Enhancement of the ncdP microtubule motor mutant by mutants of αTub67C

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SUMMARY

Ncd is a kinesin-related microtubule motor protein required for chromosome segregation in Drosophila oocytes and early embryos. In tests for interactions with other proteins, we find that mutants of αTub67C, which affect an oocyte- and early embryo-specific α-tubulin, enhance meiotic nondisjunction and zygotic loss of ncdP, a partial loss-of-function mutant of ncd. The enhancement is dominant and allele-specific with respect to αTub67C, and depends on the recessive effects of ncdP. Cytologically, embryos of αTub67C/+ show delayed meiotic divisions and defective female pronucleus formation, while meiotic spindle assembly is abnormal in embryos of ncdP/ncdP. Doubly mutant αTub67C ncdP/ncdP embryos are rescued for female pronucleus formation, but show delayed meiotic progression and defective pronuclear conjugation or fusion. Delayed completion of meiosis, together with failure of pronuclear fusion, prevents normal interactions of maternal with paternal chromosomes, enhancing the ncdP mutant phenotype. The genetics and cytology of doubly mutant embryos and the molecular defect of NcdP provide evidence for interaction of Ncd with αTub67C in vivo. These results imply that a specific α-tubulin isoform is required for normal cellular function of a kinesin motor protein.

Key words: Ncd, Kinesin microtubule motor, α-tubulin, Mutant interaction

INTRODUCTION

Several proteins have now been identified that function during the meiotic divisions in Drosophila oocytes. Nonclaret disjunctional (Ncd) is a member of the kinesin family of microtubule motors that is required for normal assembly of meiotic spindles in Drosophila oocytes. Unlike conventional kinesin, Ncd translocates toward microtubule minus, rather than plus ends (Walker et al., 1990; McDonald et al., 1990) and generates torque as it moves, causing microtubules gliding on Ncd-coated coverslips to rotate as they glide (Walker et al., 1990). Antibody staining experiments (Hatsumi and Endow, 1992a; Endow et al., 1994a; Matthies et al., 1996) and analysis of oocytes and embryos expressing Ncd as a fusion with the green fluorescent protein (GFP) (S. A. Endow and D. J. Komma, unpublished; Endow and Komma, 1996) have demonstrated localization of the Ncd motor protein to meiotic spindles in oocytes, and mitotic spindle fibers, centrosomes, and spindle poles in early embryos. Oocyte spindles of ncd null mutants exhibit multiple, diffuse, or broad poles (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992b; Matthies et al., 1996), supporting the hypothesis that the motor is required for assembly of bipolar meiotic spindles (Verde et al., 1991; Hatsumi and Endow, 1992a; Hyman and Karsenti, 1996). Early mitotic spindles of null mutants show centrosome loss, precocious splitting of centrosomes, and chromosome loss at metaphase (Endow et al., 1994a; Endow and Komma, 1996), indicating that the Ncd motor functions to maintain spindle pole integrity in mitosis and may act to prevent chromosome loss by maintaining chromosome attachment to the spindle in metaphase.

Other kinesin proteins are thought to help position chromosomes for meiotic segregation (Zhang et al., 1990; Murphy and Karpen, 1995; Afshar et al., 1995) or, in mitosis, to separate centrosomes for spindle formation (Enos and Morris, 1990; Hagan and Yanagida, 1990; Heck et al., 1993), assemble bipolar spindles (Saunders and Hoyt, 1992), or maintain counteracting forces needed to prevent spindle collapse (Saunders and Hoyt, 1992).

Binding of kinesin motors to different isoforms of tubulin for force generation and movement on microtubules may contribute to the regulation of kinesin motor protein activity. Multiple forms of α- and β-tubulin are present in many eukaryotic cells, and are thought to be needed for assembly of microtubules for specific cellular functions (Fuller et al., 1988; Hoyle and Raff, 1990). Oocytes and early embryos of Drosophila contain three α-tubulins: αTub67C, αTub84B and αTub84D. One of these, αTub67C, is expressed only in the ovary (Kalfayan and Wensink, 1982) and is specific to nurse cells, oocytes and early embryos, comprising ~25% of the α-tubulin in early embryos (Matthews et al., 1989). αTub67C is highly divergent compared to other α-tubulins (Kalfayan and Wensink, 1981) with major differences at the N- and C-termini (Theurkauf et al., 1986). Mutants of αTub67C have been recovered that cause instability of the protein, resulting in reduced αTub67C levels in embryos (Matthews et al., 1993). The abnormal spindles and chromosome clusters of mutant embryos indicate that αTub67C is required for normal meiotic and mitotic divisions of oocytes and embryos, paralleling the requirement for Ncd.

To obtain information regarding functional interactions of
the Ncd motor with tubulin proteins in vivo, we analyzed genetic interactions between ncd mutants and tubulin mutants. Our results provide evidence that functional interactions of Ncd with microtubules in vivo require the oocyte- and early embryo-specific α-tubulin, αTub67C. These observations constitute the first evidence that functional interactions of a kinesin protein with microtubules require an isotype-specific α-tubulin.

