindles having normal appearance, none show tripolar spindles typical of a *msps* or *d-tacc* single mutant. This epistasis suggests a close functional relationship between NHK-1 and Msps or D-TACC, although understanding its molecular basis requires further study. This cytological and genetic evidence suggests that NHK-1 may play a role in spindle formation in addition to karyosome formation. It is difficult to distinguish spindle function from chromosome function in female meiosis because these two are intimately connected in acentrosomal spindle formation. Identification of critical effectors and regulators is now essential for a precise understanding of NHK-1 function in female meiosis.

Possible roles of NHK-1 in mitosis

Identification of new alleles and the RNAi study in S2 cells indicates that NHK-1 is also essential for mitotic progression. Compared with female meiosis, defects in NHK-1 manifest themselves quite differently in mitosis. Chromosome alignment is compromised because of inefficient microtubule attachment to kinetochores in mitosis, whereas the kinetochore–microtubule attachment seems normal in female meiosis. The polarity of spindles is disrupted in mitosis, whereas multiple bipolar spindles are formed in female meiosis.

Why do defects of the same molecule have apparently different consequences in two types of cell division? It is likely that this difference is attributable to the presence of centrosomes in mitosis and their absence in female meiosis. As centrosomes are dominant microtubule-organizing centers in mitosis, the overall spindle morphologies may appear very different even if they have an identical underlying defect. Alternatively, but not exclusively, part of the difference may be a result of the multifunctional nature of NHK-1. As NHK-1 is a protein kinase, it is likely to have multiple substrates and functions during mitosis and meiosis. Therefore, prominent defects in a particular system are determined by which substrates are most sensitive to underphosphorylation.

NHK-1 as a regulator of mitosis and meiosis

Coordinated regulation of various mitotic events is essential for mitotic progression. Mitotic kinases, with notable examples being Polo and Aurora, play crucial roles in the regulation of mitotic progression. However, the roles and regulation of these kinases have yet to be revealed, and more kinases regulating mitosis need to be identified. Our screening for mutants defective in female meiosis has led to the identification of the conserved kinase NHK-1, which is required for mitotic and meiotic progression. Coincidentally, like NHK-1, Polo and Aurora kinases were originally identified as female sterile mutants in *D. melanogaster* (Sunkel and Glover, 1988; Glover et al., 1995). They were later shown to regulate a wide range of mitotic and meiotic processes (Barr et al., 2004; Ducat and Zheng, 2004; Glover, 2005). Mitotic- or meiotic-specific phosphorylation of NHK-1 suggests that NHK-1 itself is regulated by other mitotic kinases and therefore may play a part in coordinating mitotic or meiotic progression.

Materials and methods

D. melanogaster genetics

Standard techniques of fly manipulation were followed (Ashburner, 1989). All stocks were grown at 25°C in standard cornmeal media. w^{1118} was used as wild type. Details of mutations and chromosome aberrations can be found in Lindsley and Zimm (1992) or at http://flybase.org (Drysdale et al., 2005). Double mutants between *nhk-1^trip* and *msps^MJ208*, *d-tacc^stella*, or *ncd^1* were constructed by recombinations using visible markers and checked by complementation of single mutations. New *nhk-1* alleles were isolated from *EP(3)863* by P element excision as follows. Jumpstarter males *yw;nhk-1^EP/TM3, P(Δ2–3)*, were crossed with *yw; EP(3)863/TM6C* females. From each cross, only one *w⁻* revertant male, *yw;(nhk-1^EP)/TM6C*, in which the P element was excised, was individually crossed to *yw;nhk-1^trip/TM6C* females. A total of 138 independent excision events (*w⁻* revertants) were examined, and 28 chromosomes that showed female sterility over *nhk-1^trip* were further examined. Genomic DNA from homozygotes or hemizygotes over *Df(3R)ro80b* was used for molecular analysis by PCR. The level and size of the NHK-1 protein in the mutants were compared with wild type by immunoblotting.

