Anastral Meiotic Spindle Morphogenesis: Role of the Non-Claret Disjunctional Kinesin-like Protein

Heinrich J.G. Matthies,* Heather B. McDonald,‡ Lawrence S.B. Goldstein,* and William E. Theurkauf§

*Division of Cellular and Molecular Medicine, University of California at San Diego, San Diego, California 92093-0683; ‡Department of Genetics, University of Washington, Seattle, Washington 98195-0001; and §Department of Biochemistry and Cell Biology and the Institute for Cell and Developmental Biology, State University of New York at Stony Brook, Stony Brook, New York 11794-5215

Abstract. We have used time-lapse laser scanning confocal microscopy to directly examine microtubule reorganization during meiotic spindle assembly in living *Drosophila* oocytes. These studies indicate that the bipolarity of the meiosis I spindle is not the result of a duplication and separation of centrosomal microtubule organizing centers (MTOCs). Instead, microtubules first associate with a tight chromatin mass, and then bundle to form a bipolar spindle that lacks asters. Analysis of mutant oocytes indicates that the Non-Claret Disjunctional (NCD) kinesin-like protein is required for normal spindle assembly kinetics and stabilization of the spindle during metaphase arrest. Immunolocalization analyses demonstrate that NCD is associated with spindle microtubules, and that the centrosomal components γ-tubulin, CP-190, and CP-60 are not concentrated at the meiotic spindle poles. Based on these observations, we propose that microtubule bundling by the NCD kinesin-like protein promotes assembly of a stable bipolar spindle in the absence of typical MTOCs.

CYTOLOGICAL studies suggest that meiotic spindle morphogenesis in a variety of systems is mechanistically distinct from mitotic spindle assembly in typical somatic cells of higher eukaryotes (for reviews see Rieder et al., 1993; Vernos and Karsenti, 1995; McKim and Hawley, 1995). Three features that differentiate many meiotic systems from typical mitotic cells are a central role for chromatin in triggering spindle assembly (however, see Vernos et al., 1995), the presence of microtubules throughout the cytoplasm, and an absence of pronounced astral microtubule organizing centers (MTOCs)[1]. Immunocytochemical analyses of fixed tissue indicate that the *Drosophila* female meiotic spindle shares these conserved features and suggest a pathway for spindle assembly in which cytoplasmic microtubules first associate with the condensed meiotic chromosomes to form an apolar array, and then reorganize into a bipolar spindle (Theurkauf and Hawley, 1992).

While immunocytochemical analyses have provided insight into the mechanisms of spindle assembly and chromosome segregation during *Drosophila* female meiosis (Theurkauf and Hawley, 1992; McKim et al., 1993; Hatsumi and Endow, 1992*a*), these studies provide no direct information on the kinetics of spindle morphogenesis. In vivo analyses in other systems have allowed elegant studies of the temporal control of meiosis and highlighted the dynamic nature of meiotic chromosome segregation (Nicklas, 1967, 1974, 1989; Nicklas and Staehly, 1967; Nicklas and Koch, 1969). We have therefore developed a technique that allows the direct observation of spindle assembly in living *Drosophila* oocytes. In this study, we use this technique to characterize spindle morphogenesis in wild-type oocytes and to evaluate the effect of the Non-Claret Disjunctional (NCD) kinesin-like protein on spindle assembly kinetics and stability.

Mutational analyses of meiotic chromosome segregation in *Drosophila* females have identified genes required for several key meiotic functions, including homologue pairing and recombination, sister chromosome adhesion, and meiotic spindle assembly (for reviews see Miyazaki and Orr-Weaver, 1994; Hawley, 1989; Hawley et al., 1993). Among the genes identified by these studies, *ncd* appears to play a particularly critical role in meiosis I spindle function. Mutations at the *ncd* locus lead to high levels of nondisjunction and loss of both exchange and nonexchange chromosomes, predominantly during the first meiotic division, but also in the early mitotic divisions (Davis, 1969). Cytological studies of fixed *ncd* mutant oocytes reveal a low frequency of relatively normal bipolar spindles, and more frequent multipolar or apolar microtubule arrays (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992*a*). While these observations indicate that the *ncd* gene product plays a role in spindle organization and function, neither the source of the cytological variability nor the precise role of NCD in the spindle assembly pathway is known.

Address all correspondence to William E. Theurkauf, Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794-5215. Tel.: (516) 632-8596. Fax: (516) 632-8575. e-mail: theurk@mcbsgi.bio.sunysb.edu.

1. *Abbreviations used in this paper*: MTOC, microtubule organizing center; NCD, Non-Claret Disjunctional; NEB, nuclear envelope breakdown.

Molecular, biochemical, and immunocytochemical analyses suggest a mode of action for NCD during spindle assembly. The *ncd* locus encodes a kinesin-like protein with minus-end–directed microtubule motor activity (Endow et al., 1990; McDonald and Goldstein, 1990; McDonald et al., 1990; Walker et al., 1990), and a microtubule-bundling activity (McDonald et al., 1990; Chandra et al., 1993). Bundling activity may be due in part to the tail of NCD, which contains an ATP-insensitive microtubule-binding domain (Chandra et al., 1993). In addition, NCD has been immunolocalized to spindle microtubules in tissue-culture cells and early embryos and to isolated meiotic spindles from *Drosophila* oocytes (Hatsumi and Endow, 1992*b*, Endow et al., 1994). These observations suggest that NCD mediates microtubule–microtubule interactions in the meiotic spindle.

The analyses of living and fixed oocytes presented here support a chromatin-driven spindle assembly pathway and strengthen the conclusion that conventional centrosomes are not involved in forming the meiotic spindle poles (Theurkauf and Hawley, 1992). Analyses of mutant oocytes indicate that NCD plays a semi-redundant role in drawing microtubules together during spindle pole morphogenesis, and an essential role in stabilizing the highly tapered poles in mature oocytes that are arrested in meiotic metaphase I (Mahowald and Kambysellis, 1980). Spindle instability appears to account for the cytological variability of meiotic figures observed in *ncd* mutant oocytes (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992*a*). We suggest that the NCD kinesin-like protein serves to catalyze and to stabilize bipolar spindle organization by bundling spindle microtubules. This function may be essential to an evolutionarily conserved anastral meiotic spindle assembly pathway, but redundant during mitotic spindle assembly in the presence of typical MTOCs.

Materials and Methods

Antibody Production

To study the distribution and behavior of NCD, we prepared two rabbit antisera. Peptides corresponding to amino acids 217–232 and 671–685 of NCD were chemically synthesized by the Harvard microsequencing facility and coupled to keyhole limpet hemocyanin, and the peptide–keyhole limpet hemocyamin conjugates were used to immunize rabbits (BAbCO, Berkeley, CA). These peptides correspond to a region of the putative coiled-coil domain and the last 16 COOH-terminal amino acids of NCD. Antibodies were affinity purified on full-length NCD coupled to Affi-gel blue. The affinity-purified antibodies recognize full-length and appropriate truncated forms of NCD (glutathione-*S*-transferase fusion proteins); N195; and N280 (Stewart et al., 1993) (data not shown).

