Mitch – a rapidly evolving component of the Ndc80 kinetochore complex required for correct chromosome segregation in *Drosophila*

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Summary

We identified an essential kinetochore protein, Mitch, from a genetic screen in *D. melanogaster*. Mitch localizes to the kinetochore, and its targeting is independent of microtubules (MTs) and several other known kinetochore components. Animals carrying mutations in *mitch* die as late third-instar larvae; mitotic neuroblasts in larval brains exhibit high levels of aneuploidy. Analysis of fixed *D. melanogaster* brains and *mitch* RNAi in cultured cells, as well as video recordings of cultured *mitch* mutant neuroblasts, reveal that chromosome alignment in *mitch* mutants is compromised during spindle formation, with many chromosomes displaying persistent mono-orientation. These misalignments lead to aneuploidy during anaphase. Mutations in *mitch* also disrupt chromosome behavior during both meiotic divisions in spermatocytes: the entire chromosome complement often moves to only one spindle pole. Mutant mitotic cells exhibit contradictory behavior with respect to the spindle assembly checkpoint (SAC). Anaphase onset is delayed in untreated cells, probably because incorrect kinetochore attachment maintains the SAC. However, mutant brain cells and *mitch* RNAi cells treated with MT poisons prematurely disjoin their chromatids, and exit mitosis. These data suggest that Mitch participates in SAC signaling that responds specifically to disruptions in spindle microtubule dynamics. The *mitch* gene corresponds to the transcriptional unit CG7242, and encodes a protein that is a possible ortholog of the Spc24 or Spc25 subunit of the Ndc80 kinetochore complex. Despite the crucial role of Mitch in cell division, the *mitch* gene has evolved very rapidly among species in the genus *Drosophila*.

Supplementary material available online at
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Key words: Aneuploidy, Spindle checkpoint, Chromosome congression, Mono-oriented chromosomes

Introduction

Among the cellular structures most responsible for the accurate distribution of chromosomes during cell division are the centromeres and their associated kinetochores. Centromeres are the sites where sister chromatids are most closely attached during the period between chromosome replication and anaphase onset (reviewed in Chan et al., 2005; Craig and Choo, 2005). Centromeres also elaborate kinetochores: proteinaceous structures containing microtubule (MT) motors and other molecules that connect chromosomes to the spindle (such as CENP-E (Putkey et al., 2002), power chromosomal movements (such as dynein) (Savoian et al., 2000), and disassemble MT plus ends (such as MCAK) (Hunter et al., 2003). Kinetochores are also the seat of the spindle assembly checkpoint (SAC), which prevents the onset of anaphase until the kinetochores are stably attached to the spindle. Several molecular components of the SAC are specifically present on kinetochores that are not attached to the spindle (reviewed by Lew and Burke, 2003).

Many kinetochore proteins were first discovered in genetic screens, particularly in the yeast *Saccharomyces cerevisiae.* Mutations that disrupted the fidelity of chromosome segregation led to the discovery of kinetochore proteins, such as Ctf19, Mcm21 and Cin8 (Hoyt et al., 1992; Poddar et al., 1999; Spencer et al., 1990). Most of the canonical SAC proteins were originally identified through screens for mutations that prevented cells from arresting mitosis in the presence of spindle poisons (Hoyt et al., 1991; Li and Murray, 1991). Although many yeast kinetochore components have proven to be conserved in higher eukaryotes, others have no identifiable metazoan homologs (reviewed by Meraldi et al., 2006). Because yeast kinetochores are imperfect models for kinetochores in multicellular eukaryotes, we have conducted genetic screens for mutants that disrupt mitotic chromosome segregation in *Drosophila melanogaster.* The utility of this approach is illustrated by our identification of a multisubunit complex containing the proteins Zw10, Rod and Zwilch that associates with kinetochores and is conserved throughout metazoan, but not yeast, lineages (Scaerou et al., 2001; Williams et al., 2003). Here, we describe a new kinetochore protein, Mitch (mitotic chaos), which is required for correct chromosome alignment and segregation during meiosis and mitosis in *Drosophila*.

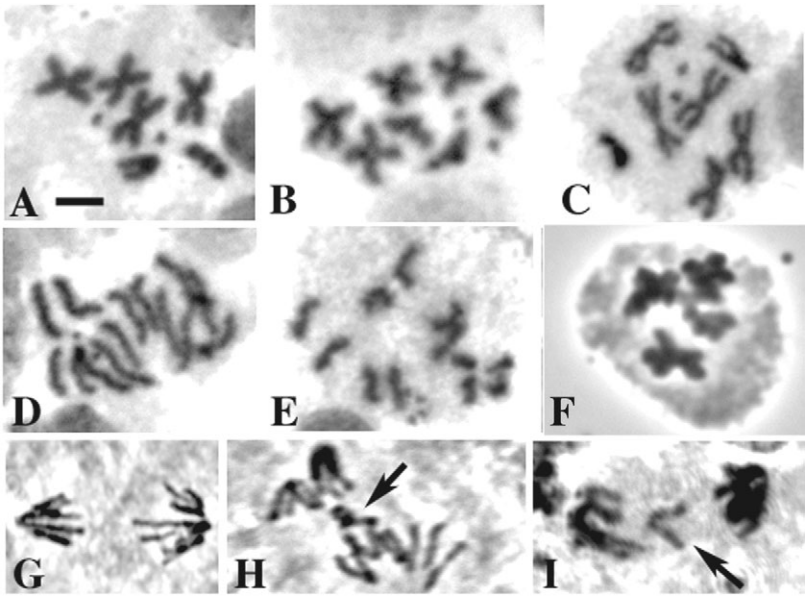

Fig. 1. Mitotic defects in *mitch* mutants. (A-I) Larval brain neuroblasts from wild-type (A,G), *mitch*^{1} (B,D,F,H), and *mitch*^{2}/*mitch*^{19B} (C,E,I) were stained with orcein to visualize chromosomes. (A-E) Brains treated with colchicine and hypotonic solution. In contrast with normal 6A,XX or 6A,XY chromosome complements in wild-type (A; here from a male), *mitch* mutants contain many aneuploid cells, as seen in (B) 6A,XXX and (C) 7A,XY. (A indicates the number of autosomes, including the dot-like fourth chromosomes.) (D,E) Many *mitch* mutant cells treated with colchicine also exhibit PSCS in which the sister chromatids have become detached from each other even at the centromere. (F-I) Untreated brains. (F) Many mitotic figures in untreated *mitch* mutant brains have overcondensed chromosomes. During anaphase, when chromatids in wild type normally migrate equally to the poles (G), lagging chromatids are visible in *mitch* mutants (arrows; H,I). Bar, 5 μm.

Results

Mutations in *mitch* disrupt chromosome congression and segregation during mitosis and meiosis

Our laboratory recently screened for mitotic mutants among a large collection of ethylmethanesulfonate (EMS)-mutagenized *Drosophila* stocks (Koundakjian et al., 2004) in which homozygosity for EMS-treated third chromosomes caused lethality during third instar larval or pupal stages (see Materials and Methods). To find mitotic mutants, brains isolated from dying third instar larvae were treated with colchicine, then fixed and stained with orcein, because these conditions are particularly favorable for visualizing chromosome number and morphology (Gatti and Goldberg, 1991). The brains of larvae homozygous for a particular mutagenized third chromosome, bearing the mutation we now call *mitch*^{1}, displayed high levels of aneuploidy (Fig. 1B, compare with wild-type in Fig. 1A; Table 1). Substantial levels of aneuploidy also occurred in the brains of larvae carrying mutations for other alleles and interallelic combinations of *mitch* that we subsequently obtained (Fig. 1C; Table 1). Our analysis of the chromosome complements of aneuploid cells suggested that all chromosome types were equally subject to missegregation in these mutant strains (data not shown).