**MATERIALS AND METHODS**

**Drosophila** mutants

Mutants used in this work are described by Lindley and Zimm (1992). ncdD was originally isolated as an EMS-induced dominant female-sterile mutant but now shows only weak semi-dominant and recessive effects on mitotic chromosome segregation, and is normally wild type for mitotic chromosome distribution (Komma et al., 1991). The X chromosome and chromosome 2 in the ncdD stock were replaced with Oregon R chromosomes by mating to balancer lines. The atm67C and atm67B mutants, atm67C, atm67B, and atm67C, were obtained from the Bloomington Stock Center. The X chromosome, chromosome 2, and the tip of chromosome 3 distal to ca in the atm67B mutant stocks were replaced with Oregon R chromosomes by recombination and mating to balancer stocks. Recombination with the Oregon R chromosome 3 removed the lethal associated with atm67C (Matthews et al., 1993). The original or recombiant atm67C chromosomes were recombined with the ncdD chromosome to produce the atm67C ncdD genotypes, followed by replacement of the X and chromosome 2 with Oregon R chromosomes, as described (Komma and Endow, 1995).


**Embryo viability and chromosome segregation tests**

Embryo viability and X chromosome segregation tests were carried out as described (Endow et al., 1994a; Komma et al., 1991) by mating wild-type or mutant females to y w P/Y males. Single-pair matings were transferred to new vials on days 3-7, eggs were counted, and offspring from each vial were scored for phenotype. Regular offspring were + females and B3 males. Nondisjunction of X chromosomes in oocytes results in X and null-X eggs that give rise to XX/B3Y females and X/0 males carrying a paternal X chromosome. X/0 males in excess of XX/B3Y females are attributed to meiotic loss (Sturtevant, 1929). Gynandromorphs are X chromosome mosaics that arise following chromosome loss in early mitosis. + males carrying a maternal X chromosome are due to paternal X or Y chromosome loss, resulting in nullo-X,Y sperm. Exceptional y w female offspring of atm67C+ females are attributed to fusion of paternally-derived haploid nuclei, or failure of haploid nuclei to segregate, in haploid embryos that arise following defective female pronucleus formation (Komma and Endow, 1995). Calculations of total embryos and frequencies of gametic nondisjunction and loss were corrected for inviability of half of the nondisjunction and meiotic loss embryos as 0Y or X/X0 embryos. Minute (haplo-4) offspring were scored but were excluded from calculations of chromosome mis-segregation because of their highly variable recovery.

**Statistical analysis**

Chromosome segregation data were analyzed using χ² tests for data in which expected values were ≥5 in tests of the null hypothesis that the offspring under comparison were produced by females from the same population. Expected frequencies were calculated as averages of the observed frequencies. For data in which an expected value was <5, statistical tests were carried out by assuming a Poisson distribution. The probability of observing i offspring in a class, Pi(i), is (e⁻ᵐ mᵢ/i!) for a standard Poisson distribution, where m and i are the numbers expected and observed, respectively. m is calculated as (sample size) x (expected frequency).

**Antibody staining of oocytes and embryos**

Oocytes were collected, dechorionated, fixed in formaldehyde/EGTA without taxol, and vitelline membranes were removed by hand, as described previously (Hatsumi and Endow, 1992b). In some experiments, oocytes were activated in hypotonic saline prior to fixation (Hatsumi and Endow, 1992b). Staining with monoclonal anti-α-tubulin and polyclonal anti-Ncd antibodies, followed by DAPI (4',6-diamidino-2-phenylindole) was as described (Hatsumi and Endow, 1992a; Endow et al., 1994a). The α-tubulin antibody (Chemicon International Inc.) cross-reacts with all isofroms of α-tubulin in *Drosophila* and the Ncd antibody is specific for the nonconserved N-terminal tail of Ncd. The Ncd antibody cross-reacts with a single major band on a western blot of *Drosophila* proteins (Hatsumi and Endow, 1992a) and shows no specific cross-reactivity with spindles of oocytes or embryos of the canld null mutant (Hatsumi and Endow, 1992a; Endow et al., 1994a). Control embryos of canld for the present experiments were stained by the α-tubulin antibody but showed no staining with the Ncd antibody.

Wild-type embryos were collected at 30 minute intervals and mutant embryos, at 30 minute, 60 minute or 90 minute intervals. Following dechorionation, vitelline membranes were removed and embryos were fixed in methanol/EGTA without taxol and stained with antibodies and DAPI, as described (Hatsumi and Endow, 1992b; Endow et al., 1994a). In some experiments, embryos were stained with monoclonal anti-histone antibody (1:200 dilution) (Chemicon International Inc.), followed by FITC-conjugated anti-mouse IgG (1:250 dilution, 6 µg/ml) (Vector Lab, Inc.) to visualize the chromosomes.

**Confocal microscopy**

Images of antibody-stained spindles and chromosomes were collected with a Bio-Rad MRC 600 laser scanning confocal imaging detector mounted on a Zeiss Axioshot microscope, using a x63/1.4 NA Planapochromat objective.