Lysates from wild-type ovaries contained antigens of the expected molecular weight, while antigen was not detectable in ovary lysates from *ncd* mutant flies lacking the message encoding for the NCD protein (Yamamoto et al., 1989; data not shown). Based on these results, we conclude that the antisera are specific to the NCD protein and verify that the mutation (ca^{nd}) used in this study is null for NCD.

Drosophila Stocks

Oregon R, Canton S, and yw stocks were used as sources of wild-type control oocytes. No morphological or kinetic differences in spindle assembly were observed between these stains. Homozygous *ncd* mutants were recovered from either a ca^{nd} homozygous stock or a ca^{nd}/TM3 balanced stock. Flies were fed standard corn meal and molasses fly food and maintained at constant temperature and humidity.

Western Blotting

Egg chambers were mass isolated as described by Theurkauf and Hawley (1992). For Western blotting, oocytes were homogenized in hot Laemmli sample buffer, boiled, and frozen. Western blotting was carried out according to standard procedures using polyvinylidene difluoride membranes (Bio Rad Laboratories, Hercules, CA) (Harlow and Lane, 1988).

Production of Recombinant NCD

NCD and truncations thereof were produced in *Escherichia coli* as previously reported (Stewart et al., 1993). Full-length NCD was purified by Pipes precipitation (McDonald et al., 1990) or by standard chromatographic techniques including chromatofocusing, phosphocellulose, and gel filtration columns. N195 and N280 were purified on glutathione beads as previously reported (Stewart et al., 1993).

Immunocytochemistry of Drosophila Oocytes

Oocytes were prepared, fixed, and immunostained as described by Theurkauf (1994*b*). Alpha-tubulin (Sigma Chemical Co., St. Louis, MO) and core histone (Chemicon Intl. Inc., Temecula, CA) antibodies were used at 1:500-fold dilution and NCD antibodies were used at 1:100-fold dilution. Rabbit anti-γ tubulin antibody (provided by Y. Zheng, University of California, San Francisco) was used at 1:1,000 and anti-CP-60 antibody (Kellogg et al., 1989; provided by K. Oegema, University of California, San Francisco) was used at 1:500. Oocytes were incubated with primary antibodies for 4 h, washed six times for 15 min, and then incubated for 4 h with appropriate fluorescently labeled secondary antibodies. The labeled oocytes were then washed as above, mounted, and observed by confocal microscopy. Images were processed on a Silicon Graphics workstation using Voxel View by Vital Images (Fairfied, IA).

Production of Fluorochrome-conjugated Bovine Brain Tubulin

Bovine brain tubulin was isolated by conventional methods and labeled with either fluorescein or tetramethyl-rhodamine by the procedure of Kellogg et al. (1988). To insure that the labeled protein is active, microtubules were "cycled" three times as described in Kellogg et al. (1988).

Time-lapse Confocal Microscopy

Time-lapse laser confocal microscopy was used to analyze microtubule organization in living oocytes as previously described (Theurkauf, 1994*a*), and microtubule reorganization in living embryos was examined using the following modifications to the procedure: embryos collected on apple juice agar plates were transferred to double-stick tape, and chorions were manually removed (Ashburner, 1989). Dechorionated embryos were then placed on a 22 mm × 44 mm coverglass coated with double-stick tape adhesive (dissolved in heptane and painted on the coverglass). After briefly drying the embryos over Dryrite dessicant (5–10 min), the embryos were covered with halocarbon oil. Microinjections were done on the stage of the confocal microscope. For the present studies, several confocal microscopes were used, including BioRad's 600 and 1000 models (Bio Rad Laboratories) and an RCM8000 (Nikon Inc., Garden City, NY). All of the confocal attachments were used in conjunction with inverted microscopes using Nikon objectives (Optiphot or Diaphot; Nikon Inc.). Images were stored either directly on the hard drive or on a optical memory disc recorder (OMDR, LVN3000R; Sony, Montvale, NJ) or video systems (TQ3038; Panasonic, Secaucus, NJ).

To observe meiotic spindle assembly, 3-d-old flies were placed on fresh food for 3–6 d and ovaries were dissected under halocarbon oil. Rhodamine-labeled bovine brain tubulin was then pressure injected into late stage 12 or early stage 13 oocytes (King, 1970), in close proximity to the nucleus. Oocytes were observed with a ×40 fluor or a ×60 plan apo lens. Images were recorded at 1–2-min intervals until signs of nuclear envelope breakdown were apparent, and then collected at 4–10-s intervals.

Results

Spindle Assembly in Living Oocytes

Previous analyses of fixed material suggested that the *Drosophila* female meiotic spindle assembles by a path-

way that does not rely on the duplication and separation of centrosomal MTOCs (Theurkauf and Hawley, 1992). Mitosis and meiosis are dynamic processes, however, and analysis of fixed samples cannot provide direct data on the temporal sequence of spindle morphogenesis. To directly observe the pathway of spindle morphogenesis, we initiated in vivo analyses of spindle assembly in wild-type oocytes and early embryos. Analyses of meiotic spindle assembly used a time-lapse confocal microscopic technique developed to examine microtubule dynamics during axial patterning in stage 8–9 egg chambers (Theurkauf, 1994*a*). For the present studies, stage 12–13 oocytes were microinjected with fluorochrome-conjugated bovine brain tubulin. After microinjection, these late-stage oocytes mature and arrest in metaphase of the first meiotic division, thus allowing direct observation of nuclear envelope breakdown and spindle assembly. To analyze mitotic spindle morphogenesis, early embryos were microinjected with fluorescent tubulin conjugates as described by Kellogg et al. (1988), and then analyzed using time-lapse confocal microscopy.