To view all the stages of mitosis in mutant brains, we investigated the *mitch* mutant phenotype in the absence of colchicine, visualizing mitotic figures with orcein (Fig. 1F-I) or by staining fixed brains for DNA and tubulin (Fig. 2). Three major types of aberration were observed. First, many *mitch* mutant cells contained moderately overcondensed chromosomes (Fig. 1F, Fig. 2D, Table 1), suggesting delays in mitotic progression. Second, the chromosomes were generally unable to congress correctly to the equator of the spindle: in contrast with wild-type (Fig. 2A), few *mitch* cells contained a well-formed metaphase plate (Fig. 2C). Third, the majority of anaphases in *mitch* mutant cells displayed lagging chromosomes and the unequal allocation of chromosomes (Fig. 1H,I; Fig. 2D); such defects are not seen in wild type (Fig. 1G, Fig. 2B). Since tubulin staining revealed relatively normal bipolar spindles, the abnormal chromosome distribution seen in fixed *mitch* neuroblasts is unlikely to be caused by disruptions of the spindle apparatus (Fig. 2).

To better understand the *mitch* mutant phenotype, we obtained time-lapse DIC recordings of cultured mutant brain cells (Fig. 3; see also supplementary material Movies 1-5). These movies verified that chromosomes in *mitch* mutants fail to congress correctly during prometaphase or to segregate faithfully at anaphase. Particularly notable was the persistence of mono-oriented chromosomes around the spindle poles (Fig. 3B,C; supplementary material Movies 2 and 3). In all of our

Table 1. Mitotic parameters in *mitch* mutant brains

Genotype	No. of brains (no. of cells)	OC (%)	MI	Met:Ana
Untreated				
Wild-type	5 (314)	0.3	1.04	7.3
mitch^{1}	4 (298)	55.7	0.91	12.5
mitch^{1}/*Df*	6 (107)	70.2	0.33	7.2
mitch^{1}/*mitch*^{2}	2 (95)	21.5	0.45	12.6
mitch^{2}/*Df*	4 (176)	61.9	0.86	12.5
mitch^{19B}	5 (18)	82.3	0.19	17.0
asp^{1}	2 (211)	87.8	2.10	28.6
mitch^{1};*asp*^{1}	3 (206)	58.3	1.02	8.0

Genotype	No. of brains (no. of cells)	Ane (%)	PSCS (%)	MI
Colchicine-treated				
Wild-type	4 (545)	0.1	0.9	2.8
mitch^{1}	6 (278)	27.3	38.4	1.1
mitch^{1}/*Df*	5 (62)	43.5	41.9	n.d.
mitch^{1}/*mitch*^{2}	3 (226)	14.1	28.0	n.d.
mitch^{2}/*Df*	10 (386)	14.5	29.7	n.d.
mitch^{19B}	8 (32)	37.5	50.0	n.d.
asp^{1}	2 (470)	n.d.	1.9	4.4
mitch^{1};*asp*^{1}	3 (82)	n.d.	25.7	0.8
Taxol-treated				
WT	5 (897)	n.d.	5.8	3.0
mitch^{1}	5 (183)	n.d.	44.3	1.6

Ane (%), proportion of anaphases in percent; PSCS (%), precocious sister chromatid separation in percent; MI, mitotic index; OC (%), frequency of cells containing overcondensed cromosomes in percent; Met:Ana, prometaphase/metaphase to anaphase/telophase ratio; n.d., not determined.

recordings of mutant neuroblasts, at least two chromosomes were mono-oriented for periods averaging about 1 hour (range, 25 minutes to >2 hours). The contrast with wildtype, in which full congression and stabilization of chromosomes at the metaphase plate is achieved in less than 4 minutes, is striking.

Mutations of *mitch* not only cause defects in mitosis, but also in the two meiotic spermatocyte divisions. Bivalent congression during meiosis I and II was clearly disrupted in *mitch* mutant spermatocytes (Fig. 4B-C,I; compare with wild-type in Fig. 4A,H). Chromosome segregation during anaphase and telophase of both meiotic divisions (wild type shown in Fig. 4D,E,J) was also defective. Approximately 85% of ana/telophase I figures exhibited an unequal distribution of chromosomes, and in one-third of these cases, one daughter cell received the entire chromosome complement while the other received no chromosomes (Fig. 4F,G). A large percentage of ana/telophases in meiosis II were also unequal (Fig. 4K) and, as expected from the asymmetrical first division, many of the resulting cells did not contain any chromosomes at all (Fig. 4L). Our findings are consistent with previous reports in other *Drosophila* mutant strains that meiosis can occur in the absence of chromosomes (Bucciarelli et al., 2003).

Depletion of Mitch in *Drosophila* S2 tissue culture cells using RNA interference (RNAi) resulted in mitotic phenotypes similar to those seen in *mitch*-mutant larval brains. At metaphase, cells treated with *mitch* double-stranded (dsRNA) exhibited defects in chromosome congression, including apparently mono-oriented chromosomes near the spindle poles (supplementary material Fig. S1). Depletion of Mitch by *mitch* dsRNA was verified by antibody staining (data not shown).

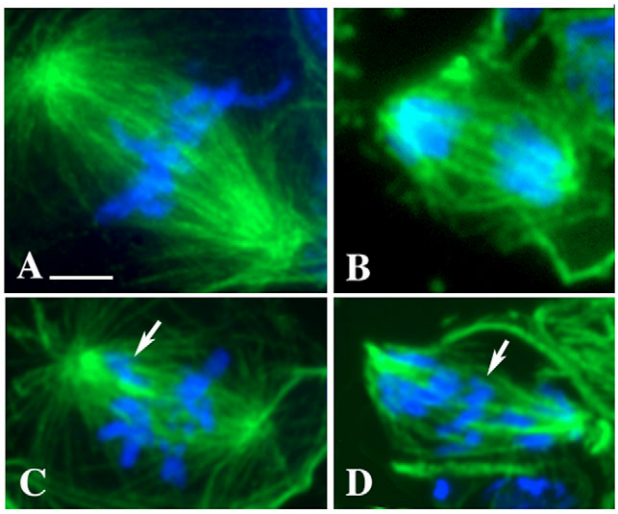

Fig. 2. Chromosome misalignment in *mitch* mutants. (A-D) Brain neuroblasts from wild-type (A,B) and *mitch*^{1} (C,D) were stained for chromosomes (blue) and microtubules (green). At metaphase (A,C), chromosomes are correctly aligned at the metaphase plate in wild-type (A), whereas the chromosomes are mis-oriented in *mitch* mutants (C) with some chromosomes abnormally situated near the pole (arrow). At anaphase (B,D), the normal even distribution of chromatids (B) is disrupted in *mitch* mutants (D) so as to produce lagging chromosomes (arrow); moderate chromosome overcondensation is also apparent in this mutant anaphase. In all *mitch* mutant mitotic figures, the spindles are morphologically normal. Bar, 5 μm.

Furthermore, in *mitch* RNAi cells incubated with colchicine, the spindle checkpoint was defective, with precocious sister chromatid separation (PSCS) occurring in 39% of cells (n=739), in contrast to 0.7% PSCS in control cells receiving no dsRNA (n=520) (supplementary material Fig. S2). Cells incubated with dsRNA from genes with no role in mitosis do not cause these effects (Yu et al., 2004).

Untreated *mitch* mutant neuroblasts exhibit a functional SAC

Wild-type larval brain cells treated with spindle poisons exhibit a mitotic arrest (c-metaphase) in which sister chromatids remain attached at the centromeres, whereas cyclin B levels remain elevated for extended periods (Gonzalez et al., 1991). The disruption of MTs creates unattached kinetochores, which generate spindle assembly checkpoint (SAC) signals that delay anaphase onset (Basu et al., 1999). SAC components such as Bub3 and Mad2 strongly localize to kinetochores when checkpoint signaling is ‘on’ in wild-type cells with disrupted spindles (Basu et al., 1998; Logarinho et al., 2004; Williams et al., 2003).