**Embryo squashes for chromosomes**

Chromosomes in fixed embryos were visualized as described (Komma and Endow, 1995). Briefly, antibody-stained embryos were mounted in 10 mM Tris-HCl, pH 7.9 + 1 mM EDTA (TE) on Denhardt-treated slides (Brach and Haase, 1978) using siliconized coverslips. Embryos were staged under fluorescence and squashed in situ, coverslips were removed, and embryos were post-fixed up to 1 hour in cold EtOH. Slides were mounted in TE containing 5 µg/ml DAPI and chromosomes were photographed onto 4” x 5” Tri-X film (Kodak 4164 Pan Professional Film). Negatives were scanned into digital images using a Sharp JX-320 scanner.

**Image processing**

Image contrast was adjusted using Bio-Rad SOM or COMOS software or Adobe Photoshop, and images were printed using a Tektronix Phaser HSDX printer. For meiotic spindles or polar bodies that were not in the same focal plane, confocal images collected in successive focal planes were copied to ‘layers’ of one image (Adobe Photoshop v. 3.0) and merged using the ‘screen’ mode option and 100% opacity.
RESULTS

\(\alpha\text{Tub67C}\) mutants enhance ncd\(^D\) meiotic nondisjunction

ncd\(^D\)/ncd\(^D\) females produce frequent X/X/Y and X/0 (B\(^3\) female and y w male) offspring caused by nondisjunction of the X chromosome in meiosis; the frequency of these offspring was 0.108 in the cross shown in Table 1. Genetic tests for interactions between ncd\(^D\) and \(\alpha\text{Tub67C}\) mutants showed significantly increased frequencies of nondisjunctional offspring in progenies of \(\alpha\text{Tub67C}1\) ncd\(^D\)/ncd\(^D\) and \(\alpha\text{Tub67C}3\) ncd\(^D\)/ncd\(^D\) females compared with ncd\(^D\)/ncd\(^D\) females, 0.194 and 0.149, respectively, as shown in Table 1. Zygotic loss of the X chromosome was also significantly increased in these progenies with frequencies of 0.021 for \(\alpha\text{Tub67C}1\) ncd\(^D\)/ncd\(^D\) and 0.015 for \(\alpha\text{Tub67C}3\) ncd\(^D\)/ncd\(^D\), compared with 0.001 for ncd\(^D\)/ncd\(^D\). The meiotic nondisjunction of \(\alpha\text{Tub67C}2\) ncd\(^D\)/ncd\(^D\) females did not differ significantly from that of ncd\(^D\)/ncd\(^D\) females (\(\chi^2=1.12\), 1 d.f., 0.5\(>\)P>0.1) in the data shown in Table 1, nor did zygotic X chromosome loss (P=0.06). The enhancement of ncd\(^D\) meiotic nondisjunction and zygotic loss by \(\alpha\text{Tub67C}\) is therefore allele-specific with respect to \(\alpha\text{Tub67C}\). The allele-specific enhancement of ncd\(^D\) by \(\alpha\text{Tub67C}\) mutants was observed in repeated experiments carried out over a period of 3 years, although the magnitude of the effect was greater in earlier experiments than those reported in Table 1 (e.g. see data for \(\alpha\text{Tub67C}3\) ncd\(^D\)/ncd\(^D\) reported by Komma and Endow, 1995, to document recovery of exceptional androgenetic offspring). The decreased effect in recent experiments is presumably due to the accumulation of modifiers in the stocks.

Table 1 also shows that ncd\(^D\)/ncd\(^D\) enhances the production of exceptional androgenic y w females by \(\alpha\text{Tub67C}1\)/+ and \(\alpha\text{Tub67C}3\)/+, but not by \(\alpha\text{Tub67C}1\)/+. These exceptional females are attributed to fusion of paternally-derived haploid cleavage nuclei, or failure of newly replicated haploid chromosomes to segregate, in embryos defective for female pronucleus formation (Komma and Endow, 1995).

\(\alpha\text{Tub67C}1\) ncd\(^D\)/+ females heterozygous for ncd\(^D\) and \(\alpha\text{Tub67C}1\), \(\alpha\text{Tub67C}2\), or \(\alpha\text{Tub67C}3\) did not show a significant increase in meiotic chromosome nondisjunction, compared with ncd\(^D\)/+ females (Table 1). The zygotic loss of \(\alpha\text{Tub67C}6\) ncd\(^D\)/+ females is also not significantly elevated relative to \(\alpha\text{Tub67C}1\)/+ females (zygotic loss of \(\alpha\text{Tub67C}3\) ncd\(^D\)/+ compared to \(\alpha\text{Tub67C}1\)/+, P=0.09). The genetic interaction between ncd\(^D\) and \(\alpha\text{Tub67C}\) results in elevated meiotic nondisjunction and zygotic loss since depends on the recessive rather than the weak semi-dominant effects of ncd\(^D\) (Komma et al., 1991). The meiotic nondisjunction of \(\alpha\text{Tub67C}\) females is significantly decreased compared with nondisjunction of \(\alpha\text{Tub67C}\) females; the basis of this decrease is not certain from the present data.