Direct comparison of in vivo microtubule reorganization during meiosis and mitosis confirms that different spindle assembly pathways are used in these systems. During the mitotic divisions in the early embryo, centrosomal MTOCs duplicate and migrate to opposite poles of the nucleus well before nuclear envelope breakdown (NEB) (Fig. 1, *a–d*; *arrows*). As a result, the axis of the spindle can be predicted directly from microtubule organization before NEB. In the maturing oocyte, by contrast, microtubule asters were not observed before NEB ($n = 15$; Fig. 1, *e–h*). At the onset and just after NEB, microtubule asters were occasionally observed either close to the nuclear membrane or in areas adjacent to the spindle ($n = 2$). However, in the two cases in which microtubule asters were observed, they played no clear role in spindle assembly and were not associated with the spindle poles. After NEB, fluorescent tubulin enters the nucleus, and a compact tubulin-excluding region within the nuclear area was observed. This appears to correspond to the condensed mass of chromosomes called the karyosome (Mahowald and Kambysellis, 1980). Between 11 and 15 min after NEB, microtubules are first observed around the karyosome (12 ± 4 min; $n = 15$ [mean ± SD; number of observations]). At this stage, the future spindle axis cannot be predicted from microtubule organization (Fig. 1 *f*). This is followed by the appearance of thicker fibers that probably represent microtubule bundles. These bundles often appear to associate laterally with the karyosome and predict the spindle axis (Fig. 1 *g*, *arrows*). Between 20 and 25 min after NEB (23 ± 6 min, $n = 15$; Fig. 1 *h*, *arrows*), these microtubule bundles are drawn together, and a clearly bipolar structure is established. These observations are consistent with an absence of well-organized centrosomal MTOCs (see below) and a central role for chromatin in meiotic spindle morphogenesis (Theurkauf and Hawley, 1992; McKim et al., 1993).

To ascertain the stability of the metaphase-arrested spindle in mature oocytes, samples were observed during spindle assembly and again at various points up to 8 h later ($n = 3$). Consistent with the functional requirement for a stable spindle apparatus, the shape, orientation, and position of the spindle within the egg remained relatively constant.

Female Meiotic Spindles Lack Immunocytochemically Identifiable Centrosomes

The in vivo analyses described above suggested an absence of conventional centrosomes from the pole of the female meiotic spindle. We therefore attempted to immunocytochemically assay for centrosomes in this system, using antibodies to two previously characterized centrosomal proteins, γ-tubulin and CP-60 (Zheng et al., 1995; Oakley and Oakley, 1989; Oakley et al., 1990; Kellogg et al., 1989; Kellogg and Alberts, 1992; Raff et al., 1993). Double-label analysis of microtubule and centrosomal antigen distributions indicate that neither of these proteins is concentrated at the poles of the highly tapered meiotic spindle (Fig. 2). In contrast, both proteins are present at the poles of mitotic spindles in the germarium during early oogenesis (Fig. 2; CP-60 data not shown) and in syncytial blastoderm stage embryos (Kellogg et al., 1989; Theurkauf, W.E., unpublished results). Both the mature oocytes and the germaria were isolated, fixed, and labeled simultaneously. The lack of spindle pole staining in the mature oocytes is therefore not due to differences in tissue isolation or immunolabeling. Similar results were obtained with oocytes fixed in formaldehyde or formaldehyde followed by methanol, indicating that the absence of centrosomal antigen immunolocalization to the meiotic spindle poles is unlikely to be an artifact of a specific fixation procedure (data not shown). Although rigorous conclusions are difficult to draw from negative results, these observations support the contention that spindle morphogenesis during female meiosis is not dependent on centrosome function.

Role of the NCD Kinesin-like Protein in Spindle Morphogenesis

Previous studies indicate that the product of the *ncd* gene, a kinesin-like protein with minus-end–directed motor activity, is required for meiotic spindle function in the oocyte (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992*a*). We have reevaluated the effects of an *ncd* mutation on spindle morphogenesis using both immunocytochemical techniques and in vivo analyses. For these studies, we have used the ca^{nd} mutation. The ca^{nd} chromosome carries a deletion that removes a portion of the *ncd* gene and is therefore functionally null for NCD (Yamamoto et al., 1989). As observed previously, we found that populations of fixed ca^{nd} oocytes display a wide range of abnormal spindle configurations (Fig. 3); (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992*a*). Apparently normal spindles were occasionally observed (Fig. 3, *a* and *b*), in addition to multipolar and unusually wide "frayed" spindles (Fig. 3, *c* and *d*). Microtubule bundles were also observed around individual bivalents (Fig. 3, *e* and *f*). This variability could reflect differences between individual oocytes (see Davis, 1969; Baker and Hall, 1976) or a general instability of meiotic spindle structure.