The persistence of mono-oriented chromosomes in *mitch* mutant neuroblasts suggested the presence of unattached kinetochores that maintain SAC signaling and consequently cause mitotic delays or arrest. We found that the SAC is indeed operational in *mitch* cells. First, as previously mentioned, the majority of chromosomes in *mitch* cells become overcondensed (Fig. 1F and Fig. 3, Table 1), as occurs when wild-type cells are delayed in metaphase for extended periods by MT poisons (Gatti and Goldberg, 1991; Gonzalez et al., 1991). Second, the ratio of cells in prometaphase/metaphase to those in anaphase/telophase was generally twofold higher in *mitch* mutant versus wild-type brains (Table 1). Unexpectedly, in *mitch* mutants the mitotic index was not elevated relative to wild-type cells, and was in fact reduced in the strongest allelic combinations (Table 1). We attribute this low mitotic index to the death of mutant cells that were delayed in division for long periods. In this respect, Acridine Orange staining revealed a substantial increase in the number of apoptotic cells in *mitch* mutant brains (data not shown). The most direct evidence that *mitch* mutants contain a functional SAC comes from time-lapse observations of living cells (Fig. 3A-C; Movies 1-3). Of the *mitch* cells recorded that eventually entered anaphase, the average time between nuclear envelope breakdown and anaphase onset was 39±22 minutes compared with 11±3 minutes in wild type; these numbers underestimate the severity of the defect because several of the *mitch* mutant cells observed by us never entered anaphase after 1-2 hours of continuous recording.

SAC does not function in *mitch* mutant neuroblasts with disrupted spindles

Given the evidence above that the SAC is operational in untreated *mitch* neuroblasts, we were surprised to find that spindle poisons fail to activate SAC signaling in *mitch* mutant cells (as they do in wild-type cells). Instead of arresting in mitosis, colchicine-treated *mitch* cells prematurely enter anaphase. For example, in contrast to wild-type cells in which colchicine leads to an increased mitotic index (MI) from 1.04 to 2.8 (see Table 1), colchicine does not elevate the MI in *mitch* mutant cells (0.91 in the absence of colchicine and 1.1 in its

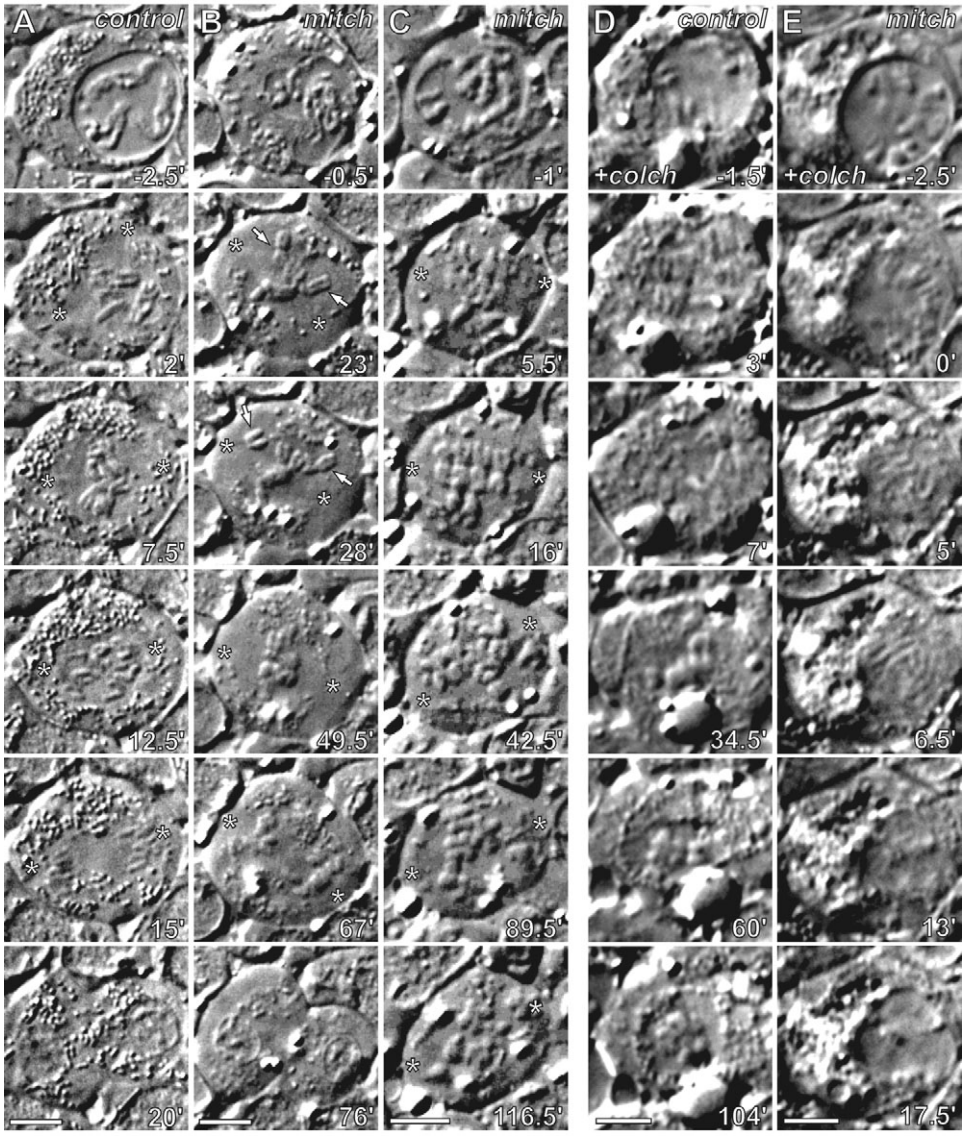

Fig. 3. Mitotic progression in living control and *mitch* neuroblasts. (A) Time-lapse series of an untreated wild-type neuroblast. At the start of the recording, the chromosomes are beginning to condense during prophase. Nuclear envelope breakdown (NEB; time 0) occurs 2.5 minutes later, and the chromosomes rapidly align at the metaphase plate within the next 7 minutes. The cell entered anaphase 12 minutes after NEB, which was followed by chromosome decondensation and reformation of the nuclear envelope during telophase and organization of the cleavage furrow during cytokinesis. (B) Time-lapse series of an untreated *mitch* neuroblast in which two chromosomes (arrows) remained mono-oriented for extended periods. Anaphase was much delayed, beginning only 1 hours after NEB; telophase and cytokinesis soon followed. (C) Time-lapse series of another untreated *mitch* neuroblast in which the chromosomes were distributed along the longitudinal axis of the spindle and remained misaligned in prometaphase for more than 2 hours, without entering anaphase. Note the overcondensation of the chromosomes due to prolonged mitotic arrest. Asterisks indicate the position of the centrosomes inferred by DIC as the focus center of a clear zone; these asterisks thus define the virtual longitudinal axis of the spindle. (D) Time-lapse series of a wild-type neuroblast entering mitosis in the presence of 50 μM colchicine. This cell remained in c-mitosis for more than 2 hour (when the recording was stopped) and consequently the chromosomes look overcondensed. (E) Time-lapse series of a *mitch* neuroblast entering and exiting mitosis in only 13 minutes in the presence of 50 μM colchicine. The chromosomes appear to disjoin at 6.5 minutes, but no anaphase movement was subsequently observed. The chromosomes decondensed and the nuclear envelope completely reformed around a single nucleus by 17 minutes. See movies in the supplementary material for clearer visualization. Bar, 5 μm.

