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Centrosome and spindle function of the *Drosophila* Ncd microtubule motor visualized in live embryos using Ncd-GFP fusion proteins

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SUMMARY

The Ncd microtubule motor protein is required for meiotic and early mitotic chromosome distribution in *Drosophila*. Null mutant females expressing the Ncd motor fused to the *Aequorea victoria* green fluorescent protein (GFP), regulated by the wild-type *ncd* promoter, are rescued for chromosome segregation and embryo viability. Analysis of mitosis in live embryos shows cell cycle-dependent localization of Ncd-GFP to centrosomes and spindles. The distribution of Ncd-GFP in spindles during metaphase differs strikingly from that of tubulin: the tubulin staining is excluded by the chromosomes at the metaphase plate; in contrast, Ncd-GFP forms filaments along the spindle microtubules that extend across the chromosomes. The existence of Ncd-GFP fibers that cross the metaphase plate suggests that Ncd interacts functionally with chromosomes in metaphase. Differences are no longer observed in anaphase when the chromosomes have moved off the metaphase plate. A mutant form of Ncd fused to GFP also localizes to spindles in live embryos. Mutant embryos show frequent centrosome and spindle abnormalities, including free centrosomes that dissociate from interphase nuclei, precociously split centrosomes, and spindles with microtubule spurs or bridges to nearby spindles. The precociously split and free centrosomes indicate that the Ncd motor acts in cleavage stage embryos to maintain centrosome integrity and attachment to nuclei. The frequent spindle spurs of mutant embryos are associated with mis-segregating chromosomes that partially detach from the spindle in metaphase, but can be recaptured in early anaphase. This implies that the Ncd motor functions to prevent chromosome loss by maintaining chromosome attachment to the spindle in metaphase, consistent with the Ncd-GFP fibers that cross the metaphase plate.

Key words: Ncd, Microtubule motor, Centrosome, Spindle, Mitosis, GFP, Kinesin

INTRODUCTION

Many mutants have been identified that are required for normal mitotic divisions in *Drosophila* (reviewed by Gatti and Baker, 1989; Glover, 1989). Analysis of these mutants has led to the identification of a cell-cycle regulatory protein (Edgar and O'Farrell, 1989), mitotic kinases (Llamazares et al., 1991; Glover et al., 1995) and phosphatases (Axton et al., 1990; Mayer-Jaekel et al., 1993), and a myosin light chain, required for cytokinesis (Karess et al., 1991). The proteins required for mitosis and identified by mutant analysis include members of the kinesin family of microtubule motor proteins. Ncd, a meiotic kinesin that functions in early mitosis (Komma et al., 1991; Endow et al., 1994a), is mutated by the classical mutation, *cand* (Lewis and Gencarella, 1952; Yamamoto et al., 1989), and KLP61F (Heck et al., 1993), a mitotic kinesin required for centrosome separation in embryonic and larval tissues, was identified through a P element mutagenesis screen.

The discovery of microtubule motor proteins with a clear involvement in mitosis has attracted great interest, since motor proteins could account for many of the movements of the spindle and chromosomes in mitosis. Microtubule motors bind to and move unidirectionally on microtubules, and have been proposed to generate the forces required for spindle assembly and maintenance, attachment of the chromosomes to the spindle, and movement of chromosomes toward opposite poles (reviewed by Bloom and Endow, 1995). Evidence also exists that kinesin motors can facilitate microtubule depolymerization (Endow et al., 1994b; Lombillo et al., 1995a,b; Walczak et al., 1996), raising the possibility that the motors modulate microtubule dynamics during mitosis. Although only two mitotic kinesin proteins in *Drosophila* have been reported to date, at least four other mitotic kinesins, CENP-E (Yen et al., 1992), MCAK (Wordeman and Mitchison, 1995), Xklp2 (Boleti et al., 1996) and MKLP1 (Nislow et al., 1992), have been identified in *Xenopus*, Chinese hamster and human cells, and have functions that appear to differ from those of Ncd and KLP61F. These kinesins are also expected to exist in *Drosophila*.

Ncd is an unusual member of the kinesin family that moves with the opposite polarity on microtubules as kinesin, toward microtubule minus ends, present at spindle poles. The Ncd motor is required for chromosome segregation during meiosis in oocytes where it has been proposed to function in assembly of bipolar meiotic spindles (Hatsumi and Endow, 1992a,b). Ncd also functions during mitosis in early embryos – mutants

of *ncd* cause chromosome loss that has been attributed to the first two or three cleavage divisions (Sturtevant, 1929; Portin, 1978; Nelson and Szauter, 1992) and antibody-stained embryos show centrosomal and spindle pole abnormalities in cycle 1-9 cleavage divisions (Endow et al., 1994a). Results of antibody staining experiments have led to the hypothesis that the Ncd motor functions at the spindle poles in mitosis, acting to maintain centrosome attachment to poles and prevent centrosome splitting until late anaphase, paralleling the proposed role of the motor in establishing and maintaining poles in oocyte meiotic spindles.

To obtain further information regarding Ncd mitotic function in early embryos, plasmids for expression of the Ncd motor protein fused to the *A. victoria* green fluorescent protein (GFP) were constructed and transformed into *Drosophila*. The Ncd-GFP fusion protein, under the regulation of the wild-type *ncd* promoter, localizes to centrosomes and spindle fibers of mitotic spindles in early embryos. Localization of the Ncd-GFP protein to spindles permits spindle assembly and dynamics to be monitored during mitosis in live embryos. A mutant form of Ncd fused to GFP also localizes to spindles and was used to follow mitotic spindles in the absence of Ncd motor function. The observations reveal that the Ncd microtubule motor performs an active role during mitosis in early embryos. The motor acts to maintain centrosome integrity and attachment to nuclei, contributes to midbody stability, and helps to prevent chromosome loss during the early mitotic divisions.

MATERIALS AND METHODS

ncd-gfp transgenes

Drosophila carrying 2 or 4 copies of the *ncd-gfp* or *ncd-gfp** transgene (S. A. Endow and D. J. Komma, submitted) and homozygous for the ca^{nd} null mutant of *ncd* were used in this study. The *ncd-gfp* transgenes are denoted *F24M1* and *F24M3*, and the *ncd-gfp** transgenes, *M3M1* and *M9F1*. The *gfp** refers to the gene encoding the $S_{65} \rightarrow T$ mutant GFP that shows ~6-fold increased fluorescence compared to wild-type GFP (Heim et al., 1995). Embryos of *Drosophila* homozygous for ca^{nd} and a <u>ncd</u>-*gfp** transgene (S. A. Endow and D. J. Komma, unpublished), *M4F1* or *M4F2,* that encodes a null mutant of *ncd*, were also examined. The <u>ncd</u> mutant corresponds to the *ncd* mutant, ncd^2 (Lindsley and Zimm, 1992), originally recovered by EMS mutagenesis.

Tests for rescue of a null mutant by the *ncd-gfp* transgenes

Tests of the ability of the *ncd-gfp*, *ncd-gfp** and <u>ncd</u>-*gfp** transgenes to rescue the ca^{nd} null mutant of *ncd* for chromosome segregation and embryo viablity were carried out as described previously (Komma et al., 1991; Endow et al., 1994a).

Imaging of GFP in live embryos by confocal microscopy

Embryos were collected at 30 minute intervals by inverting bottles of *Drosophila* onto grape juice agar plates and aged on the collection plates for 1.5 hours at room temperature to allow development to syncytial blastoderm stage. Chorions were removed manually by rolling the embryos on a piece of double-stick tape on a microscope slide and dechorionated embryos were removed to a drop of light halocarbon oil (Halocarbon Oil 27, Sigma Chemical Co.) on the slide. In some cases the oil was bubbled briefly with O_2 prior to use. A coverslip fragment was mounted onto two layers of double-stick tape placed on either side of the embryos, and the embryos were staged under visible light. Pre-cellularized embryos with pole cells or pole cell buds, indicating cycle 8-13, were examined immediately by laser scanning confocal microscopy.

The confocal scanning system consisted of a krypton/argon laser attached to a Bio-Rad MRC 600 scanning unit mounted on a Zeiss Axioskop microscope. The Bio-Rad BHS or GR2 filter block, or a custom GFP block was used to image GFP or GFP*. The BHS filter block contained 488/10 excitation, 510LP dichroic and OG 515LP emission filters, and the GR2, a 522/32BP emission filter (used in conjunction with a 488/10 excitation filter and trichroic filter block). The custom confocal GFP filter block contained 488/10 excitation, Q498LP dichroic and HQ518/40BP emission filters (Chroma Technology Corp.). A ×63/1.4 NA Planapochromat objective was used to collect images. Images were collected into stack files of 60 images at 15 or 16.5 second intervals using the Bio-Rad COMOS time-lapse feature with 2 or 3 Kalman-averaged slow scans per image.

No differences in cellular localization were observed between Ncd-GFP and Ncd-GFP* but a major difference in their characteristics is that the Ncd-GFP* fluorescence in *Drosophila* embryos is brighter than that of Ncd-GFP by an estimated ~6- to 7-fold, consistent with the ~6-fold greater fluorescence reported previously for the $S_{65}{\rightarrow}T$ mutant compared to wild-type GFP (Heim et al., 1995). Confocal images of Ncd-GFP*-decorated spindles were of higher quality than those of Ncd-GFP and could be collected under conditions less damaging to the embryos (higher neutral density filter, smaller pinhole setting). Ncd-GFP* is therefore preferable to Ncd-GFP for these imaging studies. Ncd-GFP in this report refers to either wild-type GFP or the $S_{65}{\rightarrow}T$ mutant GFP* fused to wild-type Ncd.