Distribution of NCD in Mature Oocytes

Analysis of fixed oocytes indicates a role for NCD in es-

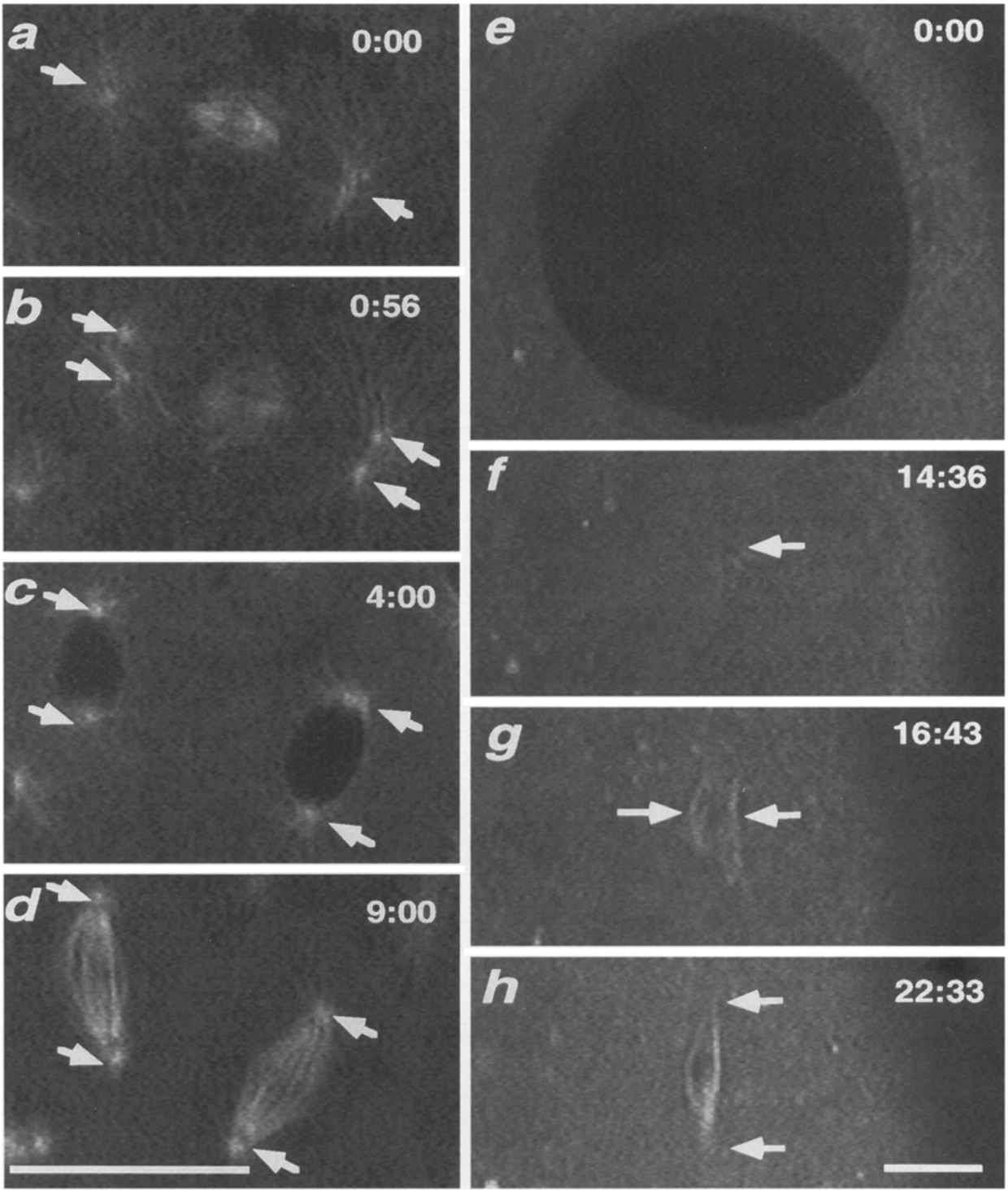

Figure 1. Microtubule reorganization during meiotic and mitotic spindle assembly in vivo. Embryos or oocytes were injected with rhodamine-conjugated tubulin and analyzed using time-lapse confocal microscopy (see Materials and Methods). (*a–d*) Microtubule reorganization during the mitotic division 10 in the syncytial embryo. (*a* and *b*) Early in interphase, centrosomal MTOCs divide and begin to migrate to opposite poles of the nucleus. (*c* and *d*) Centrosome migration is completed during late interphase, and centrosome position predicts the orientation of the spindle. (*e–h*) Microtubule reorganization as the meiotic spindle assembles during oocyte maturation. (*e*) No astral microtubule arrays are present before NEB. (*f*) 10–15 min after NEB, the first microtubules are observed in association with the karyosome. The orientation that the spindle will obtain cannot be determined from microtubule organization at this time. (*g*) Later, microtubule bundles (*arrows*) form and appear to make lateral connections with the karyosome. The orientation of these bundles predicts the spindle axis. (*h*) These bundles are drawn together to establish the mature spindle poles (*arrows*). Time = 0 for the sequence shown in *a–d* is defined by the start of centrosome separation. Time = 0 in *e–h* is NEB, as indicated by nuclear entry of fluorescent tubulin conjugate. Bars: (*a–d*) 10 μm; (*e–h*) 10 μm.

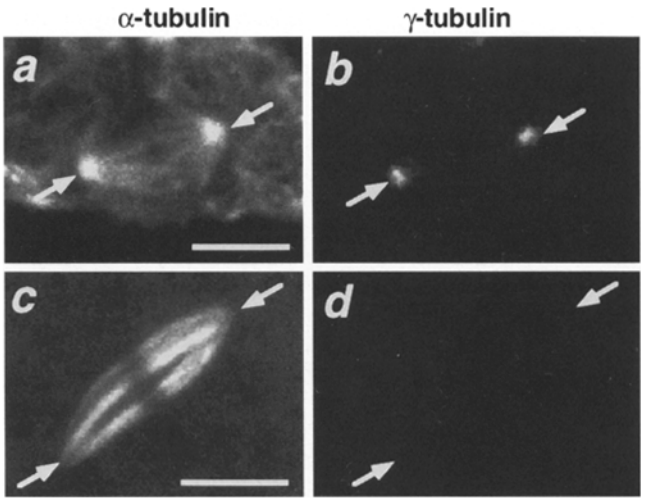

Figure 2. γ-Tubulin immunolocalizes to the poles of mitotic spindles, but is not observed at the poles of meiotic spindles. Total ovarian tissue was formaldehyde fixed and doubled immunolabeled with mouse anti–α-tubulin and rabbit anti–γ-tubulin antibodies. Microtubule distribution, as revealed by the anti–α-tubulin antibody, is shown in *a* and *c*; γ-tubulin localization is shown in *b* and *d*. Oocyte development is initiated by four mitotic divisions within the germarium. (*a* and *b*) In the mitotic cells of the germarium, γ-tubulin accumulates at the spindle poles. (*c* and *d*) In the mature oocyte, however, γ-tubulin is not detectable at the poles of the highly tapered meiotic spindle. Germaria and mature oocytes were fixed and immunolabled simultaneously, and identified cytologically within this mixture of material. Bars: (*a* and *b*) 10 μm; (*c* and *d*) 10 μm.

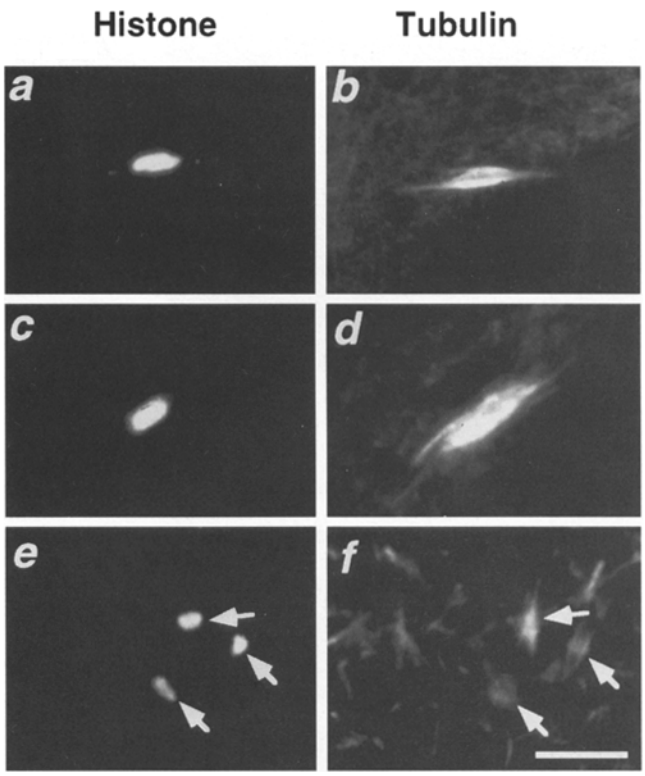

Figure 3. Spindle structures in fixed mutant oocytes lacking the *ncd* gene product. Mutant oocytes were fixed and immunolabeled with fluorescent anti–α-tubulin and anti-core histone antibodies to reveal microtubules (*a*, *c*, and *e*) and chromatin (*b*, *d*, and *f*). (*a* and *b*) Within the population of fixed oocytes, apparently normal meiotic figures were occasionally observed. (*c–f*) More typically, spindles had broad frayed poles (*c* and *d*), or no organized spindle was found and dispersed chromosomes in association with small microtubule bundles were observed (*e* and *f*). Bar, 10 μm.