Tests of \(\alpha\text{Tub67C}1\) ncd\(^D\)/+ or \(\alpha\text{Tub67C}1\) ncd\(^D\)/ncd\(^D\) females carrying null alleles of ncd resulted in female sterility and were therefore uninformative with regard to the ncd allele specificity for the interaction with \(\alpha\text{Tub67C}\). No other partial loss-of-function alleles of ncd were available for testing for interactions with the \(\alpha\text{Tub67C}\) mutants.

Enhancement of ncd\(^D\) meiotic nondisjunction and zygotic loss by \(\alpha\text{Tub67C}\) requires \(\alpha\text{Tub67C}\)

Tests of Df(3L)Ac1, a deficiency that uncovers \(\alpha\text{Tub67C}\), for

<table>
<thead>
<tr>
<th>Female parent</th>
<th>Offspring</th>
<th>Total gametes</th>
<th>Gametic X nd</th>
<th>X loss</th>
<th>Zygotic X loss</th>
<th>Total X mis-seg</th>
<th>Total embryos</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>s/+</td>
<td></td>
<td>616</td>
<td>2</td>
<td>516</td>
<td>1</td>
<td>1,138</td>
<td>0.005</td>
<td>0.994</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1)/+</td>
<td></td>
<td>1,004</td>
<td>1</td>
<td>935</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1.953</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1) ncd(^D)/+</td>
<td></td>
<td>624</td>
<td>1</td>
<td>595</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>1.233</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1) ncd(^D)/ncd(^D)</td>
<td></td>
<td>396</td>
<td>57</td>
<td>304</td>
<td>30</td>
<td>19</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1)/+</td>
<td></td>
<td>588</td>
<td>16</td>
<td>562</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>1.209</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1) ncd(^D)/+</td>
<td></td>
<td>482</td>
<td>1</td>
<td>459</td>
<td>2</td>
<td>1</td>
<td>947</td>
<td>0.004</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1) ncd(^D)/ncd(^D)</td>
<td></td>
<td>115</td>
<td>8</td>
<td>137</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>280</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1)/+</td>
<td></td>
<td>284</td>
<td>238</td>
<td>2</td>
<td>1</td>
<td>525</td>
<td>&lt;0.002</td>
<td>0.004</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1) ncd(^D)/+</td>
<td></td>
<td>656</td>
<td>1</td>
<td>549</td>
<td>5</td>
<td>12</td>
<td>2</td>
<td>1.231</td>
</tr>
<tr>
<td>(\alpha\text{Tub67C}1) ncd(^D)/ncd(^D)</td>
<td></td>
<td>369</td>
<td>42</td>
<td>271</td>
<td>16</td>
<td>12</td>
<td>9</td>
<td>777</td>
</tr>
<tr>
<td>ncd(^D)/+</td>
<td></td>
<td>645</td>
<td>632</td>
<td>2</td>
<td>1</td>
<td>1.281</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>ncd(^D)/ncd(^D)</td>
<td></td>
<td>762</td>
<td>572</td>
<td>715</td>
<td>38</td>
<td>2</td>
<td>1</td>
<td>1.660</td>
</tr>
</tbody>
</table>

The table shows offspring of matings between females of the indicated genotype and y w B\(^3\) B\(^{fly}\) males in tests of X chromosome segregation. Regular offspring are + females and B\(^3\) males. Meiotic nondisjunction or loss of the X gives rise to B\(^3\) (X/X/Y) females and y w (X/0) males. Gynandromorphs (gyn) (X/X-X/0 mosaics) arise upon zygotic loss of the maternal X; occasional y gynandromorphs are produced by zygotic loss of the paternal X. + (X/0) males are due to meiotic loss of the paternal X chromosome. y w females are patroclinous exceptions and have been reported previously (Komma and Endow, 1995). \(\alpha\text{Tub67C}1\) and \(\alpha\text{Tub67C}3\) cause dominant enhancement of the effects of ncd\(^D\) on meiotic nondisjunction and zygotic chromosome loss. Viability of the \(\alpha\text{Tub67C}1\) ncd\(^D\)/ncd\(^D\) and \(\alpha\text{Tub67C}3\) ncd\(^D\)/ncd\(^D\) mutant embryos is also reduced compared with embryos of ncd\(^D\)/ncd\(^D\). \(\alpha\text{Tub67C}\) causes a less severe dominant enhancement of ncd\(^D\)/ncd\(^D\) than \(\alpha\text{Tub67C}1\), and its recessive mutant effects are also less severe than those of \(\alpha\text{Tub67C}1\) (Matthews et al., 1993). The basis of the approximate 2:1 ratio of nondisjunctional X/X/Y to X/0 offspring in the crosses of ncd\(^D\)/ncd\(^D\) females is not known. This is not a general characteristic of ncd\(^D\) (e.g. see Table 3). Calculations of total embryos and gametic nondisjunction and loss were corrected for inviability of half of the nondisjunctional and loss embryos as 0.5 or X/X/Y embryos. Total adults (not shown) included M (haplo-4) offspring. The data shown for \(\alpha\text{Tub67C}1\) ncd\(^D\)/ncd\(^D\) and ncd\(^D\)/ncd\(^D\) were pooled from two experiments.