presence; Table 1). Additionally, in the presence of colchicine, *mitch* mutant cells display a high incidence of PSCS, which is a hallmark for exit from mitosis (Fig. 1D,E and Table 1). Neither wild-type (Fig. 5A; Table 1) nor *mitch* heterozygote brains (data not shown) show significant levels of PSCS. Colchicine-treated *mitch* cells with PSCS also show other signs of mitotic exit such as low levels of cyclin B (see Fig. 5B and compare with Fig. 5A in which the chromosomes are still attached) and low levels of Bub3 and Mad2 staining at kinetochores (Fig. 5C-E). Most convincingly, we found that in live cell recordings that colchicine-treated *mitch* mutant neuroblasts exited mitosis after only 18.1±4.5 minutes (Fig. 3E; supplementary material Movie 5), which is roughly the same amount of time that untreated wild-type cells spend in prometaphase/metaphase. By contrast, three of four control live wild-type cells remained blocked in mitosis for more than 3 hours after colchicine exposure, although one cell adapted to the SAC and exited mitosis after 1 hour (Fig. 3D; supplementary material Movie 4). All of these findings are consistent with the idea that colchicine-treated *mitch* cells prematurely enter anaphase because they inappropriately adapt to the SAC, either because the SAC is nonfunctional or because Mitch is specifically required for SAC function in the absence of spindle MTs.

It has been suggested that various components of the SAC detect different aspects of kinetochore/spindle interactions, with some components monitoring MT occupancy at kinetochores and others sensing tension across attached kinetochores (Logarinho et al., 2004; Waters et al., 1998). One explanation for the presence of a functional SAC in untreated,

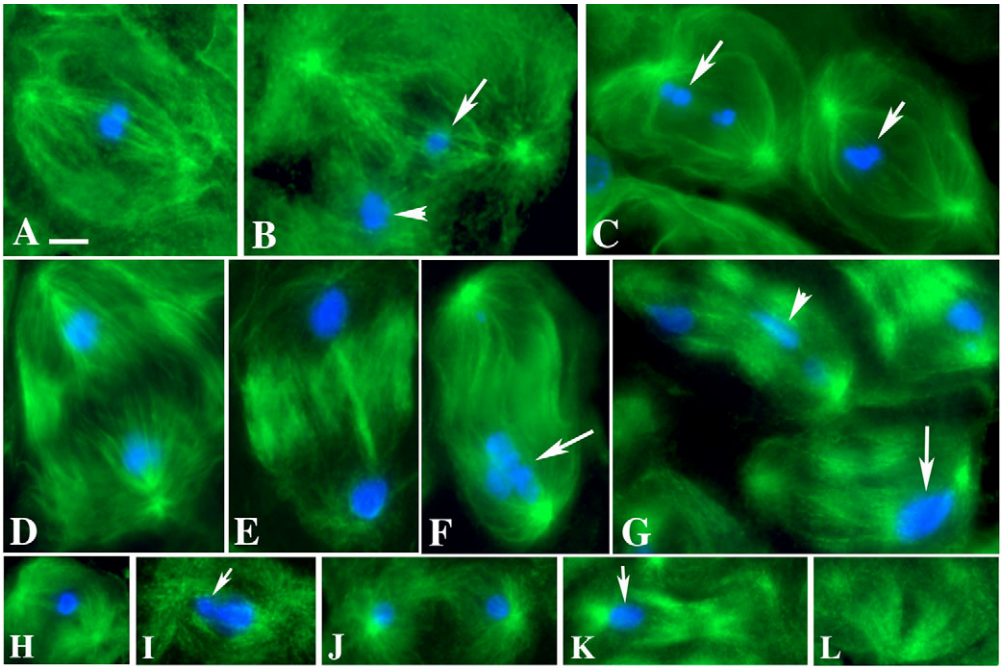

Fig. 4. *mitch* mutations disrupt chromosome segregation in both male meiotic divisions. (A-L) Testes from wild-type (A,D,E,H,J), *mitch*^{1} (B,F,L), and *mitch*^{1}/*mitch*^{2} (C,G,I,K) were stained to visualize chromosomes (blue) and microtubules (green). At metaphase I (A,B,C), bivalents in the *mitch* mutants are positioned away from the center of the cell, either off the main axis of the spindle (arrowhead) or, more frequently, on the spindle axis but closer to one pole than the other (arrows). At anaphase/telophase I (D-G), *mitch* bivalents often migrate only to one pole (arrows in F,G), though sometimes lagging chromosomes are observed along with unequal segregation (arrowhead, G). These phenotypes are reiterated in the second meiotic division (H-L): chromosome mis-alignment during metaphase (I; see arrow) followed by complete nondisjunction (K; arrow). Telophase II figures lacking chromosomes are also observed (L). Bar, 5 μm.

but not in colchicine-treated, *mitch* mutant cells is that *mitch* mutations specifically interfere with the ability of the checkpoint to detect the absence of MT attachments. To test this, we incubated wild-type and *mitch*-mutant brains with taxol, which stabilizes MTs (Williams and Goldberg, 1994). With this treatment, kinetochore-MT interactions are maintained, but the centromeres are not under tension (Waters et al., 1998). We observed high levels of PSCS in taxol-treated mutants, and also noted that the mitotic index was similar to that in colchicine-treated *mitch* mutants, yet significantly lower than that in drug-treated wild-type brains (Table 1). These data imply that the SAC in *mitch* mutants is either non-functional or prematurely overridden not only when MTs are absent, but also when MTs are present but their dynamics compromised.

Finally, to test whether the SAC bypass in *mitch* mutants is specific for MT poisons or instead a more general response to spindle perturbation, we examined larvae carrying mutations in both *mitch* and *abnormal spindle* (*asp*). In the absence of a functional *asp* gene, cells exhibit spindle abnormalities that normally delay anaphase onset and mitotic exit, even though many of the kinetochores become attached to the spindle (Gonzalez et al., 1998). We found, however, that in untreated

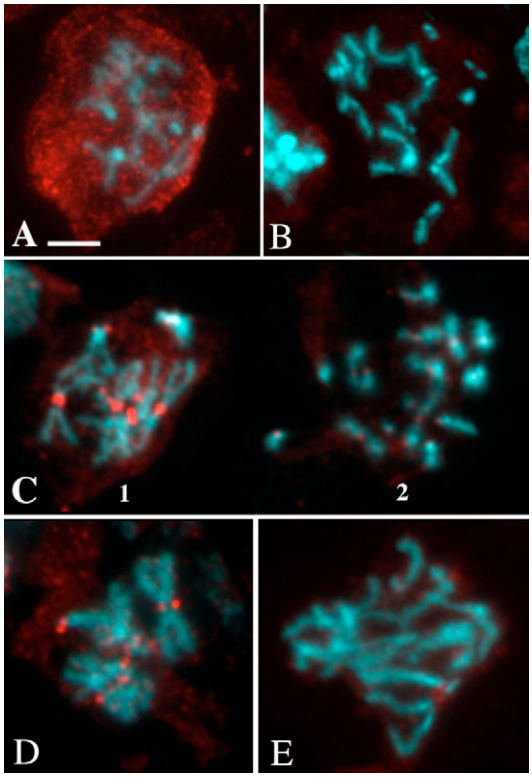

Fig. 5. The spindle checkpoint is compromised in *mitch* mutant mitotic cells treated with microtubule poisons. Brains from *mitch*^{1} were treated with colchicine and hypotonic solution and examined for cyclin B (A,B; red), Bub3 (C; red), or Mad2 (D,E; red) and chromosomes (blue.) High levels of cyclin B are present in cells containing attached sister chromatids (A) whereas they are drastically lowered in PSCS cells (B). Two cells next to each other in the same field (C) show a difference in the distribution of Bub3 with respect to sister chromatid separation (PSCS). In cell 1, high levels of Bub3 are present at the kinetochores of the attached sister chromatids, but very low levels of Bub3 exist at kinetochores of PSCS cells (cell 2). Mad2 is present at much higher levels at the kinetochores of attached chromosomes (D) than at the kinetochores in PSCS cells (E). Bar, 5 μm.

mitch^{1} *asp*^{1} double mutant brains the anaphase frequency was appreciably increased with respect to that in *asp*^{1} brains (Table 1). Moreover, a significant level of PSCS was apparent in *mitch*^{1} *asp*^{1} double-mutant brains, but not those of *asp*^{1} single mutants (Table 1). These mitotic phenotypes resemble those previously reported for *zw10 asp* and *rod asp* double mutants (Basto et al., 2000); mutations in *zw10*, *rod* and *zwilch* also bypass the spindle checkpoint in the presence of MT poisons (Basto et al., 2000; Williams et al., 2003). However, unlike *mitch*, these latter genes are also required for a functional SAC in untreated neuroblasts.