tablishing or maintaining spindle structure (Fig. 3) (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992*a*). Consistent with a structural role for NCD, the protein has been immunolocalized to isolated meiotic spindles that have been purified from wild-type ovaries (Hatsumi and Endow, 1992*b*). However, the distribution of NCD within the oocyte itself has not been reported. Because the mature oocyte is filled with short microtubules, this information is critical to determining if NCD is restricted to spindle microtubules, or it associates with all of the microtubules within the oocyte (Theurkauf and Hawley, 1992). We therefore examined the distributions of NCD, microtubules, and chromatin in whole mount oocytes. No detectable NCD immunoreactivity is observed in *ncd* null mutant oocytes (Fig. 4), indicating that the staining patterns discussed below are specific. We observe NCD on meiotic spindle fibers but not on free cytoplasmic microtubules (Fig. 5, *g* and *h*). NCD thus appears to be specifically targeted to the spindle microtubules. In addition, the distributions of NCD and spindle microtubules do not appear to be identical. Early in spindle assembly, NCD is present on all of the short microtubules that associate with the karyosome (Fig. 5, *a–d*). As a bipolar microtubule organization is established, however, NCD immunoreactivity is observed from the poles to where microtubule bundles interact with the chromatin mass, while relatively little NCD is observed on microtubules in the center of the spindle that were not in direct contact with the chromatin (Fig. 5, *e–h*). These studies suggest that the NCD actively redistributes to a subset of microtubules as the spindle matures.

Spindle Assembly Dynamics in ncd Mutant Oocytes

To determine the effect of *ncd* loss-of-function on spindle assembly dynamics, we analyzed microtubule reorganization during maturation in living mutant oocytes. The initial stages of spindle assembly in ca^{nd} mutant oocytes were similar to wild type: nuclear morphology and position were indistinguishable from wild-type, and microtubules were observed in association with the karyosome 9–13 min (11 min ± 1.4 min, $n = 8$) after NEB. The kinetics of bipolar spindle generation, however, was dramatically altered in the mutants. The time required to assemble a bipolar spindle was extremely variable, and in four of eight cases, bipolar structures were not observed during the course of the time-lapse recording. In the remaining four examples, the time required for bipolar spindle assembly was significantly longer than observed in wild type (37, 52, 55, and 97 min after NEB). In addition, the spindles that formed in these oocytes were invariably unstable and would actively disorganize into either apolar or monopolar arrays, or transiently disorganize and then reform (Fig. 6).

In the four *ncd* mutant oocytes in which no clear spindle poles developed, the karyosome was found to move within the ooplasm. In one particularly dramatic case, persistent motility of the karyosome was detected (Fig. 7). In this ex-

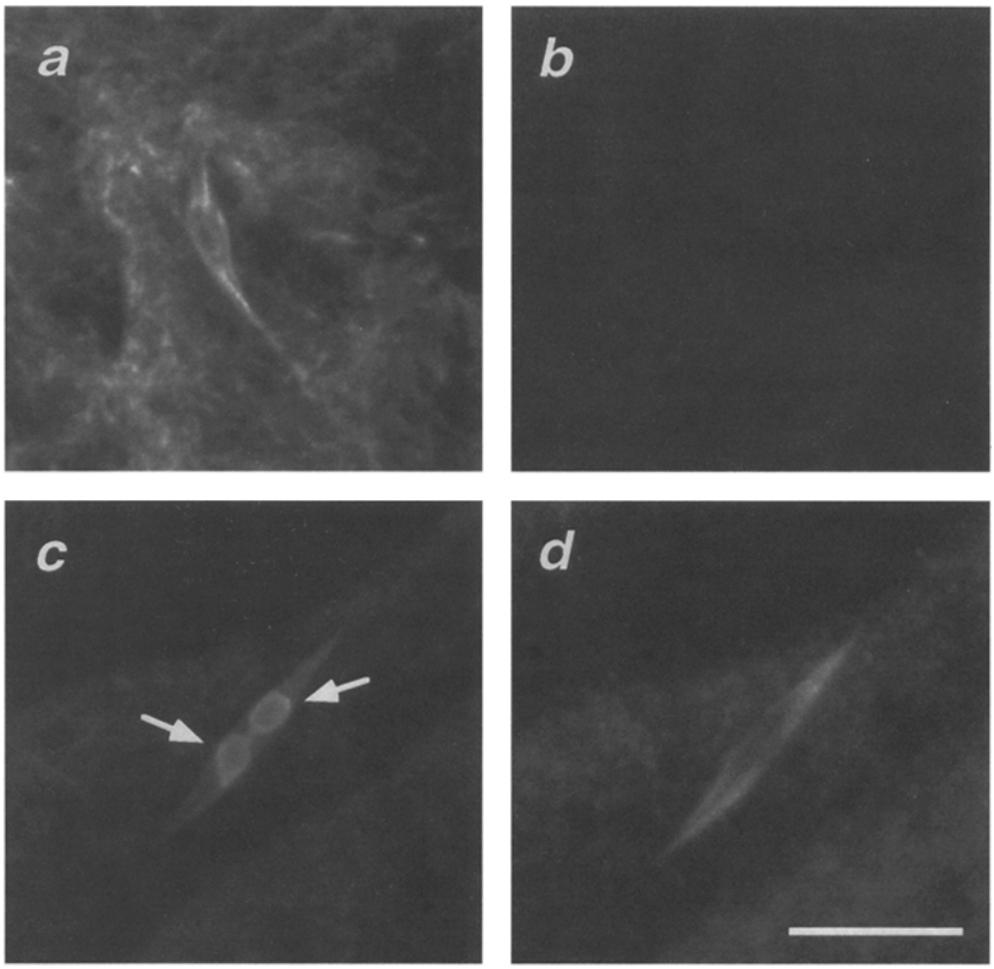

Figure 4. Specificity of anti-NCD antibodies in whole mount oocytes. NCD was immunolocalized in homozygous ca^{nd} (*ncd* null, *b*) and wild-type (*d*) oocytes. To reveal spindle structure, the oocytes were also labeled for tubulin (*a*) or tubulin and chromatin (*c*). (*a* and *b*) No NCD immunoreactivity was detected on spindle microtubules in ca^{nd} mutant oocytes. (*c* and *d*) By contrast, in wild type NCD was found on the spindle and was generally coincident with the microtubule pattern. However, the NCD protein was present at lower concentrations in the chromatin-containing center of the spindle (*c* and *d*). Oocytes were immunolabeled with either a rabbit polyclonal antibody to NCD and an mAb to tubulin (*a* and *b*), or antibody to NCD and a mixture of mAbs to tubulin and anti-core histones (*c* and *d*). Use of the mixture of mAbs allowed imaging of NCD protein with one fluorescein-conjugated secondary antibody (*d*) and microtubules and chromatin with a Texas red–conjugated secondary antibody (*d*). Bar, 10 μm.

ample, microtubules appeared to trail behind the main chromatin mass, and the karyosome changed direction several times after colliding with other large organelles. These observations demonstrate that NCD affects the efficiency of bipolar meiotic spindle assembly and plays an essential role in stabilizing the organization and position of the bipolar microtubule array after it is established.