*aIncludes 1 y gynandromorph.

n, nondisjunction.
interactions with ncdp showed significantly increased frequencies of meiotic and zygotic chromosome loss observed in progeny of Df(3L)A C1 ncdp/+ and Df(3L)A C1 ncdp/ncdp females, compared to ncdp/+ and ncdp/ncdp females (Table 2). The increased zygotic chromosome loss observed in progeny of αTub67C ncdp/ncdp females mutant for αTub67C3 as αTub67C1 and ncdp can therefore be attributed to loss of αTub67C1 function.

Nondisjunctional offspring are not increased in frequency in progeny of Df(3L)A C1 ncdp/ncdp compared to ncdp/ncdp females, but instead the frequency of these offspring is suppressed. Deficiency of αTub67C therefore enhances the meiotic and zygotic chromosome loss of ncdp and suppresses the meiotic nondisjunction. This indicates that the enhanced meiotic nondisjunction of ncdp by αTub67C1 and αTub67C3 requires the αTub67C1 or αTub67C3 mutant protein and excludes the possibility that the effect is a consequence of reduced levels of αTub67C due to protein instability. This interpretation is also consistent with the allele specificity of the interaction.

Enhancement of ncdp meiotic nondisjunction or zygotic loss is not observed for a deficiency of αTub84B

Tests of Df(3R)Sce2, a deficiency that uncovers αTub84B, were carried out to determine whether the interaction between ncdp and αTub67C was specific for αTub67C or could be observed for other α-tubulin genes. αTub84B encodes an α-tubulin that is constitutively expressed in Drosophila tissues throughout development, and is found in all tissues including oocytes and embryos. Df(3R)Sce2 ncdp/ncdp females, heterozygous for the αTub84B deficiency and homozygous for ncdp, showed no significant differences compared to ncdp/ncdp females with respect to meiotic chromosome nondisjunction (χ²=0.14, 1 d.f., 0.9>P>0.5) or zygotic loss (P=0.18) (Table 3). These results show that the enhancement of gametic and zygotic chromosome loss of ncdp by Df(3L)A C1 is not observed for Df(3R)Sce2. The genetic interaction between ncdp and αTub67C is therefore unlikely to be a consequence of reduced α-tubulin levels in general, but instead appears to be specific for αTub67C.

Oocytes of αTub67C3/+ complete the meiotic divisions but are defective in female pronucleus formation

Polar bodies and mitotic spindles were examined cytologically in early embryos of αTub67C3/+ females and compared with polar bodies and spindles of wild-type embryos and αTub67C wild-type mutant embryos to determine the effects of ncdp and αTub67C on assembly of these microtubule-containing structures. Microtubules were stained with anti-tubulin and anti-Ncd antibodies, and chromosomes were stained with DAPI or anti-histone antibody.

Following completion of the oocyte meiotic divisions, the female pronucleus forms from the inner-most of the four haploid nuclei (Sonnenblick, 1950). The three remaining nuclei condense into chromosomes and assemble into polar bodies that consist of 1 or 2 haploid sets of chromosomes sur-

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**Table 2. Enhancement of ncdp meiotic nondisjunction by αTub67C mutants requires αTub67C**

<table>
<thead>
<tr>
<th>Female parent</th>
<th>Offspring</th>
<th>Total gameset</th>
<th>Gametic</th>
<th>Zygotic</th>
<th>Total X</th>
<th>Total embryos</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3L)A C1/+</td>
<td>+ y</td>
<td>263</td>
<td>1</td>
<td>0.003</td>
<td>0.003</td>
<td>1.551</td>
<td>0.500</td>
</tr>
<tr>
<td>Df(3L)A C1 ncdp/+</td>
<td></td>
<td>432</td>
<td>7</td>
<td>0.002</td>
<td>0.018</td>
<td>1.687</td>
<td>0.663</td>
</tr>
<tr>
<td>Df(3L)A C1 ncdp/ncdp</td>
<td></td>
<td>265</td>
<td>11</td>
<td>0.002</td>
<td>0.041</td>
<td>2.270</td>
<td>0.426</td>
</tr>
<tr>
<td>ncdp/+</td>
<td>+ y</td>
<td>645</td>
<td>2</td>
<td>&lt;0.001</td>
<td>&lt;0.003</td>
<td>1.454</td>
<td>0.883</td>
</tr>
<tr>
<td>ncdp/ncdp</td>
<td>+ y</td>
<td>762</td>
<td>38</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>3.523</td>
<td>0.505</td>
</tr>
</tbody>
</table>

The table shows offspring from X chromosome segregation tests of females heterozygous for a deficiency, Df(3L)A C1, that uncovers αTub67C. The Df(3L)A C1/+ females were wild type, heterozygous, or homozygous for ncdp. Data for ncdp/+ and ncdp/ncdp females from Table 1 are shown for comparison. Df(3L)A C1 enhances the effect of ncdp on gametic and zygotic loss of the X, but suppresses the effect of ncdp on meiotic nondisjunction.