The *mitch* gene corresponds to CG7242

By deletion mapping, we delimited the *mitch*^{1} mutation to the region of chromosome 3R between the centromere-proximal breakpoints of *Df(3R)ry1608* (87D4-6) and *Df(3R)ry614* (87D2-4) (supplementary material Fig. S3) (Leonhardt et al., 1993). We determined the position of these breakpoints at the DNA level by quantitative autoradiography and single-embryo PCR (see Materials and Methods), delimiting *mitch* to a 30-kb region that contains five predicted genes and three previously characterized lethal complementation groups (supplementary material Fig. S3). A mutation in one of these complementation groups [*l(3)87Da*^{2}; hereafter *mitch*^{2}] was found to be allelic to *mitch*^{1}. The brains of third instar *mitch*^{1}/*mitch*^{2} larvae exhibited the same mitotic defects previously described (Table 1), and most of these animals subsequently died during pupal stages. A few *mitch*^{1}/*mitch*^{2} escapers survived to adulthood but were sterile; the testes of heteroallelic males displayed meiotic defects similar to those shown in Fig. 4. Consistent with previous observations of other *l(3)87Da* alleles that are no longer extant (Hilliker and Chovnick, 1981; Hilliker et al., 1980), the large majority of heteroallelic escapers were female (>80%), for reasons that remain unknown.

Sequencing of the exons in the five candidate genes revealed that *mitch*^{2} has a nonsense mutation at the beginning of the third exon of the gene CG7242 that truncates the original 222-amino-acid-long gene product to 129 residues. To date, we have been unsuccessful in identifying the molecular lesion associated with *mitch*^{1}, but we suspect involvement of a mutation affecting CG7242 gene expression because the protein product is no longer detectable by immunofluorescence (see Fig. 6).

To verify the identification of CG7242 as *mitch* and to obtain additional mutant alleles, we generated deletions from imprecise excisions of a P element that inserted into the short intergenic region between CG7242 and its neighbor *granny smith* (*grsm*, CG7340; see Materials and Methods and supplementary material Fig. S1). Although this P element is inserted only 63 nucleotides upstream of the presumptive CG7242 transcription initiation site, it is without obvious phenotypic effect in homozygotes or in hemizygotes bearing the P element and the deficiency *Df(3R)ry75*. Several of the imprecise excisants are lethal in homozygotes and, moreover, failed to complement *mitch*^{1}and *mitch*^{2}; they did, however, complement alleles of the two other essential genes in the

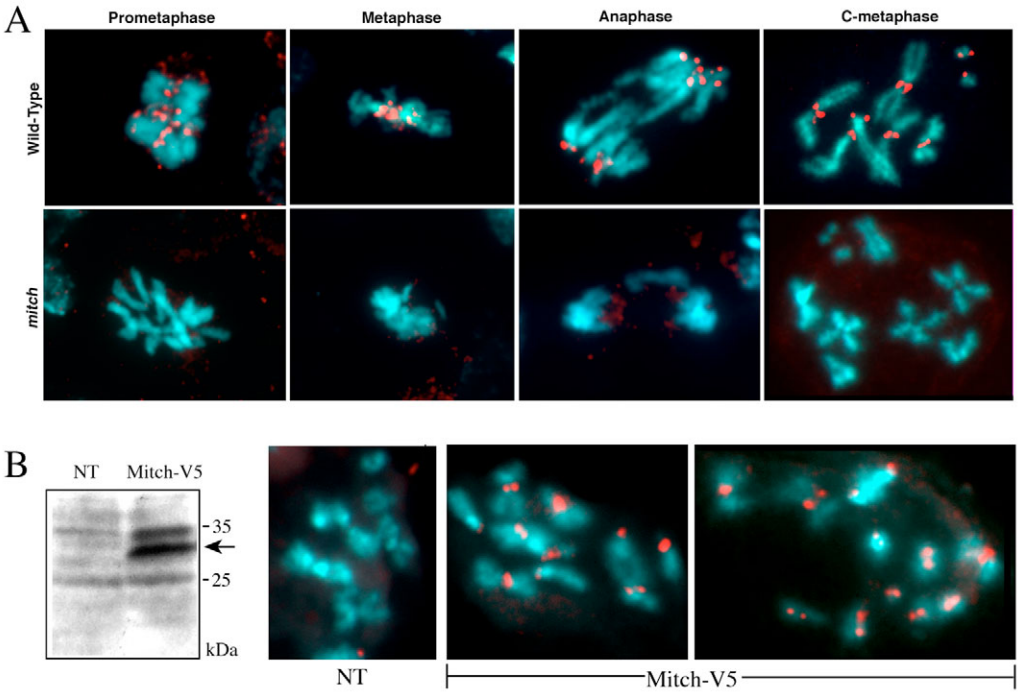

Fig. 6. Mitch localizes to the kinetochore during mitosis. (A) Wild-type (top row) and *mitch*-mutant (bottom row) brains were stained with anti-Mitch antibodies (red). In wild-type, Mitch localizes to kinetochores from prometaphase through late anaphase and likewise in chromosome spreads of cells arrested in metaphase by colchicine (c-metaphase). The kinetochore staining of anti-Mitch antibodies is absent in mitotic figures from *mitch* mutants. (L-R on the bottom row: *mitch*^{1}, *mitch*^{2}/*Df*, *mitch*^{2}/*Df*, *mitch*^{1}). (B) Epitope-tagged Mitch. Western blot (left panel) of *Drosophila* tissue culture cells non-transfected (NT) or transfected with *mitch-V5*, and probed with anti-V5 antibody, identifying the 30 kd Mitch-V5 protein. (Right panels) Indirect immunofluorescence of cells incubated with anti-V5 antibody. Mitch-V5 was absent in non-transfected cells (NT), but localized to the kinetochore in transfected cells (here seen in c-metaphase). Bar, 5 μm.

region. Two of these excisions, *mitch*^{19B} and *mitch*^{22}, remove the majority of CG7242 coding sequences, yet do not extend into any other nearby gene (supplementary material Fig. S1). The *mitch*^{19B} and *mitch*^{22} deletions cause mitotic phenotypes similar to those associated with *mitch*^{1} and *mitch*^{2} (Table 1). Other excisants that remove the entirety of the neighboring CG7340 gene are viable as homozygotes and exhibit normal mitosis (C.K., unpublished). Considered together, these data equate *mitch* with CG7242 and *l(3)87Da.* The COILS2 program (Lupas et al., 1991) predicts with high probability that Mitch contains at least one coiled-coil domain (amino acids 45-99) corresponding to the second exon of CG7242. Searches of databases with Mitch sequences did not uncover any significant matches with other proteins.