Discussion

A Model for Drosophila Meiotic Spindle Assembly

Accurate mitotic and meiotic chromosome segregation depends upon microtubule reorganization into a functional bipolar spindle. While many features of meiotic and mitotic spindle function are likely to be shared, the mechanism that generates bipolarity in many meiotic systems may differ from that of typical mitotic cells. The bipolar organization of typical mitotic spindles is established by the duplication and separation of centrosomal MTOCs, while bipolarity in many meiotic spindles appears to be established through more direct microtubule–chromatin interactions (see McKim and Hawley, 1995; Vernos and Karsenti, 1995; Rieder et al., 1993). The results of in vivo analyses and immunolocalization reported here, combined with previous cytological analyses of *Drosophila* oocyte maturation (Theurkauf and Hawley, 1992; Hatsumi and Endow, 1992*a*), indicate that chromatin plays a critical role in organizing the meiosis I spindle in *Drosophila* females, and that conventional centrosomal MTOCs are not present. *Drosophila* female meiosis thus appears to represent a genetically and cytologically tractable system for the analysis of a chromatin-driven meiotic spindle assembly pathway that may function in vertebrates, insects, and plants (Rieder et al., 1993).

We have divided meiotic spindle assembly in *Drosophila* oocytes into four phases: (*a*) chromosome condensation; (*b*) NEB and microtubule association with chromatin; (*c*) microtubule bundling into a bipolar structure; and (*d*) metaphase I arrest and spindle stabilization (Theurkauf and Hawley, 1992).

In the first stage of meiotic spindle assembly, chromosomes condense into a tight mass called the karyosome. At this stage, astral MTOCs are not associated with the oocyte nuclear envelope, as is the case in typical mitotic cells, including the early syncytial *Drosophila* embryo (Fig. 1). In two cases, we did observe asterlike microtubule structures at or after NEB, but neither did they predict the future spindle axis nor appear to directly participate in spindle assembly. The lack of microtubule asters, combined with the absence of the centrosomal antigens γ-tubulin and CP-60 at the meiotic spindle poles (Fig. 2), indicates

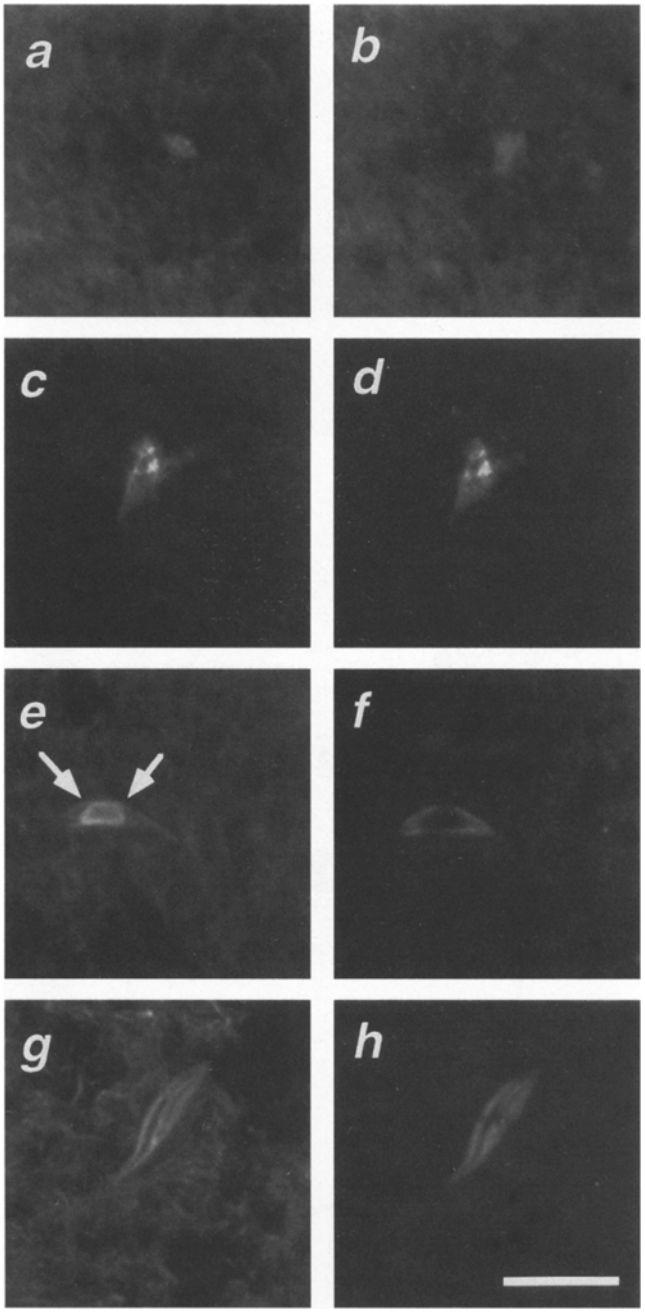

Figure 5. Microtubule and NCD reorganization during spindle assembly. (*a* and *b*) Shortly after NEB, both microtubules (*a*) and NCD (*b*) associate with the karyosome. (*c* and *d*) Somewhat later, short bundles of microtubules (*c*) are observed and largely colocalize with NCD (*d*). (*e* and *f*) Microtubules then reorganize to form a bipolar structure (*e*), and NCD generally colocalizes with spindle microtubules, but appears to be excluded from the spindle midzone (*f*). (*g* and *h*) Exclusion of NCD from the midzone is also apparent in the fully formed spindle, where microtubule staining is continuous across the center of the spindle (*g*), while NCD staining is interrupted at the center of the spindle (*h*). Cytoplasmic microtubules are not labeled by the NCD antibody, indicating that this protein is specifically targeted to spindle microtubules (compare microtubules in *g* with NCD in *h*). Bar, 10 μm.