Rhodamine-tubulin and rhodamine-histone injections

Embryos were collected at 30 minute intervals, aged on collection plates for 15-60 minutes, then dechorionated and aligned end-to-end on a narrow strip of double-stick tape on a microscope slide. After desiccation in a Petri dish containing anhydrous $CaSO_4$ for 6.5 minutes for rhodamine-tubulin or 5.5 minutes for rhodamine-histone injections, embryos were covered with halocarbon oil (Series 700; Halocarbon Products, Corp.) that had been bubbled briefly with O_2 just prior to use and injected midway down the embryo, as described (Minden et al., 1989). The tubulin, from bovine brain, was conjugated with tetramethylrhodamine (Hyman et al., 1991) (Molecular Probes, Inc.) and was demonstrated previously to incorporate into spindles of early *Drosophila* embryos (Sullivan et al., 1990). The histones were bovine H2A/H2B histones, conjugated with tetramethylrhodamine (Valdes-Perez and Minden, 1995) (a gift from J. Minden) and shown previously to incorporate into chromatin of early *Drosophila* embryos (Minden et al., 1989). After injection, embryos were aged in moisture chambers for 15-60 minutes prior to observation. For confocal microscopy, a coverslip fragment was mounted onto 3 layers of double-stick tape placed on either side of the embryos. Rhodamine and GFP dual images were collected using the Bio-Rad GR2 filter block and a neutral density filter that transmitted 10% or 3% of the laser light.

Antibody staining of fixed embryos

Embryos carrying 4 copies of the *ncd-gfp* transgene and homozygous for ca^{nd} were collected for 30 minutes, aged 1.75 hours on collection plates and fixed with methanol-EGTA (Hatsumi and Endow, 1992b) or formaldehyde-EGTA (Theurkauf, 1992) in the absence of taxol. Staining of fixed embryos, following rehydration and permeabilization, was with an anti-Ncd antibody specific for the nonconserved tail region of Ncd (Hatsumi and Endow, 1992a), followed by a rhodamine-conjugated anti-α-tubulin antibody, as described (Hatsumi and Endow, 1992b; Endow et al., 1994a). Embryos were stained with DAPI prior to observation under fluorescence, and images were collected using laser scanning confocal microscropy.

Digital images

IBM PC confocal image files were opened as stacks, converted to

single images, and saved as TIFF or PICT files using the public domain program for Macintosh, NIH Image v 1.59. Image contrast and size was adjusted and images were pseudo-colored using Adobe Photoshop v 3.0.4. Figs 1, 4 and 7 were made using the Crop and Make Montage stack macros of NIH Image. The color image in Fig. 2 was made by merging a layer containing the rhodamine-tubulin spindle image with the Ncd-GFP spindle image, pseudo-colored green, using the Difference option of Adobe Photoshop v 3.0.4. Micron bars were drawn on the images with Adobe Photoshop using a 10 or 20 μm bar overlaid on the images with Bio-Rad COMOS for calibration. Files were viewed or made into movies with Adobe Premiere 4.0 or NIH Image.

RESULTS

Movies of Figs 1-8 can be viewed on the World Wide Web (http://www.cityscape.co.uk/users/ag64/jcscont.htm).

Ncd-GFP localizes to mitotic spindles in early embryos

Females carrying 4 copies of *ncd-gfp**, 2 each of the *M3M1* and *M9F1* insertions, regulated by the wild-type *ncd* promoter, show essentially complete rescue of the ca^{nd} null mutant of *ncd* for both chromosome segregation and embryo viability (Table 1). Two copies of either insertion gives only partial rescue. These results are similar to those obtained previously for *ncd-gfp*: 4 copies of *ncd-gfp*, 2 each of *F24M1* and *F24M3*, rescues ca^{nd} for both chromosome segregation and embryo viability (S. A. Endow and D. J. Komma, unpublished), while 2 copies of either insertion gives only partial rescue. These results demonstrate that 4 copies of the Ncd-GFP fusion protein can replace the function of wild-type Ncd in *ncd-gfp* or *ncd-gfp** females.

Examination of mitotic spindles in early embryos showed that the Ncd-GFP fusion protein is localized to mitotic spindle fibers and spindle poles, including centrosomes, as reported for wild-type Ncd (Hatsumi and Endow, 1992a; Endow et al., 1994a). Spindles of *ncd-gfp* M3M1; M9F1* (4-copy) embryos and *M3M1* (2-copy) embryos appeared normal (Fig. 1), although occasional *M3M1* embryos with abnormal spindles could be observed. The association of Ncd-GFP with the mitotic spindle persists through the syncytial blastoderm divisions of early *Drosophila* embryos and can be observed as late as cycle 14 in gastrulating embryos. Spindles present in pole cells were also associated with Ncd-GFP.

Centrosomal localization of Ncd-GFP is cell cycle-dependent

The centrosomal localization of Ncd-GFP is illustrated in Fig. 1, which shows images taken from a time-lapse series of a cycle 9 *M3M1/M3M1;*ca^{nd}/ca^{nd} embryo carrying 2 copies of the *ncd-gfp** transgene. The images were collected without contrast enhancement and a linear contrast adjustment was applied to the entire montage to preserve relative contrast between images. Stages of the mitotic cycle were assigned by comparison to double images of spindles and chromosomes collected from *ncd-gfp** embryos injected with rhodamine-conjugated histones to label the chromatin. During interphase (frames 1 and 2), Ncd-GFP is associated with centrosomes and is also present in the cytoplasm. The nuclei exclude Ncd-GFP and can be detected as dark spheres that contrast with the uniform fluorescence of the cytoplasm. The centrosomes become very bright with Ncd-GFP fluorescence at nuclear envelope breakdown (frame 3), recognized by a sudden decrease in contrast between the nucleus and cytoplasm, and during spindle assembly in early metaphase (frame 4), and remain brightly fluorescent in mid-metaphase (frames 5-7) and late metaphase/early anaphase (frame 8). During mid- and late anaphase (frames 9 and 10), the centrosomal fluorescence diminishes and remains diminished during telophase (frames 11 and 12) and into the next interphase. The difference in intensity of centrosome fluorescence demonstrates that Ncd-GFP localization to centrosomes is dependent on the stage of the mitotic cycle.

Ncd-GFP forms fibers in metaphase and anaphase spindles

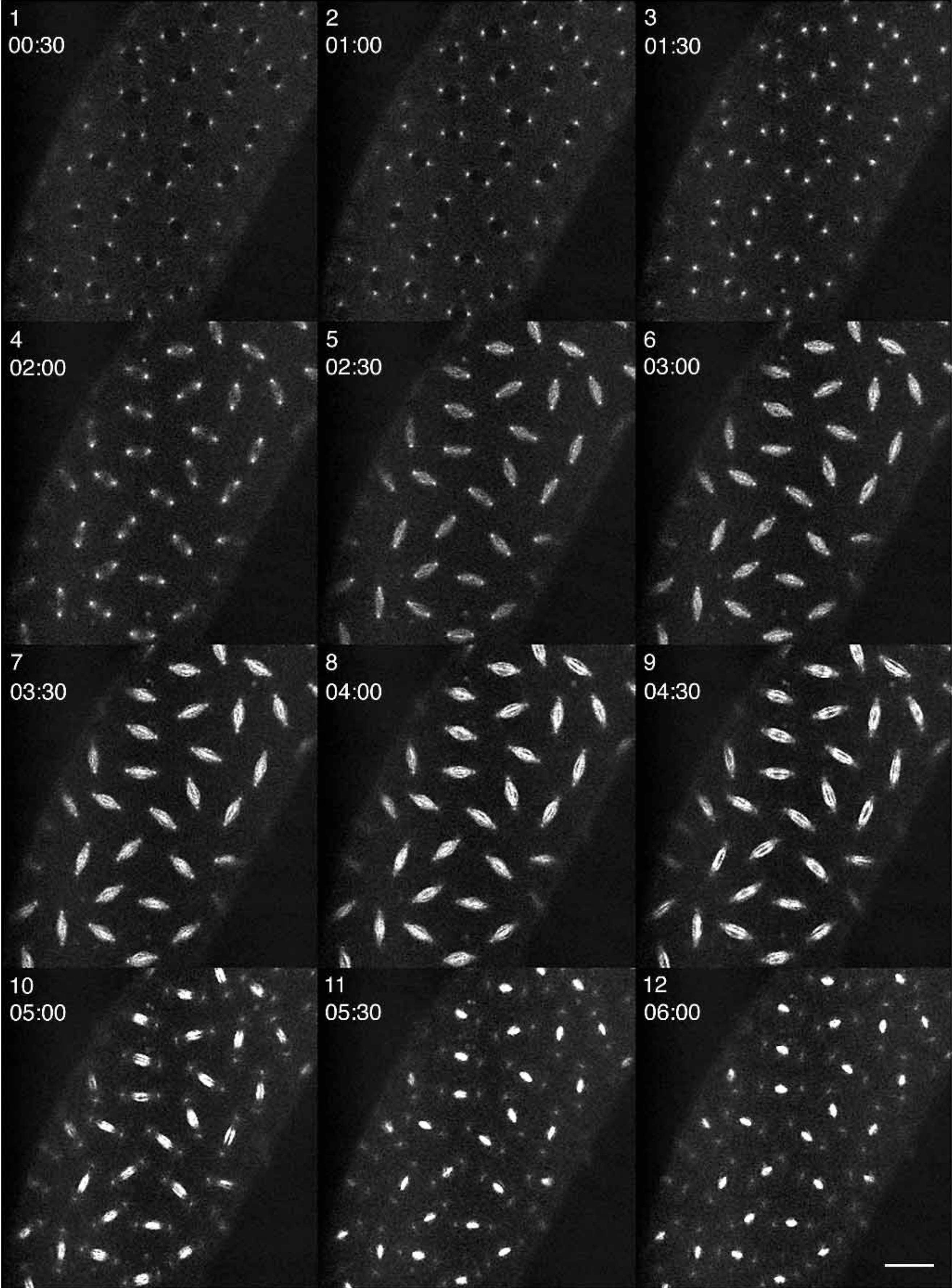
Ncd-GFP is also associated with spindle microtubules in a cell cycle-dependent manner, as shown in Fig. 1. During early and mid-metaphase (frames 4-7) the spindle fibers become bright with Ncd-GFP fluorescence and remain bright in late metaphase/early anaphase (frame 8) and mid-anaphase (frame 9). The fluorescence is redistributed to the developing midbody in late anaphase (frame 10), and associated with the midbody in telophase (frames 11 and 12).