Mitch associates with kinetochores during cell division

To determine the intracellular location of the Mitch protein, we prepared antibodies against both native and denatured forms of Mitch (see Materials and Methods). Affinity-purified antibodies against both forms were used to localize Mitch in larval brain neuroblasts by immunofluorescence. In wild-type colchicine-treated cells, all four of these antibodies specifically stain the kinetochores (Fig. 6A). Mitch is located between CID (the centromeric histone H3 variant) in the inner kinetochore/centromeric region, and Zwilch, which is part of the Zw10-Rod complex in the outer kinetochore (Fig. 7) (Blower and Karpen, 2001; Williams et al., 2003). Mitch staining corresponds very closely to that of Bub3 (Fig. 7) (Basu et al., 1998). In all *mitch* mutant strains examined, the kinetochores in larval neuroblasts failed to stain with any of the anti-Mitch antibodies (Fig. 6A), so the kinetochore staining in wild-type cells indeed reflects the intracellular location of Mitch. The assignment of Mitch to the kinetochore was verified by the targeting of epitope-tagged Mitch protein to kinetochores in *Drosophila* tissue culture cells (Fig. 6B; see also Fig. 8A).

The association of Mitch with kinetochores is cell-cycle dependent because Mitch immunofluorescence signals are only detected in dividing cells from prometaphase to late anaphase. This implies that Mitch is a transient kinetochore protein recruited to the centromere/kinetochore only during cell division. Interestingly, unlike most SAC proteins which leave the kinetochore at anaphase (Basu et al., 1999; Basu et al., 1998), Mitch remains at the kinetochores throughout the metaphase-to-anaphase transition. The localization of Mitch in meiosis is similar to that seen in mitosis. Mitch localizes to the kinetochores from prometaphase I through anaphase I, then from prometaphase II through anaphase II (data not shown).

The kinetochore targeting function of Mitch is independent of other known kinetochore components

We next asked whether the association of Mitch with kinetochores was affected in animals mutant for genes encoding either other known kinetochore proteins, including *zw10*, *rod*, *zwilch*, *bubR1*, *bub3*, *polo* and *cenp-meta* (Basu et al., 1999; Basu et al., 1998; Llamazares et al., 1991; Williams et al., 2003; Yucel et al., 2000), or certain spindle components, such as *asp* and *mast/orbit* (Gonzalez et al., 1998; Maiato et al., 2002). Mitch was correctly targeted to kinetochores in all of the mutants we examined (data not shown). Mitch localization is dependent, however, upon the presence of CID (the *Drosophila* orthologue of the centromere-specific H3-like protein CENP-A) and the DNA-binding centromere protein CENP-C. In the tissue culture cells treated with dsRNA

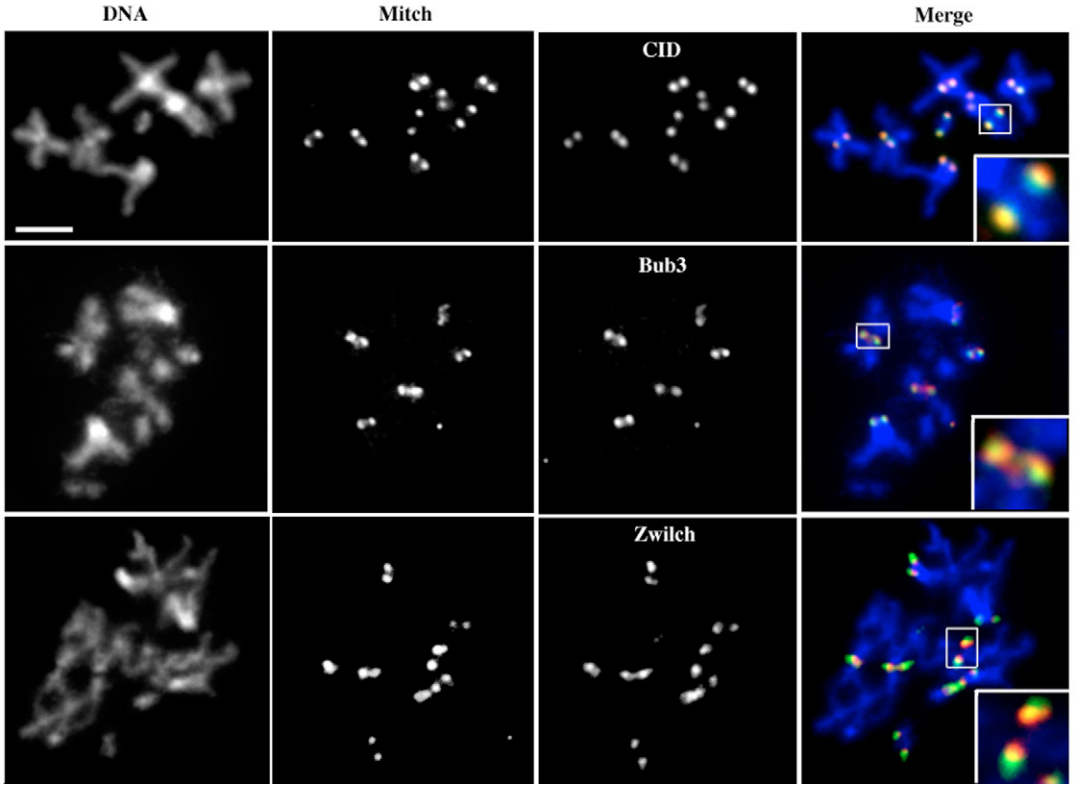

Fig. 7. Mitch is located between CID and Zwilch at the kinetochore. Wild-type brains treated with colchicine and hypotonic solutions were examined simultaneously for DNA, Mitch, and another kinetochore antigen (CID, Bub3, or Zwilch). The colocalization of Mitch (red) with these other kinetochore proteins (green) was observed in the merged images (far right column; also see magnified insets). Mitch appears to be distal to CID localization, but proximal to Zwilch. Mitch approximately colocalizes with the Bub3 protein (center row). Bar, 5 μm.

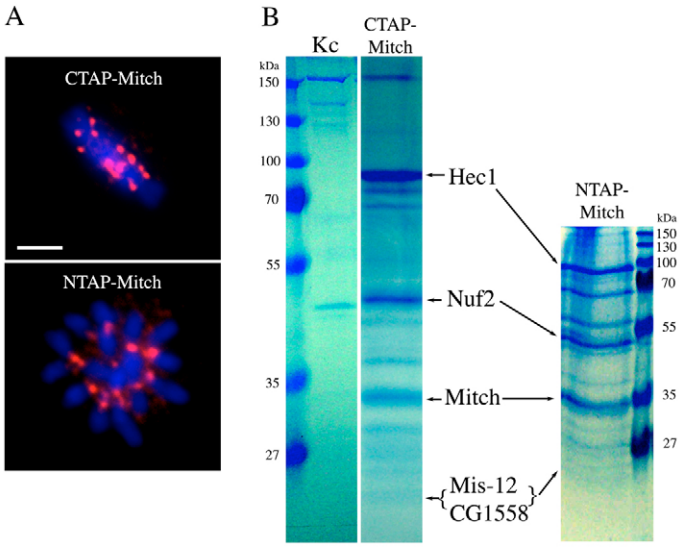

Fig. 8. Mitch is a component of the Ndc80 complex. (A) Localization of TAP-tagged Mitch protein expressed in *Drosophila* Kc tissue culture cells. Mitch fused with protein-A–CBP either at the N-terminus (NTAP) or at the C-terminus (CTAP) localizes to the kinetochores of the chromosomes (blue). Bar, 10 μm. (B) Mitch co-purifies with Hec1, Nuf2, Mis-12 and CG1558 (arrows). SDS-PAGE gels were stained with Coomassie Blue and protein bands were identified by mass spectrometry. Other bands on the gel represent proteins that are either highly abundant (tubulin, actin), also present in the control (Kc cells without the tagged construct), or breakdown products of the proteins present in the complex.

corresponding to CID or CENP-C, in which the centromere localization of these proteins was eliminated, Mitch failed to reach the kinetochores (supplementary material Fig. S4).