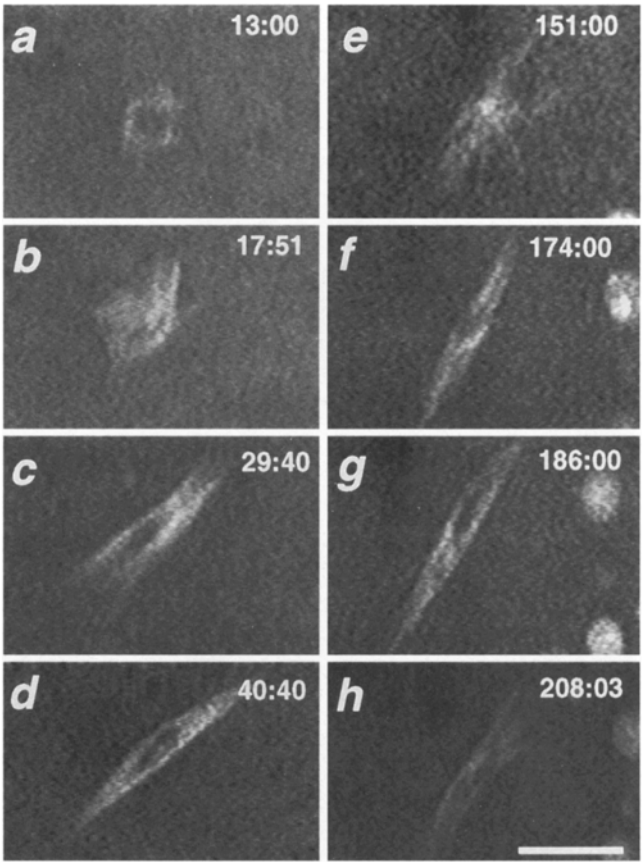

Figure 6. Spindle assembly in the absence of NCD function. (*a*) In oocytes lacking NCD, the initial association of microtubules with the karyosome is morphologically and kinetically similar to wild type. The process of spindle pole assembly, however, is dramatically affected by loss of NCD. (*b–d*) In this example, assembly of a morphologically normal bipolar was not observed until 40 min after NEB. In wild-type oocytes, this process requires only 20–23 min (see Fig. 1). (*e–h*) In addition, the spindle is unstable and degenerates to form an apolar or multipolar array (*e*), then reforms a relatively normal bipolar organization (*f* and *g*), and then degenerates again (*h*). Spindle instability may explain much of the cytological variability observed in fixed material (see Fig. 3). Bar, 10 μm.

that centrosomal MTOCs do not play a key role in spindle morphogenesis or orientation.

During the second stage in spindle assembly, both NCD and microtubules begin to associate with the karyosome and form an apolar array. This could represent microtubule nucleation by the chromosome arms and kinetochores, leading to the short microtubule spurs that are observed projecting from the karyosome. Alternatively, the cytoplasm contains many randomly scattered short microtubules at this stage, and microtubules that spontaneously assemble within the cytoplasm could be captured by chromatin.

In the third phase, microtubules are bundled into a bipolar array. In *ncd* mutants, this process often fails completely, and microtubules remain randomly arrayed around the karyosome (Fig. 7). In other instances, spindle formation is prolonged, and requires two- to eightfold longer than wild type (Fig. 6). These observations indicate that

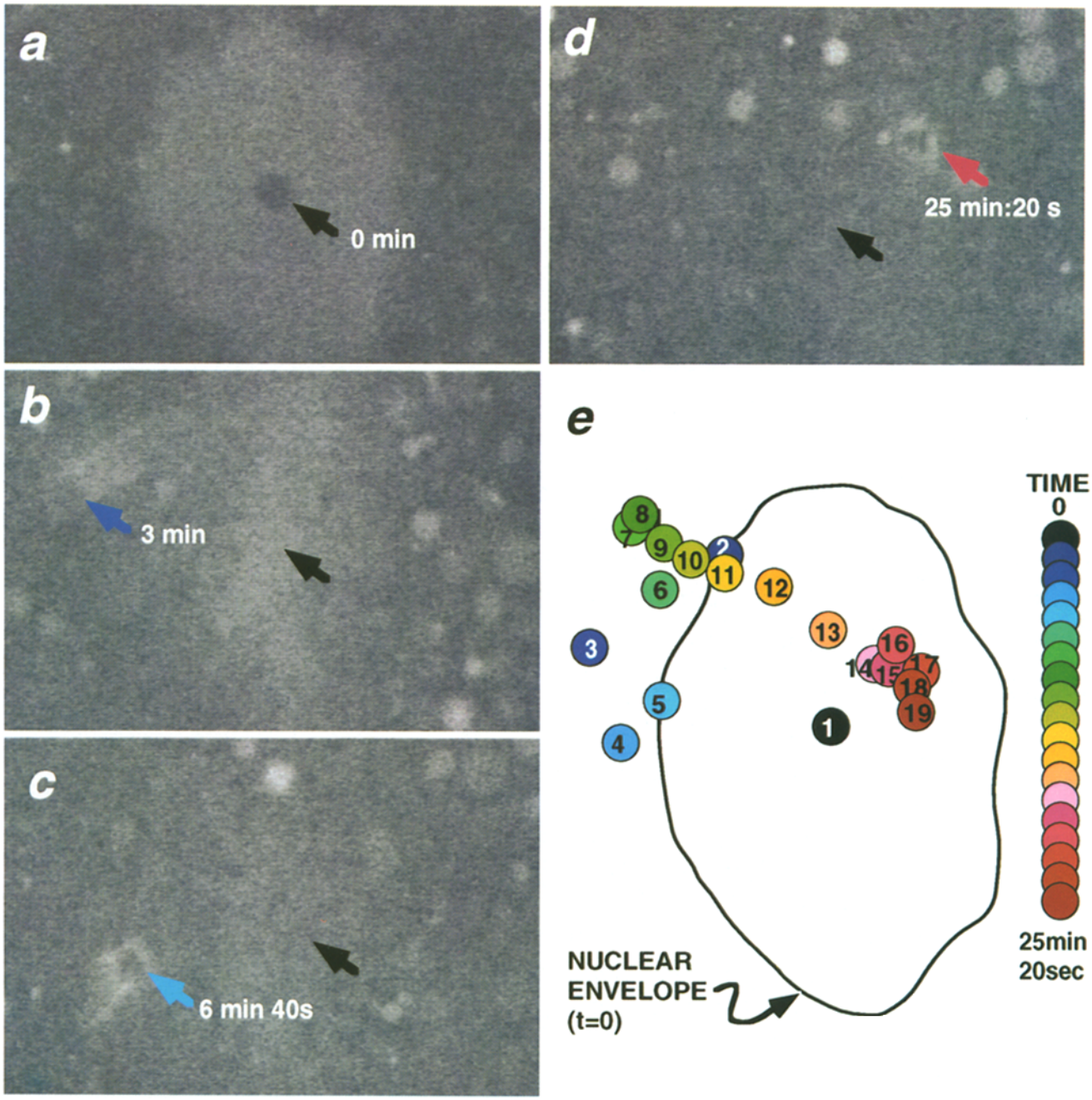

Figure 7. Karyosome motility in oocytes lacking the NCD protein. In a subset of *ca*nd mutant oocytes, a bipolar spindle is never established, and the karyosome and associated microtubules migrate through the ooplasm. (*a*) At NEB, the position of the karyosome is similar to wild type. (*b–d*) After NEB, the karyosome and associated microtubules move through the ooplasm. In each panel, the position of the karyosome at NEB is indicated by the black arrow, and the position of the karyosome at the indicated time is given by the color-coded arrow. (*e*) Karyosome movement during the course of the recording. Karyosome position was determined at 80-s intervals beginning at NEB, and the position at each of these time points is indicated by a color-coded circle. The color code is shown at right; time = 0 is indicated by the black circle, and the final time point is indicated by the red circle.