*Includes 1 y+ gynandromorph.

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**Table 3. A deficiency of αTub84B does not enhance ncdp meiotic nondisjunction or zygotic loss**

<table>
<thead>
<tr>
<th>Female parent</th>
<th>Offspring</th>
<th>Total gameset</th>
<th>Gametic</th>
<th>Zygotic</th>
<th>Total X</th>
<th>Total embryos</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3R)Sce2+/+</td>
<td>+ y</td>
<td>907</td>
<td>4</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>3.094</td>
<td>0.565</td>
</tr>
<tr>
<td>Df(3R)Sce2 ncdp/+</td>
<td></td>
<td>645</td>
<td>10</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>2.187</td>
<td>0.562</td>
</tr>
<tr>
<td>Df(3R)Sce2 ncdp/ncdp</td>
<td></td>
<td>249</td>
<td>10</td>
<td>0.085</td>
<td>0.002</td>
<td>2.349</td>
<td>0.250</td>
</tr>
<tr>
<td>*ncdp/ncdp</td>
<td>+ y</td>
<td>534</td>
<td>23</td>
<td>0.081</td>
<td>&lt;0.001</td>
<td>2.168</td>
<td>0.583</td>
</tr>
</tbody>
</table>

The table shows offspring from X chromosome segregation tests of females heterozygous for a deficiency, Df(3R)Sce2, that uncovers αTub84B. The Df(3R)Sce2+/+ females were wild type, heterozygous, or homozygous for ncdp. Df(3R)Sce2 shows no enhancement of the effect of ncdp on meiotic nondisjunction or zygotic chromosome loss, although viability of embryos doubly mutant for Df(3R)Sce2 and ncdp is significantly reduced relative to that of ncdp.

<sup>*</sup>Females were y<sup>2</sup> w<sup>67B</sup>; ncdp mated to +/B<sup>67B</sup>y+ males. X/0 males due to maternal X loss were therefore + in phenotype and X/0 males due to paternal X loss were y w. nd, nondisjunction.
Mutants of αTub67C enhance ncd

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rounding by microtubules. A wild-type polar body is shown in Fig. 1A. Separate spindles assemble around the maternal and paternal chromosomes, which lie in close apposition to one another, forming the first cleavage division spindle (Fig. 1B). The maternal and paternal chromosomes remain separate from one another until the end of the first mitotic division.

Embryos of αTub67C females, homozygous for αTub67C2 or αTub67C3, typically contained a single large polar body with >4N chromosomes (Fig. 1C), indicating that the chromosomes had failed to undergo the meiotic divisions, or had undergone the meiotic divisions but the resulting haploid nuclei had fused with one another, and had replicated in the polar body. The meiotic defect, or defect in behavior of the meiotic products, results in failure to form a female pronucleus. Consistent with this interpretation, a single small mitotic spindle containing centrosomes and associated with long astral microtubules was observed in the αTub67C mutant embryos (Fig. 1D). The presence of centrosomes indicated that the spindle was associated with paternal chromosomes since oocyte meiotic spindles are centriolar and centrosomes for the mitotic divisions are derived from the inseminating sperm (Sonnenblick, 1950). The spindles were associated with a haploid set of chromosomes (Fig. 1D). Mutant embryos of αTub67C3 females frequently contained many small mitotic spindles associated with haploid chromosome sets (Komma and Endow, 1995) (not shown), instead of a single small spindle. The multiple small spindles have been interpreted as arising from cleavage divisions of the haploid spindles (Komma and Endow, 1995). Polar bodies and spindles of αTub67C mutant embryos were brightly stained by both anti-tubulin and anti-Ncd antibodies.

Normal-appearing meiosis II spindles were observed in early (0-30 minutes) embryos of αTub67C+/+ females (Fig. 2A), indicating that mutant embryos can complete the meiotic divisions. Meiotic progression is delayed relative to wild type, however, since meiosis I or II stages were frequently observed among 0-30 minute αTub67C+/+ mutant embryos (n=5, total=18, frequency=0.278), but were infrequent among 0-30 minutes wild-type embryos (n=6, total=74, frequency=0.081). Some αTub67C+/+ mutant embryos contained 4 microtubule-associated clusters of chromosomes (Fig. 2B) near the anterior dorsal surface of the embryo where the polar bodies lie in wild-
type embryos, while later (0-90 minutes) embryos had 2-3 such clusters. Based on their position in the embryo, the haploid number of chromosomes within each body and the polar body-like sheath of microtubules surrounding the chromosomes, these structures were presumed to be polar bodies. The number of chromosomes associated with the 2-3 polar bodies of 0-90 minutes embryos accounted for the 4 haploid sets produced by the meiotic divisions, indicating that fusion of the haploid chromosome sets could occur to reduce the number of polar bodies. This is also observed in wild-type embryos (Rabinowitz, 1941). Many αTub67C3/+ mutant embryos exhibited 2-3 polar bodies containing a total of 4 N chromosomes together with small mitotic spindles associated with haploid sets of chromosomes, as observed for αTub67C mutant embryos. The small mitotic spindles were concluded to be associated with paternal chromosomes based on the centromeres present at the spindle poles. These embryos are interpreted to arise by cleavage divisions of the paternal chromosomes following failure of the female pronucleus to form.