Similarly, during mitosis in *mitch*^{1} and *mitch*^{19B} mutants (whether in untreated, colchicine- or taxol-treated brains) we did not detect any deviations from wild-type in the localization of other centromere/kinetochore proteins, including BubR1 (Basu et al., 1999), Bub3 (Basu et al., 1998), Mad2 (Logarinho et al., 2004), 3F3/2 (Ahonen et al., 2005; Logarinho et al., 2004), Cenp-meta (Maiato et al., 2006; Williams et al., 2003; Yucel et al., 2000), Zwilch (Williams et al., 2003), CID (Blower and Karpen, 2001; Henikoff et al., 2001), dynein heavy chain [DHC (Li et al., 1994; Starr et al., 1998)], Klp10A (Rogers et al., 2004), Polo (Logarinho and Sunkel, 1998) and CENP-C (Heeger et al., 2005) (supplementary material Fig. S5). Thus, the absence of Mitch does not radically alter overall kinetochore structure. It is of further interest that some kinetochore components, such as Bub3 and DHC, localize to mono-oriented chromosomes near the poles in *mitch* mutants more strongly than to chromosomes that have congressed to the spindle equator (supplementary material Fig. S5B,F indicated by arrows). Because the levels of these proteins normally decrease dramatically upon bipolar attachment (Basu et al., 1999; Basu et al., 1998; Starr et al., 1998), this finding solidifies the previous conclusion that the non-congressed chromosomes are mono-oriented and are engaged in SAC signaling.

Mitch is rapidly evolving

One would expect a protein that is crucial for chromatid segregation to be well-conserved during metazoan evolution. However, BLAST and PSI-BLAST searches using the amino acid sequence of Mitch have not found significant homologies outside of the genus *Drosophila*. Comparison of the *mitch* sequences of *D. melanogaster* and its close relative *D. simulans* (see Materials and Methods) confirms the surprisingly rapid evolution of this essential gene. Nonsynonymous nucleotide divergence (K_a) at the *mitch* locus between these two species is 3.8%, whereas the average K_a was found to be ~1% in a survey of 45 random loci (V. DuMont and C.F.A., unpublished data).

One possible explanation for these observations is that positive selection drives the divergence of Mitch. Under this hypothesis, new amino acid variants at some positions in Mitch are favored by natural selection and, thus, tend to fix in a population more rapidly than do neutral or deleterious variants. The centromere-specific histone CID provides one well-known example of such adaptive evolution (Malik and Henikoff, 2001). In order to test this idea, we obtained *mitch* sequences from thirteen *Drosophila* species (supplementary material Fig. S6), and applied maximum-likelihood tests for positive selection of the same amino acids in different lineages as described in the Materials and Methods (see also Nielsen and Yang, 1998; Swanson et al., 2003). We were unable to reject the null-hypothesis that recurrent positive selection drives the evolution of a subset of codons in *mitch*.

We also investigated whether the rapid divergence at the *mitch* locus might partly be due to selection on different codons in different lineages, by asking whether there has been an excess of amino acid substitutions between *D. melanogaster* and *D. simulans*. We sequenced *mitch* from ten isofemale lines obtained from an African (Zimbabwe) population of *D. melanogaster*, and applied the McDonald-Kreitman test to these data using *D. simulans* as an outgroup (Begun and Aquadro, 1994; McDonald and Kreitman, 1991). This allowed us to compare the ratios of nonsynonymous to synonymous substitutions between species to the same ratio within *D. melanogaster*. We again found no evidence that selection has driven any of the amino acid sequence diversity in Mitch proteins.

Mitch is a component of the Ndc80 complex

To identify proteins that interact with Mitch, we tagged Mitch with both protein A and calmodulin-binding protein (CBP) and expressed this construct in stable transfectants of *Drosophila* Kc cells. The presence of the tagged protein at the kinetochore was verified with antibodies against the protein A tag (Fig. 8A). Through tandem affinity purification (TAP) techniques (Puig et al., 2001; Veraksa et al., 2005), followed by mass spectrometry of associated proteins, we found that Mitch primarily co-purified with subunits of the Ndc80 complex, in particular the *Drosophila* Hec1 (Ndc80) and Nuf2 homologues (Bharadwaj et al., 2004; McCleland et al., 2004; Meraldi et al., 2006) (Fig. 8B). In addition, purification of Mitch also pulled down minor amounts of the *Drosophila* Mis-12 homologue (Meraldi et al., 2006) and CG1558, the latter of which does not exhibit homology to known proteins. The identification of Mitch as a component of the Ndc80 complex is consistent with two-hybrid screen data that revealed associations between Mitch, Hec1 and Nuf2 (Giot et al., 2003), as well as with recent results from two independent affinity-purification studies of the same complex (Przewloka et al., 2007; Schittenhelm et al., 2007).

Discussion

Role of Mitch in chromosome segregation

We describe here the discovery of a new kinetochore protein, Mitch. In the absence of Mitch, chromosomes fail to become correctly aligned during spindle assembly, and persistently mono-oriented chromosomes are often seen in the vicinity of the spindle poles. The aneuploidy-producing aberrations seen in mutants during anaphase and telophase are thus likely to be secondary consequences of earlier problems in kinetochore attachment and chromosome alignment.

The persistently mono-oriented chromosomes in *mitch* mutants could be of two classes. First, the sister kinetochores could be attached to MTs emanating from the same spindle pole. Normally these 'syntelic' connections are corrected by machinery mediated by Aurora kinase that increases the turnover of kinetochore-MT attachments in the absence of tension across the centromere (reviewed in Cimini and Degrassi, 2005). Alternatively, the pole-associated mono-oriented chromosomes in *mitch* mutants could be 'monotelic', with only one sister kinetochore connected to MTs and the other kinetochore being unattached. Our data do not allow us to distinguish between these two possibilities. It is, however, important to note that these two classes of mal-attachments are not mutually exclusive: the correction of syntely requires the detachment of one kinetochore from the spindle, creating a transient monotelic chromosome.

It is also unclear whether the absence of Mitch produces mono-oriented chromosomes directly or whether its absence prevents the correction of mal-attachments normally produced during spindle assembly. A precedent for the former possibility is offered by studies of a protein complex at *S. cerevisiae* kinetochores that includes Mtw1p, Dsn1p, Nnf1p and Nsl1p (Pinsky et al., 2003). Although the precise mechanism by which this complex operates is not known, it is clear that Aurora kinase produces unattached kinetochores in the absence of Mtw1p. The absence of Mitch might also directly promote mono-orientation simply by impairing MT binding by the kinetochore, particularly because *Drosophila* kinetochores attach on average to only five MTs in male meiosis (Lin et al., 1981) and to an average of only 11 MTs in S2 cells (Maiato et al., 2006). Such a reduction in MT-binding efficiency is thought to lead to the persistence of mono-oriented chromosomes in mammalian tissue culture cells injected with antibody against CENP-E (McEwen et al., 2001). Alternatively, Mitch might be involved in correcting the mono-orientations that occur early in mitosis through a mechanism whereby kinetochores that are originally unattached to kMTs can eventually nucleate and/or organize their associated kinetochore fibers (Maiato et al., 2004).