The distribution of Ncd-GFP relative to that of tubulin was examined by collecting double images of *ncd-gfp** embryos that had been injected with rhodamine-conjugated tubulin to label microtubules. The double images showed that the distributions of Ncd-GFP and tubulin were very similar to one another. However, a striking difference was observed between the Ncd-GFP and tubulin images at metaphase. A double image of Ncd-GFP and tubulin fluorescence, taken from a time-lapse series of a cycle 10 division, is shown in Fig. 2. Spindles in metaphase show association of Ncd-GFP with prominent fibers that extend from pole to pole (Fig. 2A). The Ncd-GFP fibers show uniformly bright fluorescence across the metaphase plate, in contrast to the tubulin staining which is partially excluded from the metaphase plate region (Fig. 2B). Exclusion of tubulin staining from the metaphase plate region

Table 1. Rescue of the ca^{nd} null mutant for chromosome segregation and embryo viability by the *ncd-gfp transgene**

Female parent	Copies of ncd^+	Gametic X nd	Gametic X loss	Zygotic X loss	Total* adults	Total embryos	Total mis-seg	Embryo viability
1. ca^{nd}/ca^{nd}	0	0.238	<0.007	0.056	144	1,277	0.294	0.113
2. *M3M1/M3M1;* ca^{nd}/ca^{nd}	2	<0.001	0.052	0.020	1,105	1,746	0.072	0.633
3. *M9F1/M9F1;* ca^{nd}/ca^{nd}	2	<0.001	0.063	0.008	876	1,798	0.071	0.487
4. *M3M1/M3M1; M9F1/M9F1;*ca^{nd}/ca^{nd}	4	0.001	0.005	<0.001	3,235	3,480	0.006	0.930
5. +/+	2	<0.001	<0.001	<0.001	2,047	2,315	<0.001	0.884

*Including M offspring.
Mis-seg, mis-segregation; nd, nondisjunction; +, Ore R.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was also observed in live wild-type embryos injected with rhodamine-tubulin (not shown). The difference in Ncd-GFP and tubulin distribution in metaphase spindles is emphasized in the overlay in Fig. 2C, which shows the coincidence of Ncd-GFP and tubulin fluorescence in purple, and Ncd-GFP alone in green.

The region of the metaphase plate that excludes tubulin, but not Ncd-GFP, contains the metaphase chromosomes. This was determined by examining mitotic divisions in *ncd-gfp** embryos injected with rhodamine-histones to label the nuclei and chromosomes. Doubly-labelled images showed that the Ncd-GFP fibers extend across the chromosomes present at the metaphase plate (Fig. 3). Differences in Ncd-GFP and tubulin distribution were no longer observed in anaphase when the spindle fibers had stretched and the chromosomes had moved off the metaphase plate.

To determine whether the fibers of Ncd observed in live embryos were retained in fixed embryos, embryos carrying 4 copies of the *ncd-gfp* transgene were fixed with methanol-EGTA (Hatsumi and Endow, 1992b; Endow et al., 1994a) or formaldehyde-EGTA (Theurkauf, 1992) and stained with anti-tubulin and anti-Ncd antibodies, as described (Hatsumi and Endow, 1992a,b). Metaphase spindles, examined by laser scanning confocal microscropy, showed almost identical distributions of Ncd-GFP and tubulin, as reported previously for Ncd and tubulin (Hatsumi and Endow, 1992a; Endow et al., 1994a). Fixation with formaldehyde preserved somewhat more of the mid-region staining of both Ncd-GFP and tubulin than methanol, but neither formaldehyde nor methanol fixation resulted in the prominent fibers of Ncd-GFP. The fibers of Ncd-GFP were, however, observed in spindles of live embryos collected from the same bottles.

A mutant *ncd-gfp* transgene shows loss of *ncd* function

A gene fusion between a mutant form of *ncd*, denoted <u>ncd</u>, and *gfp** has been constructed, transformed into *Drosophila* and transferred into ca^{nd} females (S. A. Emdow and D. J. Komma, unpublished). The *<u>ncd</u>-gfp** transgene fails to rescue the high levels of meiotic chromosome mis-segregation and embryo inviability of the ca^{nd} null mutant of *ncd*, but the mutant protein is expressed and localizes to meiotic spindles.

The *<u>ncd</u>-gfp** transgene also fails to rescue ca^{nd} for mitotic chromosome mis-segregation. The frequencies of mitotic chromosome loss for *<u>ncd</u>-gfp** *M4F1/M4F1;*ca^{nd}/ca^{nd} and *M4F2/M4F2;*ca^{nd}/ca^{nd} females, homozygous for ca^{nd} and one or the other of two independent *<u>ncd</u>-gfp** insertions, were 0.083 and 0.086, respectively. These frequencies are not significantly different from the 0.056 of ca^{nd}/ca^{nd} females (Table 1). The two *<u>ncd</u>-gfp** transgenes therefore cause similar frequencies of mitotic chromosome loss as ca^{nd}, behaving like functional null mutants of *ncd*.

The levels of embryo viability for the *M4F1* and *M4F2* transgenes were 0.085 and 0.113, respectively, compared with 0.113 for ca^{nd} (Table 1). The difference in embryo viability between *M4F1* and ca^{nd} is small but significant (χ^2=5.014, 1 d.f., $0.05>P>0.025$). However, embryos of *M4F1* and *M4F2* showed the same types of mitotic defects (described below) and all of the *<u>ncd</u>-gfp** mutant embryos examined (*M4F1*, n=54; *M4F2*, n=20) exhibited abnormalities. The slightly higher frequency of embryo inviability caused by *M4F1* compared to *M4F2* is probably due to an insertional position effect that results in a difference in expression of the *M4F1* transgene relative to *M4F2*. The <u>Ncd</u>-GFP* fluorescence of *M4F1* embryos appeared somewhat brighter than that of *M4F2*, consistent with this interpretation, although this was not carefully quantitated.

*<u>ncd</u>-gfp** causes spindle abnormalities and mis-segregation of chromosomes

The mutant <u>Ncd</u>-GFP* protein localizes to mitotic spindles throughout the syncytial blastoderm divisions and during cycle 14 divisions of gastrulating embryos, as observed for Ncd-GFP, and metaphase spindles showed filaments of <u>Ncd</u>-GFP* similar to those of Ncd-GFP. The midbodies of late anaphase and telophase spindles were highly fluorescent, indicating the presence of <u>Ncd</u>-GFP*, but were much more sensitive to laser irradiation than spindle midbodies of *ncd-gfp* or *ncd-gfp** embryos.

Abnormal spindles were observed in cycle 9-11 embryos, and in a stage 14 gastrulating embryo. The spindle abnormalities consisted of spurred or frayed spindles and spindles joined to adjacent spindles by microtubule spurs or branches (Fig. 4). Fig. 4 shows images of a cycle 10 division taken from a time-lapse series of an *<u>ncd</u>-gfp** *M4F1/M4F1;*ca^{nd}/ca^{nd} embryo. Several of the spindles exhibit microtubule spurs extending from the midregion or poles. The spindle spurs form in mid- or late metaphase and extend (arrowhead), retract (arrow), or form bridges in anaphase to the midregion or poles of other spindles.

The spindle abnormalities increased in frequency in late cleavage divisions as the spindles became closer together – spindles in cycle 9 showed occasional spurred spindles but no bridged spindles, which were common in cycle 10, and some embryos in cycle 11 contained tripolar or multipolar spindles. Spurred or frayed spindles were observed in all of the *<u>ncd</u>-gfp** *M4F1* and *M4F2* mutant embryos that were examined, but the frequency of abnormal spindles appeared somewhat higher in individual *M4F1* embryos, consistent with the slightly higher embryo inviability of *M4F1* compared with *M4F2*.