ncdD is defective in oocyte meiotic divisions

Whole-mount ncdD oocytes stained with anti-α-tubulin and anti-Ncd antibodies showed multipolar or broad meiotic spindles, or diffuse spindles with undefined spindle poles, as reported previously for meiotic spindles of ncd null mutants (Wald, 1936; Kimble and Church, 1983; Hatsumi and Endow, 1992b; Matthis et al., 1996). Bivalent chromosomes in non-activated or activated oocytes were frequently separated from one another and associated with separate spindles. These abnormal spindles were brightly stained by the Ncd antibody (Fig. 3), confirming association of the NcdD motor with meiotic spindles in whole-mount oocytes. The α-tubulin antibody staining (not shown) was similar to the Ncd antibody staining.

Early embryos of ncdD lacked normal polar bodies, consistent with the defective meiotic spindles observed in ncdD oocytes. Instead, oocyte chromosomes were present as free chromosomes or nuclei devoid of tubulin staining, or associated with abnormal spindles. Oocyte chromosomes in embryos of the ncd null mutant, cdD', have also been observed to lack tubulin staining or to be spindle-associated (Hatsumi and Endow, 1992b); these effects are therefore due to loss of Ncd meiotic function. Polar body-like structures were observed infrequently in early embryos of ncdD females (Fig. 4A) and were associated with loose arrays of chromosomes, unlike the focused polar body chromosomes of wild type and αTub67C mutants (Fig. 1A,C). The microtubules associated with the abnormal polar bodies of ncdD embryos were unstained or faintly stained by α-tubulin and Ncd antibodies (Fig. 4A).

Mitotic spindles of early ncdD embryos were somewhat larger in size than wild-type spindles of the same stage and were ‘ragged’ in appearance, but showed Ncd antibody staining that closely resembled the tubulin staining (Fig. 4B),
Mutants of \( \alpha \text{Tub67C} \) enhance \( ncd^D \) as in wild-type spindles (Endow et al., 1994a). The spindles present in \( ncd^D \) mutant embryos usually lacked the spurs and branches associated with free chromosomes that are characteristic of the abnormal early mitotic spindles of the \( ced^D \) null mutant (Hatsumi and Endow, 1992b) and other loss-of-function \( ncd \) mutants (Endow and Komma, 1996). The absence of spindle spurs and branches associated with the early mitotic spindles of \( ncd^D \) is consistent with the near absence of zygotic chromosome loss observed in genetic tests of \( ncd^D \) females (Komma et al., 1991).

\( \alpha \text{Tub67C}^3 ncd^D/ncd^D \) mutant embryos show delayed meiotic progression and defective pronuclear conjugation or fusion

Early (0-90 minute) embryos of \( \alpha \text{Tub67C}^3 ncd^D/ncd^D \) females frequently showed oocyte chromosomes associated with aberrant meiosis II spindle structures (\( n=4, \) total=33, frequency=0.121) (Fig. 5A). Unlike wild-type meiosis II spindles and meiosis II spindles of \( \alpha \text{Tub67C}^+/+ \) mutant embryos, spindles of \( \alpha \text{Tub67C}^3 ncd^D/ncd^D \) mutant embryos persisted after onset of the first mitotic division. Both a female and male pronucleus were observed in some mutant embryos, demonstrating rescue of female pronucleus formation compared to \( \alpha \text{Tub67C}^3/+ \). The maternal and paternal chromosomes were associated with widely separated spindles in one embryo (Fig. 5B), indicating failure of the male and female pronuclei to conjugate and fuse. In another embryo, two pairs of side-by-side telophase nuclei were associated with the same cycle 1 spindle, demonstrating that the separated pronuclei could divide, but the nuclei remained separated from one another even after completion of the first mitotic division. Many embryos of \( \alpha \text{Tub67C}^3 ncd^D/ncd^D \) females contained
small mitotic spindles with centrosomes that were associated with haploid sets of chromosomes, as observed for \( \alpha \text{Tub67C} \) and \( \alpha \text{Tub67C}^{+} \) mutant embryos. The presence of centrosomes at the poles indicated that the spindles were assembled around paternal chromosomes.