NCD plays an important but semi-redundant role in drawing microtubules together during spindle morphogenesis.

The mechanism that specifies orientation of the spindle microtubules during this phase is not known. However, we favor the speculation that the bivalent meiotic chromosomes are aligned before NEB in a polar array within a structure termed the chromocenter (Novitski, 1964; Puro and Nokkala, 1977), and that this pre-alignment specifies the future spindle axis (Hawley et al., 1993).

The last stage in the spindle assembly pathway is metaphase I arrest. This stage is characterized by a relatively long (12–18 μm), highly tapered spindle with the highest microtubule concentrations immediately adjacent to the main mass of condensed chromatin at the meta-

phase plate. In wild-type oocytes, spindle shape and position relative to the surface of the oocyte change very little during metaphase arrest. In contrast, in *ncd* mutants, metaphase arrest is characterized by spindle reorganization and dynamics. Cycles of spindle assembly and decomposition are often observed: spindles obtain the wild-type configuration, decay to monopolar or apolar arrays, and then transiently reform relatively normal bipolar microtubule structures (Fig. 6). The dynamic nature of the meiotic spindle in *ncd* mutants appears to explain the cytological variability observed in fixed material (Kimble and Church, 1983; Hatsumi and Endow, 1992*a*,*b*) (Fig. 3).

Mature stage 14 *Drosophila* oocytes can be held by the female for days before they are activated and fertilized (Mahowald and Kambysellis, 1980). Spindle stability is therefore critical to egg viability, and the *ncd* gene product appears to play an essential role in stabilizing spindle organization during meiosis I metaphase arrest. In the absence of NCD, viable zygotes may only be produced if meiotic spindle organization is relatively normal at the time the egg is activated and fertilized.

Role of NCD in Meiotic Spindle Assembly

What biochemical functions does NCD serve during assembly and maintenance of the meiotic spindle? Previous in vitro studies indicate that NCD can move towards the minus end of microtubules and is capable of microtubule bundling (McDonald et al., 1990; Walker et al., 1990; Chandra et al., 1993). If spindle microtubule orientation is conserved, then the plus ends of the *Drosophila* female meiotic spindle microtubules are at the chromosomes and the minus ends are at the poles. If this simplifying assumption is correct, then NCD could bind near the plus ends of the microtubules where they associate with chromatin and draw these microtubules together as it moves toward the minus ends, thus generating a focused anastral pole (Fig. 8).

Our in vivo analyses indicate that NCD function is necessary for normal spindle assembly kinetics. However, spindles are formed in a subset of mutant oocytes, suggesting that NCD acts in concert with another factor during spindle formation, and that this factor alone can support spindle assembly at a reduced efficiency (Fig. 8). This second spindle assembly factor cannot maintain spindle structure, however, as spindles in *ncd* mutant oocytes are unstable and continuously change configuration. The molecular nature of this second factor is not known.

Do NCD-like Proteins Have Roles in Mitotic Systems?

Null mutations in *ncd* dramatically disrupt meiotic chromosome segregation, but they have only minor effects on the first mitotic divisions in the early embryo and no detectable effect on mitotic spindle function later in development (Davis, 1969). This could reflect a specific function for NCD in the chromatin-driven spindle assembly pathway used in *Drosophila* oocytes and in other meiotic systems (Rieder et al., 1993). Alternatively, an NCD-like protein may function in concert with a redundant microtubule organizing system during mitotic spindle assembly. For example, microtubule nucleation and anchoring by the centrosome may act with NCD-related microtubule cross-

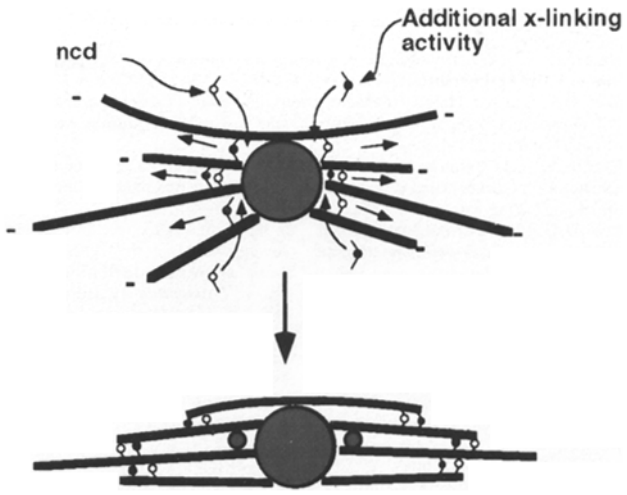

Figure 8. Model for NCD-dependent assembly of a bipolar spindle. Based on published data, the in vivo and immunocytochemical analyses reported here, and the assumption that the meiotic spindle microtubules are oriented with their plus ends at the chromatin, we propose the following model: shortly after NEB, the plus ends of cytoplasmic microtubules associate with chromatin. Bifunctional minus-end–directed microtubule motors, including NCD, then bind to the microtubules near the site of chromatin interaction. As these cross-linking motors translocate toward the microtubule minus ends, they draw the microtubules together to form a focused pole. In this model, NCD (*open circles*) supplies only a portion of the microtubule-bundling activity. The additional bundling factor(s) (*closed circles*) remain to be identified.

linkers to organize and then stabilize mitotic spindles. In the absence of NCD function, centrosomal microtubule nucleation and anchoring alone may be sufficient to stabilize the poles, although the efficiency of spindle assembly may be compromised. Either of these possibilities would explain both the critical role of NCD in the assembly and maintenance of the anastral meiotic spindle and the dispensability of this protein during the mitotic divisions.

Analyses of microsurgically dissected mitotic spindles are consistent with the presence of an NCD-related cross-linker during mitosis in somatic cells. When centrosomes are severed from the pole of mitotic spindles by micromanipulation or laser ablation, there is little effect on either anaphase chromosome movement or spindle integrity (see references in Rieder and Salmon, 1994). NCD-like proteins such as CHO2 (Kuriyama et al., 1995 and references therein [HSET gene]) could provide the stabilizing activity that allows mitotic spindle function after the removal of centrosomal MTOCs.

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