Fig. 1. Mitotic spindles visualized with Ncd-GFP* in a cleavage stage *Drosophila* embryo. Time-lapse images showing a cycle 9 division in an *ncd-gfp** *M3M1/M3M1;*ca^{nd}/ca^{nd} embryo. Images were collected without contrast enhancement and a linear contrast adjustment was applied to the entire montage to maintain relative contrast between images. Images shown are every 30 seconds; time in minutes and seconds is shown at the top of each image. Stages were assigned by comparing images to a time-lapse series collected from an *ncd-gfp** *M3M1/M3M1;*ca^{nd}/ca^{nd} embryo injected with rhodamine-histones to label the nuclei and chromosomes. Stages are as follows: frames 1 and 2, interphase; frame 3, nuclear envelope breakdown; frames 4-7, metaphase; frame 8, metaphase/early anaphase; frame 9, mid-anaphase; frame 10, late anaphase; frames 11 and 12, telophase. During interphase, Ncd-GFP is associated with centrosomes and present in the cytoplasm (frames 1 and 2). Centrosomes increase in brightness at nuclear envelope breakdown (frame 3), remain bright during metaphase (frames 4-7) and late metaphase/early anaphase (frame 8), and diminish in brightness in mid- and late anaphase (frames 9 and 10) and telophase (frames 11 and 12). Spindle fibers are bright with Ncd-GFP in metaphase and anaphase (frames 4-9), and Ncd-GFP is redistributed to the midbody in late anaphase and telophase (frames 10-12). Bar, 20 µm.

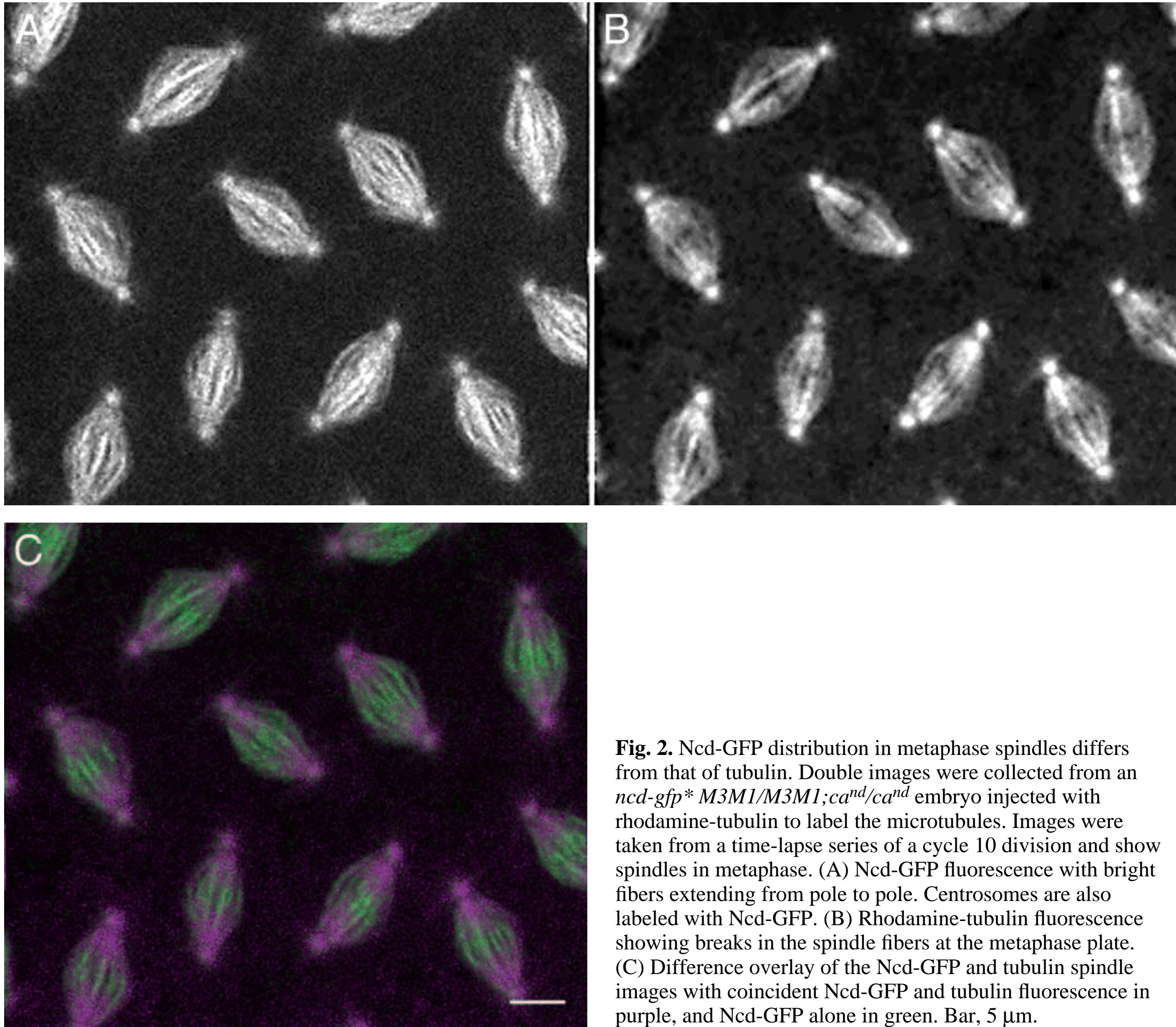


Fig. 2. Ncd-GFP distribution in metaphase spindles differs from that of tubulin. Double images were collected from an *ncd-gfp* M3M1/M3M1;ca*nd/*ca*nd embryo injected with rhodamine-tubulin to label the microtubules. Images were taken from a time-lapse series of a cycle 10 division and show spindles in metaphase. (A) Ncd-GFP fluorescence with bright fibers extending from pole to pole. Centrosomes are also labeled with Ncd-GFP. (B) Rhodamine-tubulin fluorescence showing breaks in the spindle fibers at the metaphase plate. (C) Difference overlay of the Ncd-GFP and tubulin spindle images with coincident Ncd-GFP and tubulin fluorescence in purple, and Ncd-GFP alone in green. Bar, 5 μm.

Mutant embryos with chromosomes labelled by injection of rhodamine-histones showed mis-segregating chromosomes associated with the spindle bridges or spurs. The time-lapse sequence in Fig. 5 shows a spindle spur associated with a chromosome in mid-metaphase that has partially detached from the metaphase plate (top). The chromosome reattaches (middle) but a dotlike chromosome 4 (upper arrowhead) mis-segregates along the spur of microtubules. The reattached chromosome has moved poleward but lags behind the other chromosomes in early anaphase (bottom) while the mis-segregating chromosome 4 moves off the spindle. Chromosomes frequently moved off the metaphase plate, creating a spindle spur or frayed region that recaptured the mis-segregating chromosome in early anaphase, although the tiny chromosome 4 was often lost. Spindle spurs associated with recaptured chromosomes retracted in anaphase while those that failed to recapture mis-segregating chromosomes persisted into telophase as spurred midbodies. Several bridged spindles that were observed showed chromosomes mis-segregating to poles of the two different spindles by way of the microtubule bridge. Frequent mis-segregation of chromosomes, both autosomes and chromosome 4, was observed in late cleavage stage (cycle 9-11) embryos.

Free centrosomes arise in *ncd-gfp** mutant embryos

Centrosomal defects, including free centrosomes (Fig. 6), were also observed in mutant embryos carrying the *ncd-gfp** transgene. The cycle 10 embryo shown in Fig. 6A was injected with rhodamine-conjugated histones to visualize the chromosomes. No chromatin or chromosomes were associated with the free centrosomes, although the metaphase chromosomes associated with the spindles were clearly labelled. The free centrosomes persisted into anaphase of cycle 11 without doubling. The free centrosomes in some embryos contributed to further spindle abnormalities by interacting with adjacent spindles and causing the formation of microtubule bridges, as shown in Fig. 6B.

Mutant embryos were observed during several consecutive cleavage divisions (cycles 8-11) to determine the origin of the free centrosomes. Two embryos in which free centrosomes