**DISCUSSION**

The results presented here demonstrate that \( \alpha \text{Tub67C} \) mutants cause allele-specific dominant enhancement of \( ncd^{D} \) meiotic nondisjunction and zygotic loss. The enhancement is dominant and depends on the recessive effects of \( ncd^{D} \). A deficiency that uncovers \( \alpha \text{Tub67C} \) enhanced the chromosome loss of \( ncd^{D} \), but suppressed the meiotic nondisjunction, implying that the enhanced nondisjunction of \( ncd^{D} \) by \( \alpha \text{Tub67C}^{+} \) and \( \alpha \text{Tub67C}^{3} \) requires the \( \alpha \text{Tub67C} \) protein. Chromosome mis-segregation of \( ncd^{D} \) was not affected by a deficiency of \( \alpha \text{Tub84B} \), an \( \alpha \)-tubulin that is found in all tissues including oocytes and embryos, indicating that the genetic interaction between \( ncd^{D} \) and \( \alpha \text{Tub67C} \) is specific for \( \alpha \text{Tub67C} \) rather than a consequence of reduced or altered levels of \( \alpha \)-tubulin in general. The allele-specificity of the interaction and requirement for \( \alpha \text{Tub67C} \) imply that the enhanced meiotic nondisjunction is due to protein:protein interactions. This interpretation is consistent with the molecular change in \( ncd^{D} \), a V_{336} \rightarrow F \) missense mutation in the proposed microtubule-binding region of the motor (Komma et al., 1991) that causes an ~10-fold reduced velocity of \( ncd^{D} \) in vitro motility assays relative to wild type (Moore et al., 1996).

The simplest interpretation of our genetic and cytological results is that the enhancement of \( ncd^{D} \) meiotic nondisjunction by \( \alpha \text{Tub67C} \) is caused by interaction of \( Ncd^{D} \) with the mutant \( \alpha \text{Tub67C}^{1} \) and \( \alpha \text{Tub67C}^{3} \) proteins. The \( Ncd^{D} \) motor binds to microtubules in \( \alpha \text{Tub67C} \) oocytes, but the oocyte chromosomes fail to undergo the meiotic divisions. In \( \alpha \text{Tub67C}^{+} \) mutant embryos, meiotic progression is delayed relative to wild type and the female pronucleus frequently fails to form, while \( ncd^{D} \) mutant embryos show defective meiotic spindle assembly and polar body formation. \( \alpha \text{Tub67C}^{3} \) \( ncd^{D} \) and \( ncd^{D} \) mutant embryos are rescued for female pronuclear formation but show delayed meiotic progression and defective pronuclear conjugation or fusion. The delayed assembly of meiotic spindles, together with defective pronuclear conjugation or fusion, delays or prevents interactions of maternal with paternal chromosomes and causes a worse mutant phenotype than \( ncd^{D} \) or \( \alpha \text{Tub67C}^{+} \) alone. Enhancement of the \( ncd^{D} \) mutant phenotype by \( \alpha \text{Tub67C} \) mutants can therefore be explained by enhanced interactions of \( Ncd^{D} \) with microtubules caused by mutational changes in \( \alpha \text{Tub67C} \).

These results imply that \( Ncd^{D} \) interacts with \( \alpha \text{Tub67C} \) in vivo, and provide evidence that wild-type \( Ncd \) binds to \( \alpha \text{Tub67C} \) to carry out function. Interaction of a microtubule motor protein with an isotype-specific tubulin could be important in regulating motor function at the cellular level. In particular, the question of why the \( Ncd \) motor is required for mitotic chromosome distribution in early embryos but not later in development may be due to the replacement of \( \alpha \text{Tub67C} \) in early embryos by other \( \alpha \)-tubulin isotypes (Kalfayan and Wensink, 1982; Matthews et al., 1993). The failure of pronuclear conjugation or fusion in embryos doubly mutant for \( ncd^{D} \) and \( \alpha \text{Tub67C}^{3} \) suggests that \( Ncd \) may function in wild-type embryos to move pronuclei together for fusion. \( Ncd \) could act in a manner similar to that proposed for Kar3, a minus-end kinesin microtubule motor in yeasts, which has been proposed to translocate toward the minus ends of crosslinked antiparallel microtubules that emanate from the two haploid nuclei in mating cells, moving the nuclei together for fusion (Endow et al., 1994b).

In addition to providing the first evidence for the requirement for a specific tubulin isotype for functional interactions with microtubules, the results imply that \( Ncd \) requires \( \alpha \)-tubulin for motor function in vivo. Our results do not exclude the possibility that \( Ncd \) binds to \( \beta \) as well as \( \alpha \)-tubulin to carry out function, consistent with the demonstration that a truncated \( Ncd \) motor protein can be crosslinked to both \( \alpha \) and \( \beta \)-tubulin (Walker, 1995) and with ultrastructural analysis of \( Ncd \)-decorated microtubules, interpreted as showing extensive interactions of \( Ncd \) with both tubulin subunits (Hoenger et al., 1995; Hirose et al., 1995). Besides contributing to the regulation of the motors, tubulin subunit binding interactions may be of importance in determining the polarity of translocation on microtubules of \( Ncd \) and other kinesin motors.

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