Molecular identification of the *triplet* gene

For the molecular identification of the *triplet* mutation, it was first mapped by recombination with the *rucuca* chromosome using visible and SNP markers and complementation of deficiencies. The original mutant chromosome contained one lethal mutation between *th* and *sr* on the left arm of the third chromosome and one female sterile mutation between *e* and *ca* on the right arm. Homozygous oocytes of recombinant chromosomes free from the lethal mutation gave the same meiotic phenotype as oocytes from germline clones of the original mutant chromosome. Recombinants between

e and *ca* were tested for SNPs and complementation of *triplet*. SNPs were identified by sequencing of genomic DNA amplified by PCR. SNP mapping located *triplet* to 97D. The region was further narrowed by complementation testing using deficiencies in the area. Breakpoints of the deficiencies were defined using mutations and SNPs in the regions. A tight linkage of the female sterile mutation with spindle defects in female meiosis was confirmed by cytological analysis of recombinants and the mutant chromosome over small deficiencies. All coding regions in the area were sequenced after PCR amplification. One gene encoding NHK-1 had a nonsense mutation, and no other genes contained mutations.

Molecular and immunological techniques
Standard DNA manipulation and immunological techniques were used throughout (Harlow and Lane, 1988; Sambrook et al., 1989). The primary antibodies used in this study include antibodies against α-tubulin (DM1A; Sigma-Aldrich), Msps (264; Cullen et al., 1999), D-TACC (Gergely et al., 2000; Brittle and Ohkura, 2005), NHK-1 (Aihara et al., 2004), H3-phospho-S10 (Upstate Biotechnology), CP190 (Rb188; a gift from W. Whitfield, University of Dundee, Dundee, UK; Whitfield et al., 1988), cyclin B (a gift from W. Whitfield; Whitfield et al., 1990), and Cid (Abcam). For immunoblotting, flies were heated at 100°C for 5 min or ovaries were dissected in methanol before homogenization. Peroxidase-conjugated antibodies (The Jackson Laboratory) were used as secondary antibodies for Western blot and detected by ECL kit (GE Healthcare). Protein samples from each stage of development were prepared as described in Cullen et al. (1999). To confirm phosphorylation of NHK-1, heat-treated protein extracts from adult females were incubated with or without 400 U/ml λ protein phosphatase (New England Biolabs, Inc.) for 10 min in the phosphatase buffer.

Cytological analysis
Orcein staining of brain squashes and subsequent analysis were performed as described previously (Cullen et al., 1999). Culture and RNAi of S2 cells were performed as described previously (Brittle and Ohkura, 2005; Dzhindzhev et al., 2005). Effects of RNAi were observed 5–7 d after the addition of double-stranded RNA. Nonactivated oocytes, ovarioles, larval CNSs, and S2 cells were immunostained as described previously (Theurkauf et al., 1992; Tavosanis et al., 1997; Cullen et al., 1999; Brittle and Ohkura, 2005). Activated oocytes were collected every 10 min and fixed in methanol (Cullen et al., 1999). Secondary antibodies conjugated with Cy3, Cy5, or Alexa488 (The Jackson Laboratory or Invitrogen) were used at 1:250–1:1,000 dilution. DNA was counterstained with 0.4 μg/ml DAPI (Sigma-Aldrich), 2 μg/μl propidium iodide (Sigma-Aldrich), or TOPRO3 (1:500; Invitrogen). Thin samples, such as cultured cells and brain squashes, were examined using a 100× Plan-Apochromat objective lens (1.4 NA; Carl Zeiss MicroImaging, Inc.) attached to an Axioplan2 (Carl Zeiss MicroImaging, Inc.). Images were captured by a charge-coupled device camera (Orca; Hamamatsu) using OpenLab2 (Improvision). Other images were taken using a 63× Plan-Apochromat lens (1.4 NA; Carl Zeiss MicroImaging, Inc.) attached to an Axiovert 200M (Carl Zeiss MicroImaging, Inc.) with a confocal scan head (LSM510 meta; Carl Zeiss MicroImaging, Inc.). Confocal images were presented as a maximum intensity projection of the Z-stacks. All digital images were imported to Photoshop (Adobe) and adjusted for brightness and contrast.

Online supplemental material
Fig. S1 shows depletion of NHK-1 by RNAi. Online supplemental material is available at www.jcb.org/cgi/content/full/jcb.200508127/DC1.

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