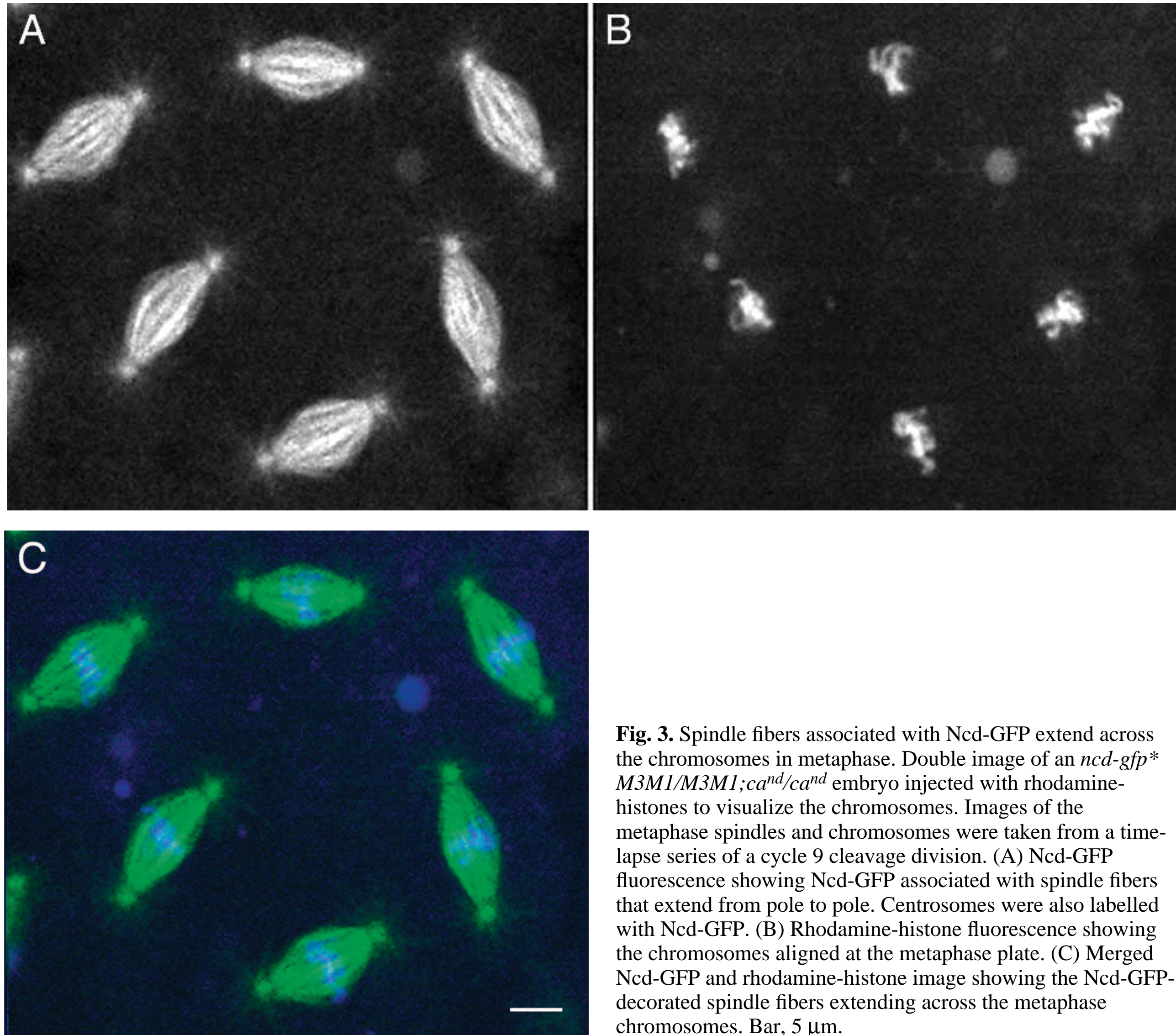


Fig. 3. Spindle fibers associated with Ncd-GFP extend across the chromosomes in metaphase. Double image of an *ncd-gfp* M3M1/M3M1;cand/cand* embryo injected with rhodamine-histones to visualize the chromosomes. Images of the metaphase spindles and chromosomes were taken from a time-lapse series of a cycle 9 cleavage division. (A) Ncd-GFP fluorescence showing Ncd-GFP associated with spindle fibers that extend from pole to pole. Centrosomes were also labelled with Ncd-GFP. (B) Rhodamine-histone fluorescence showing the chromosomes aligned at the metaphase plate. (C) Merged Ncd-GFP and rhodamine-histone image showing the Ncd-GFP-decorated spindle fibers extending across the metaphase chromosomes. Bar, 5 µm.

arose and were followed in successive cycles contained a short round spindle that first appeared different from the surrounding spindles in early metaphase and failed to form a normal midbody in telophase; the daughter nuclei receded into the interior during the following interphase leaving the centrosomes at the surface. The short round spindle in the embryo shown in Fig. 7 (frames 1-2) formed an abnormal midbody in late anaphase (frame 3) that in telophase was diminished in Ncd-GFP* fluorescence relative to other spindles (frame 4). The midbody disassembled precociously in telophase and the two daughter nuclei came back together rather than separating, and then disappeared from the surface briefly during the following (cycle 10) interphase. Just prior to nuclear envelope breakdown, two pairs of centrosomes associated with small nuclei reappeared at the surface. The centrosomes failed to assemble spindles during cycle 10 and sank into the interior during late anaphase of cycle 10.

Free centrosomes were also observed to arise in several other embryos. Spindles in *ncd-gfp* mutant embryos were frequently observed (in 14 of 74 *ncd-gfp* M4F1* or *M4F2* embryos) perpendicular rather than parallel to the surface of the embryo. All of the embryos that contained perpendicularly oriented spindles were in cycle 9. Divisions of perpendicular spindles produced only one daughter nucleus (the uppermost nucleus) at the surface of the embryo. Three perpendicularly mis-oriented spindles can be seen in the embryo in Fig. 8A. Centrosomes from two of the spindles dissociated from the daughter nucleus at the surface in interphase of cycle 10 and remained as free centrosomes during cycle 10 and 11, while the daughter nucleus of the third spindle formed a normal-appearing spindle in cycle 10.

The centrosomes (arrowheads) in the embryo shown in Fig. 8B appeared at the surface of the embryo without an associated nucleus in interphase of cycle 9. One of the centrosomes disappeared from the surface during metaphase of cycle 9, while the other persisted into cycle 10. A centrosome was also observed in one embryo dissociating from a nucleus (Fig. 8C). The dissociating centrosome (left, bottom arrowhead) in the cycle 9 embryo rose to the surface first, followed by the second centrosome and then the nucleus, and dissociated from the

nucleus during interphase, but was recaptured by the spindle in metaphase. The daughter nuclei produced by the spindle formed normal-appearing spindles in cycle 10. The apparently free centrosome in Fig. 8C (left, top arrowhead) is associated with a spindle that is perpendicular to the embryo surface. The uppermost daughter nucleus of the spindle remained at the surface and formed a normal spindle in cycle 10.

*ncd-gfp** causes precocious splitting of centrosomes

Precocious splitting of centrosomes was also observed in *ncd-gfp** mutant embryos. The mid-anaphase spindle (arrow), late metaphase spindle (arrow), and free centrosomes shown in Fig. 8A showed precocious splitting of centrosomes. This was also observed for the free centrosome in the interphase embryo and the metaphase spindle shown in Fig. 8B. Abnormally split centrosomes were typically observed associated with nuclei or spindles of mutant *M4F1* or *M4F2* embryos as early as interphase. Despite the centrosomal defects that were observed, Ncd-GFP* showed cell cycle-dependent localization to centrosomes like that observed for Ncd-GFP: the centrosomes became very bright with Ncd-GFP* at nuclear envelope breakdown and during spindle assembly in early metaphase, and showed diminished fluorescence in late anaphase and telophase.

DISCUSSION

Ncd-GFP is associated with mitotic spindles in early embryos

The Ncd microtubule motor protein has been expressed in *Drosophila* as a fusion protein consisting of wild-type Ncd fused to the green fluorescent protein from the jellyfish, *A.*

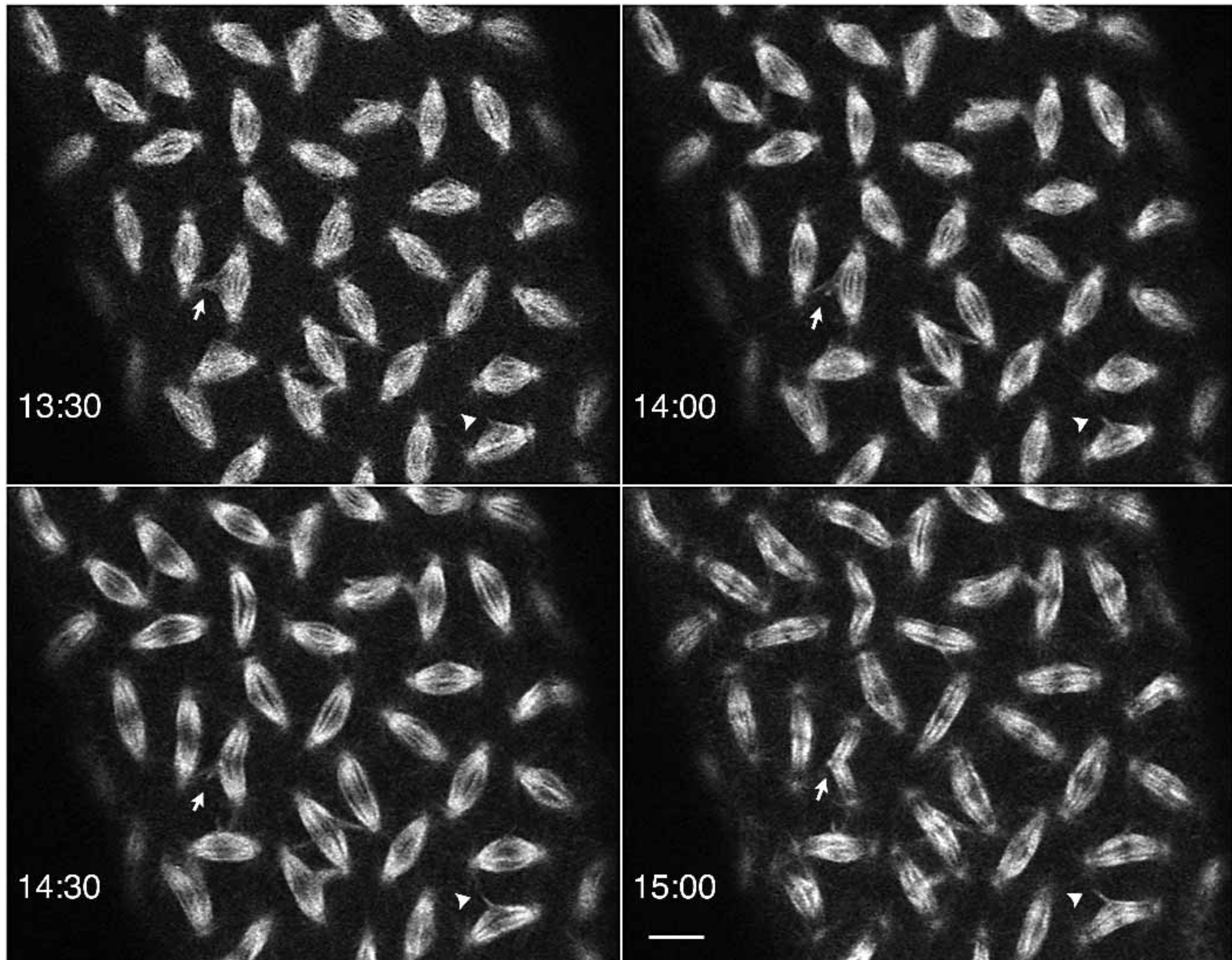


Fig. 4. Embryos mutant for *ncd-gfp** show spindle abnormalities in mitosis. Images taken from a time-lapse series of an *ncd-gfp* M4F1/M4F1;cand/cand* embryo show spindles in early to mid-anaphase from a cycle 10 division. Time in minutes and seconds is indicated at the bottom of each image. Microtubule spurs that extend from the spindle mid-region or pole can be seen. Association of the spurs with nearby spindles forms bridged spindles. Spindle spurs extend without joining to other spindles (arrowhead) or extend, then retract later in anaphase (arrow). The spurs are associated with mis-segregating chromosomes, as observed by rhodamine-histone injection of live *ncd-gfp** embryos. Bar, 10 μm.

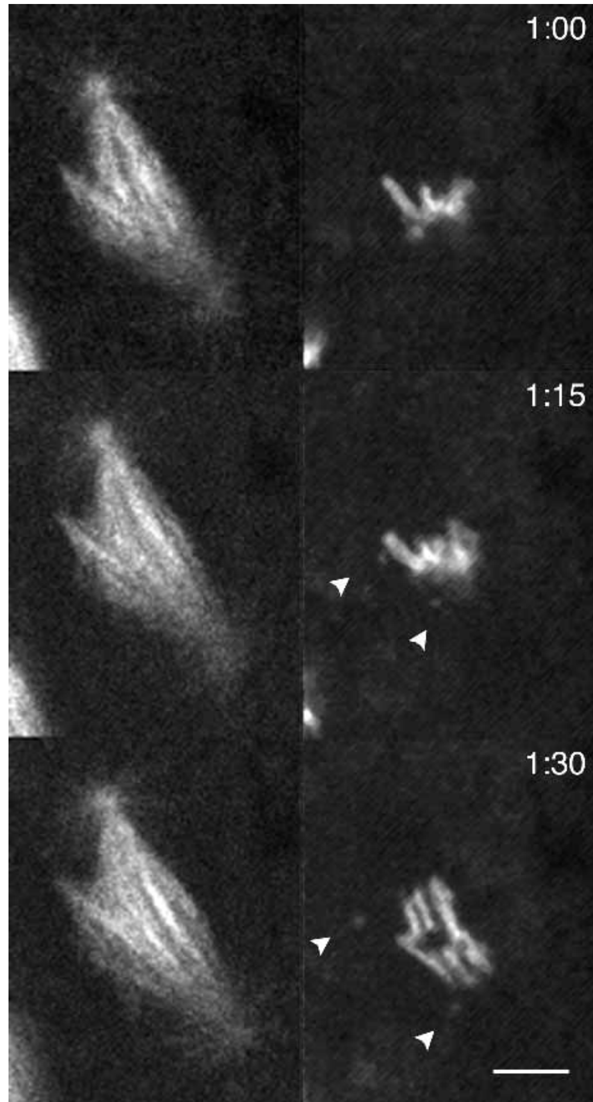


Fig. 5. Chromosome mis-segregation and loss in an ncd-*gfp** mutant embryo. The images show a spurred spindle and mis-segregating chromosomes in mid-metaphase and early anaphase of cycle 9 from a time-lapse sequence of an ncd-*gfp* M4F1/M4F1;cand/cand* embryo injected with rhodamine-histones to label the chromosomes. The spur of microtubules formed by the spindle is associated with a chromosome that has partially detached from the metaphase plate (top). The chromosome reattaches (middle) but one dotlike chromosome 4 (upper arrowhead) mis-segregates along the spindle spur while the other (lower arrowhead) moves towards a pole. The reattached chromosome lags behind the other chromosomes in moving poleward in early anaphase (bottom) while the mis-segregating chromosome 4 moves off the spindle. Time in minutes and seconds is shown at the top of each pair of images. Bar, 5 µm.

victoria. The *ncd-gfp* and *ncd-gfp** transgenes rescue the *cand* null mutant of *ncd* for chromosome segregation and embryo viability, and the Ncd-GFP fusion protein localizes to spindles of early embryos where it is associated with mitotic spindle fibers and centrosomes, as reported previously for wild-type Ncd based on antibody staining (Hatsumi and Endow, 1992a; Endow et al., 1994a). The bright fluorescence of Ncd-GFP bound to spindles permits spindles to be visualized and spindle dynamics to be monitored in live embryos.

The intensity of Ncd-GFP fluorescence in mitotic spindles was unexpected because antibody staining results suggested that Ncd perdured in the early embryo during the preblastoderm cleavage divisions (cycles 1-9) and remained detectable as late as cycle 12-13, but the motor association with mitotic spindles was greatly diminished by stage 14 (Endow et al., 1994a). The bright fluorescence of Ncd-GFP in cycle 9-14 embryos is consistent with the abundance of *ncd* RNA in embryos up to 12 hours after egg deposition (Komma et al., 1991), and implies that the greatly diminished antibody staining observed in stage 14 embryos is a result of lability of the microtubule-bound motor during fixation. Different fixation methods preserve more or less of the Ncd associated with mitotic spindles, consistent with this possibility. The motor in earlier embryos is probably also labile during fixation, but earlier embryos showed easily detectable levels of fluorescence after antibody staining (Endow et al., 1994a). Alternatively, Ncd-GFP may be more stable than the nonfusion Ncd motor protein, perduring longer than the endogenous motor.

Ncd-GFP shows cell cycle-dependent localization to centrosomes

During interphase, Ncd-GFP localizes to the centrosomes but is also present in the cytoplasm. The centrosomes become very bright with Ncd-GFP at the end of interphase, during nuclear envelope breakdown and assembly of the next round of mitotic spindles. Ncd-GFP remains associated with centrosomes throughout metaphase and anaphase, but the centrosomal localization diminishes in late anaphase and telophase. There is thus a cell cycle-dependent localization of Ncd to centrosomes as well as spindle fibers that was observed previously as diminished staining of centrosomes in late anaphase and telophase spindles by Ncd relative to tubulin antibodies (Endow et al., 1994a).

The cell cycle dependence of Ncd-GFP association with centrosomes most likely reflects the spindle pole function of the motor in early mitosis. The mutant Ncd-GFP* also shows cell cycle-dependent localization to centrosomes, with very bright centrosomal fluorescence at nuclear envelope breakdown and during spindle assembly in early metaphase, followed by diminished staining in telophase. Precocious splitting of centrosomes can be observed as early as interphase, however, resulting in doubled centrosomes. The early splitting of centrosomes in mutant embryos implies that the wild-type motor functions to maintain centrosome integrity and prevent centrosome splitting until late anaphase. An active motor is probably required for these functions, not simply the binding of the motor to centrosomes, since Ncd-GFP* can bind but is probably defective in motor activity because of a missense mutation in its ATP-binding region (S. A. Endow and D. J. Komma, unpublished).

Free centrosomes were observed frequently in mutant embryos. The free centrosomes arise by dissociation from nuclei, either daughter nuclei that have undergone a previous abnormal division or mis-oriented spindles that are positioned perpendicular rather than parallel to the cortex of the embryo. Some free centrosomes that were observed probably rose to the surface of the embryo in interphase of cycle 9 without an associated nucleus. A centrosome in one embryo was also observed

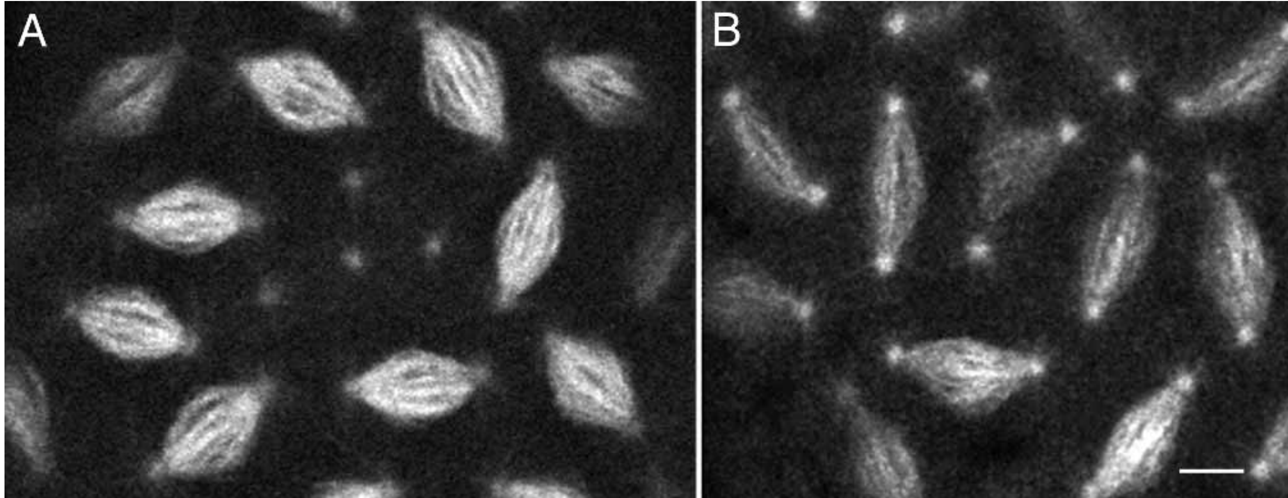


Fig. 6. Free centrosomes are present in ncd-*gfp** mutant embryos and contribute to further spindle abnormalities. The images were taken from time-lapse sequences of ncd-*gfp* M4F1/M4F1;cand/cand* embryos in mid- (A) and early metaphase (B) of cycle 10. (A) Free centrosomes in a cycle 10 embryo injected with rhodamine-histones to visualize chromosomes. The metaphase chromosomes associated with the spindles were clearly labelled with rhodamine-histones but no labelling was associated with the centrosomes. The free centrosomes persisted into anaphase of cycle 11 without doubling. (B) A free centrosome is shown bridged to an adjacent spindle that is just forming. Bar, 5 µm.

dissociating from an interphase nucleus. The apparent lability of centrosome attachment to the interphase nucleus in mutant embryos implies that the Ncd motor is required to maintain centrosome attachment to nuclei during interphase. This is consistent with the association of the Ncd-GFP and Ncd-GFP motors with centrosomes throughout the early mitotic cleavage divisions, including interphase. Localization of Ncd to centrosomes was demonstrated previously to be microtubule-dependent (Endow et al., 1994a), indicating that the motor is not an integral component of the centrosome.

The frequent mis-orientation of spindles perpendicular rather than parallel to the surface in ncd-gfp* cycle 9 mutant embryos suggests that the Ncd motor may be involved in the migration of nuclei to the cortex or orientation of nuclei during interphase of cycle 9. Microtubules have been implicated in mediating the cortical migrations, which have been proposed to be driven by sliding between antiparallel arrays of microtubules formed between the migrating nuclei and yolk nuclei (Baker et al., 1993). This model proposes the involvement of a plus-end directed microtubule motor rather than a minus-end motor like Ncd. Further studies, including examination of the actin cytoskeleton in ncd-*gfp** mutant embryos, should provide information regarding the role of Ncd in the cortical migrations, as well as possible disruption of the actin cytoskeleton, such as the actin-based metaphase furrows, in the mutant embryos.

Ncd-GFP forms filaments in mitotic spindles

The distribution of Ncd-GFP in mitotic spindles parallels that of tubulin during most of mitosis, but is strikingly different from the tubulin distribution in metaphase. Spindles in metaphase, labeled with Ncd-GFP and rhodamine-conjugated tubulin, show fibers of Ncd-GFP that extend across the metaphase plate, which partially excludes rhodamine-tubulin labelling. The same fibers that fluoresce brightly with Ncd-GFP are present in the tubulin-labelled images, but show diminished tubulin fluorescence at the metaphase plate region, while the Ncd-GFP shows uniformly bright labelling along the length of the fibers. Diminished tubulin labeling at the metaphase plate is also observed in live wild-type embryos injected with rhodamine-tubulin to visualize the spindle microtubules.

The region of the spindle excluded by tubulin contains the chromosomes, as shown in double images of Ncd-GFP-decorated spindles and chromosomes labeled with rhodamine-conjugated histones. The Ncd-GFP fibers extend across the chromosomes, enclosing them within the spindle fibers, rather than terminating at the chromosomes on the metaphase plate. The distribution of GFP fluorescence suggests that Ncd-GFP forms filaments along the spindle microtubules that continue across the kinetochore regions of the chromosomes.

Fibers of Ncd were not observed in our previous studies localizing Ncd to spindles using antibodies (Endow et al., 1994a), nor was a pronounced difference observed between the Ncd and tubulin distributions in the spindles. The possibility therefore exists that the filaments formed by Ncd-GFP are due to the GFP. However, *ncd-gfp* embryos that were fixed and stained with antibodies showed neither the fibers of Ncd nor the differences between Ncd and tubulin staining observed in live embryos. Instead, the Ncd staining in fixed *ncd-gfp* embryos resembled that of tubulin, as reported previously for wild-type embryos (Endow et al., 1994a). These results suggest that the failure to observe filaments of Ncd in our previous antibody staining experiments is due to their lability upon fixation rather than to their presence in *ncd-gfp* but not wild-type embryos.

The Ncd-GFP fluorescence in live embryos extends along the microtubule fibers so that differences between the Ncd-GFP and tubulin distributions are limited to the metaphase plate region. Ncd-GFP could bridge the gap between spindle fibers associated with kinetochores of sister chromatids by binding to polar or other microtubules that extend across the

metaphase plate, or to the chromosomes themselves, or by forming fibers by associating with other molecules of Ncd-GFP. High-resolution studies have demonstrated that the inner centromere proteins (INCENPs) relocalize in late metaphase as 'streaks' that traverse the plate, probably closely associated with microtubules (Earnshaw and Cooke, 1991). Association of Ncd-GFP with the INCENPs or other related proteins could also account for the fiberlike fluorescence that is observed. Another kinesin motor, Eg5 of *Xenopus*, has also been hypothesized to form filaments to account for its proposed mechanism of function in mitosis (Sawin et al., 1992).

The possibility that Ncd forms filaments that extend across the kinetochores of the chromosomes suggests that the motor interacts with chromosomes during mitosis. The Ncd motor is not essential for poleward movement of chromosomes in anaphase, since chromosomes move poleward in embryos of the loss-of-function *ncd-gfp** mutant. The motor could associate with kinetochore or centromeric regions of chromosomes, helping to maintain chromosome attachment to the spindle in metaphase, and facilitate poleward movement during the metaphase-anaphase transition. This would explain the frequent detachment of chromosomes from the metaphase plate and mis-segregating chromosomes observed in mutant embryos, as well as the elevated chromosome loss of mutants. Ncd interaction with chromosomes in metaphase/early anaphase is consistent with the timing of differences in Ncd-GFP and tubulin distribution along the spindle fibers: the differences are no longer apparent by mid-anaphase when the spindle fibers have stretched and the chromosomes have moved off the metaphase plate.

*ncd-gfp** causes spindle abnormalities and mis-segregating chromosomes in late cleavage divisions

Spurred or frayed spindles were observed in all of the *ncd-gfp** mutant embryos that were analyzed, and interbridged spindles

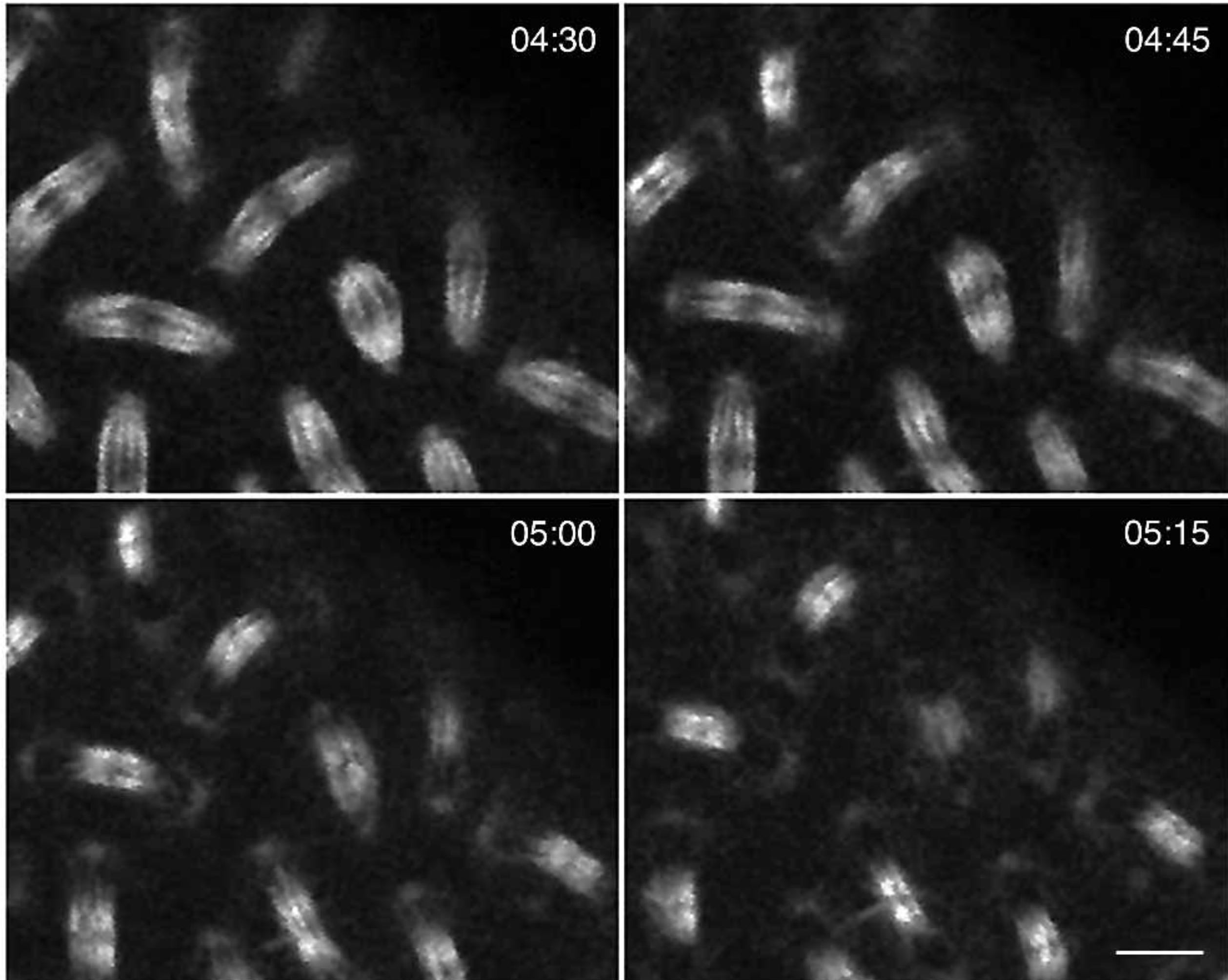
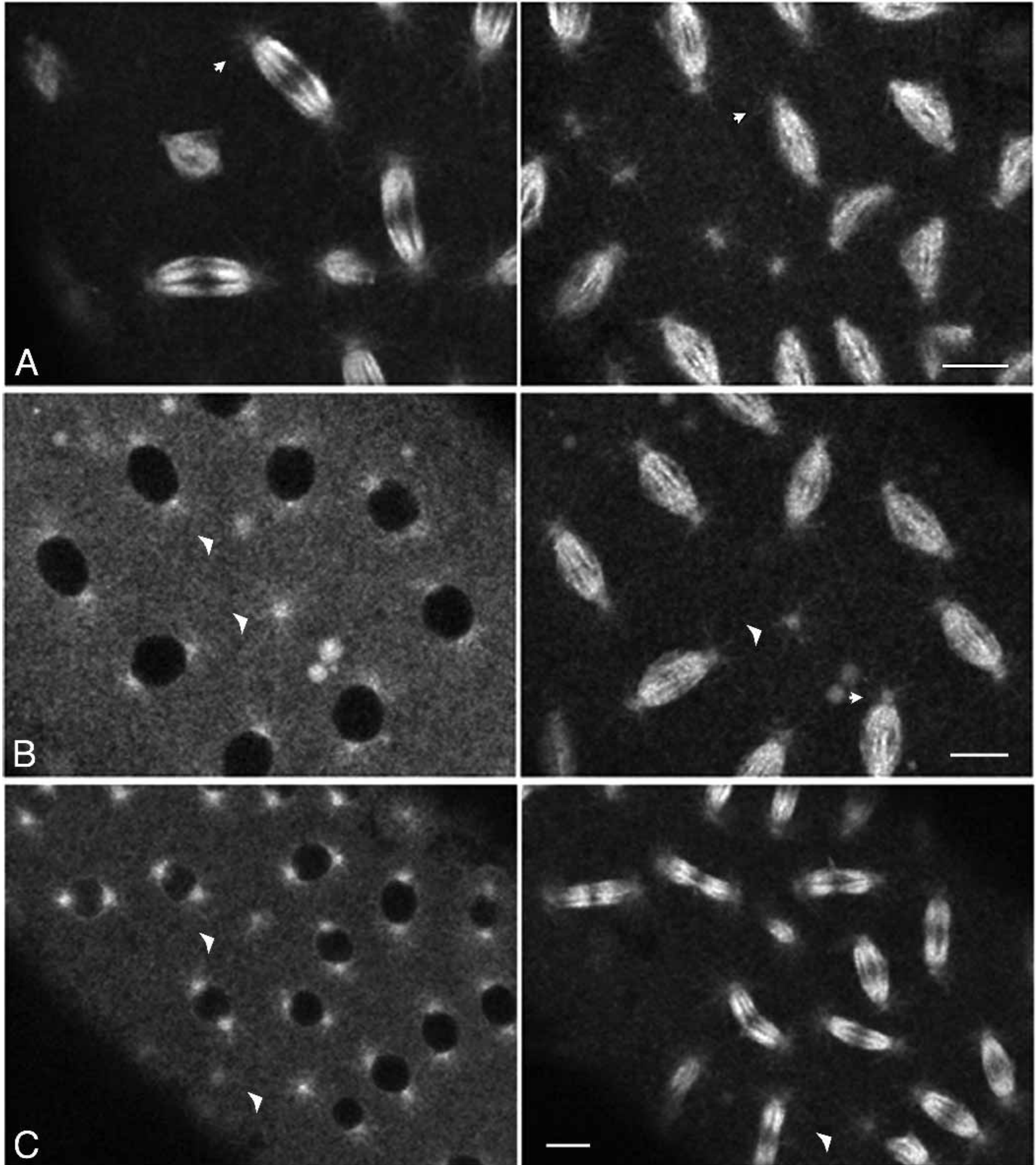


Fig. 7. Abnormal spindles in some mutant embryos result in free centrosomes in the next division. Images were taken from a time-lapse sequence of an *ncd-gfp* M4F2/M4F2;cand/cand* embryo. The short round spindle in anaphase of cycle 9 (frame 1) elongated during mid-anaphase (frame 2) but failed to form a normal midbody in late anaphase (frame 3) and telophase (frame 4). The daughter nuclei did not separate normally and disappeared from the surface in interphase. Two pairs of centrosomes associated with small nuclei reappeared just before nuclear envelope breakdown, but failed to assemble spindles, persisting as free centrosomes until late anaphase of cycle 10. Time in minutes and seconds is shown at the top of each image. Bar, 10 µm.



were observed frequently in the late cleavage divisions. The spurred spindles and mis-segregating chromosomes of *ncd-gfp** embryos demonstrate that chromosome mis-segregation and loss occurs frequently in the late as well as the early cleavage divisions. Mitotic loss in the late cleavage divisions probably contributes to the high frequency of embryo inviability observed for null and loss-of-function mutants of *ncd*.

The observation of centrosome and spindle defects, and mis-segregating chromosomes in late cleavage divisons of mutant embryos provides evidence that the Ncd motor is active in the late, as well as the early embryonic cleavage divisions. Ncd functions at the spindle poles in mitosis, maintaining centrosome attachment to nuclei and preventing centrosome splitting until late anaphase. The motor contributes to midbody stability

Fig. 8. Free centrosomes in mutant embryos arise by loss or dissociation from nuclei. Successive images were taken from time-lapse sequences of *ncd-gfp* M4F1/M4F1;cand/cand* (A,B) or *M4F2/M4F2;cand/cand* (C) embryos. (A) Three spindles in a cycle 9 mid-anaphase embryo are positioned perpendicular rather than parallel to the embryo surface (left). The centrosomes from the top two mis-oriented spindles dissociated from the daughter nucleus closer to the surface, leaving 2 pairs of free centrosomes in late metaphase of cycle 10 (right). The cortical movements of the embryo have displaced the free centrosomes downward and to the right relative to the original spindles. The free centrosomes and the centrosomes at both poles of the mid-anaphase spindle (left, arrow) and one pole of the late metaphase spindle (right, arrow) are precocious split or doubled. This is also true of the centrosomes associated with some of the unmarked spindles. (B) Two centrosomes (arrowheads) without an associated nucleus are present at the cortex of a cycle 9 interphase embryo (left). One centrosome subsequently sank into the interior, while the other centrosome remained at the surface during metaphase of cycle 9 (right) and persisted into metaphase of cycle 10. The other bright round objects are yolk granules just below the surface. The free centrosome in the interphase nucleus (left, bottom arrowhead) appears fragmented. The centrosomes at the pole of the metaphase spindle (right, arrow) and associated with some of the unmarked spindles are precociously split. (C) Dissociation of a centrosome from a cycle 9 interphase nucleus (left, bottom arrowhead). The dissociating centrosome rose to the surface first, followed by the second centrosome and then the nucleus. The centrosome was subsequently recaptured by the spindle and can be seen associated with the spindle in mid-anaphase of cycle 9 (right, arrowhead). The apparently free centrosome (left, top arrowhead) is attached to a spindle that is mis-oriented perpendicular to the embryo surface. Bars, 10 μm.

and function during segregation of daughter nuclei in late anaphase. The Ncd motor also acts to prevent chromosome loss, possibly by maintaining attachment of chromosomes to spindle fibers during the metaphase/anaphase transition and facilitating poleward movement of the chromosomes. Analysis of spindle dynamics in live embryos using partial loss-of-function *ncd* mutants, as well as mutants that affect other mitotic proteins, should provide critical information regarding the interactions of Ncd with mitotic spindle components, including other kinesin motors.

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