Role of Mitch in the spindle-assembly checkpoint

The relationship between Mitch and the SAC appears paradoxical, and suggests that the SAC is more complicated than previously envisioned. Mono-oriented chromosomes with unstable kinetochore attachments would be expected to maintain checkpoint activity – and this is what we see in both fixed and living non-drug-treated *mitch* mutant cells (Fig. 2 and Fig. 3A-C; Table 1). Surprisingly, however, the SAC is not functional in *mitch* neuroblasts exposed to colchicine or taxol, or when correct bipolar spindle assembly is compromised by lack of the MT-associated protein Asp. Under all of these conditions, *mitch* mutants prematurely disjoin their chromatids and exit mitosis (Fig. 1D,E; Table 1). That these cells enter anaphase prematurely is further indicated from the observations that cyclin B levels are low (Fig. 5A,B), and that Mad2 and Bub3 are much reduced or absent from their kinetochores (Fig. 5C-E). Indeed, direct live cell observations revealed that colchicine-treated *mitch* neuroblasts transit through mitosis with the same kinetics as untreated wild-type cells (Fig. 3D,E). A rapid escape from mitosis in the presence of spindle poisons is characteristic of *Drosophila* cells carrying mutations in genes encoding SAC components (Basu et al., 1999; Logarinho et al., 2004; Williams et al., 2003).

We conclude that Mitch is not directly required for SAC function, but it is required for maintaining a checkpoint signal if the spindle is perturbed. The inference that the SAC is in place but does not correctly signal under such conditions is consistent with our finding that checkpoint components like Bub3, BubR1 and Mad2 are associated with kinetochores in drug-treated *mitch* mutant cells before – but not after – sister chromatid separation (Fig. 5). One way to view this signaling is that Mitch plays a role in slowing the adaptation of cells to the checkpoint. Normal drug-treated cells eventually exit mitosis into the next G1; our data indicate that such adaptation to the SAC is accelerated when Mitch is absent.

The SAC monitors the accumulation of microtubules at the kinetochore and/or the resultant tension created across the centromere upon the attachment of sister kinetochores; recent results in *Drosophila* as well as in mammalian systems suggest that some checkpoint components sense MT occupancy whereas others track tension (Logarinho et al., 2004; Morrow et al., 2005). We found that the SAC fails to function in mutant brains treated with either colchicine or taxol. Since colchicine-treated cells are devoid of spindle MTs, kinetochores are by definition unattached and are not subject to tension. Kinetochores in taxol-treated cells are attached to the spindle but are not under tension (Waters et al., 1998). The most obvious common feature of colchicine and taxol treatments is the failure of microtubule subunits to become incorporated into, and removed from, kinetochore MTs. We thus posit that Mitch is required in the SAC only when the dynamic behavior of spindle MTs is compromised. Drug-induced disruptions in kinetochore MT dynamics are either not detected in *mitch* mutant cells, or the disruptions are detected but downstream events in the SAC signaling pathway are disrupted; in either case, *mitch* mutant cells would exit mitosis prematurely. Under this model, Mitch would not be required for SAC function in untreated cells because attached kinetochores have dynamic MT connections.

Mitch is not the only kinetochore-associated SAC component to display this paradoxical behavior. Other investigators previously found that in untreated larval brains of animals homozygous for mutations in *cenp-meta* (which encodes one of two *Drosophila* proteins related to the mammalian kinesin-like protein CENP-E), mitotic progression is delayed in a prometaphase-like state (i.e. the cells contain an active SAC) (Yucel et al., 2000). Importantly, however, we have shown that the SAC is not functional in *cenp-meta* mutants treated with colchicine (Williams et al., 2003). These similarities between Mitch and Cenp-meta suggest that the effects of *mitch* mutants might be mediated through Cenp-meta. In fact, since CENP-E has recently been implicated in a

novel mechanism that allows the congression of mono-oriented chromosomes (Kapoor et al., 2006), the persistent mono-orientation of chromosomes in *mitch* mutants could in theory also result from a failure of Cenp-meta to bind to, or function correctly at, the kinetochores. The former possibility appears to be excluded by our finding that Cenp-meta is targeted to kinetochores in *mitch* mutants (supplementary material Fig. S5G), but it is not inconceivable that almost all aspects of the *mitch* mutant phenotype could still be explained by indirect effects on Cenp-meta function.

Rapid evolution of the *mitch* gene

Our inability to find Mitch homologs outside of the genus *Drosophila* and our observation that nonsynonymous nucleotide divergence is considerably higher than average at the *mitch* locus led us to apply tests for positive selection of this locus within the Drosophilids. We found no evidence for positive selection on a subset of codons in Mitch, as a model of sequence evolution that allows for positive selection does not perform significantly better than models that do not allow for positive selection. Similarly, we found no evidence that selection has accelerated rates of amino acid substitution between the two species *D. melanogaster* and *D. simulans.* Divergence at this locus may therefore be the consequence of relatively weak functional constraints on the amino acid sequence of the Mitch protein.

We have found that Mitch associates strongly with Hec1 and Nuf2 (components the Ndc80 complex) and to a lesser extent with a component of the Mis12-MIND complex (Mis12). Since Hec1 and Nuf2 in other organisms are associated with the proteins Spc24 and Spc25 (Wei et al., 2006), it appears likely that Mitch corresponds functionally with Spc24 or Spc25 even though such a relationship is not obvious at the amino acid sequence level (see also Schittenhelm et al., 2007). The COILS2 program (Lupas et al., 1991) reveals that Mitch in all analyzed *Drosophila* species contains a coiled-coil domain in the N-terminal half, a characteristic also seen in Spc24 and Scp25 in other species. It will thus be of interest in the future to establish whether mutations in other components of the fly Ncd80 complex exhibit the same paradoxical behavior of the SAC we observed in *mitch* mutants.

If Mitch is indeed an ortholog of Spc24 or Spc25, it is surprising that the contradictory behavior of the spindle checkpoint described has not previously been noted. In yeast and vertebrate cells, *spc24* and *spc25* mutations also result in defective chromosome congression and alignment (Bharadwaj et al., 2004; Janke et al., 2001), presumably due to defects in kinetochore-microtubule attachments (McCleland et al., 2004). However, in budding yeast, *spc24* and *spc25* mutants are checkpoint defective, with *spc24* mutants failing to arrest in the presence or absence of nocodazole (Janke et al., 2001). Spc25-depleted human cells retain the spindle checkpoint, even though the localization of MAD1 is affected (Bharadwaj et al., 2004). *Xenopus* Spc24 and Spc25 depletion results in delocalization of Mad1 and Mad2 from the kinetochore, and abrogation of the spindle checkpoint in normally progressing cells as well as those treated with MG132, a proteasome inhibitor which normally causes metaphase arrest (McCleland et al., 2004). We find it remarkable that the relationship between these core kinetochore proteins and the spindle checkpoint is so variable in different lineages, perhaps reflecting the rapid evolution in the amino acid sequences of Spc24 and Spc25.

Materials and Methods

Fly stocks and genetic manipulations

Strains bearing lethal mutations on the third chromosome were balanced over *TM6* or *TM6B* carrying *Tubby* (*Tb*) and either *Stubble* (*Sb*) or *Humeral* (*Hu*). These markers allowed us to distinguish balancer-carrying adults (*Sb* or *Hu*) and larvae (*Tb*) from mutant homozygotes or trans-heterozygotes. The wild-type strain used in these experiments was Oregon-R. Other stocks were obtained from the Bloomington (IN) stock center.

To isolate mitotic mutants, our laboratory screened 1583 strains from the laboratory of Charles Zuker (University of California, San Diego) that carried ethylmethanesulfonate (EMS)-induced mutations on the third chromosome causing lethality during third instar larval or pupal stages (Koundakjian et al., 2004). One of these stocks contained the original *mitch*^{1} allele.

Additional mutant alleles containing small deletions of *mitch* (CG7242) were derived as imprecise excisions of the homozygous viable P element insertion *CK4A*, which is inserted between *mitch* and the adjacent gene *granny smith* (*grsm*; CG7340; supplementary material Fig. S3). The *CK4A* insertion was itself obtained by mobilizing the P-element in *l(3)S125006a* (Deak et al., 1997) using Δ2-3 transposase. The *CK4A* P element was then remobilized with Δ2-3 transposase, and the resulting progeny were tested for failure to complement the deficiency *Df(3R)ry75* (Fig. 6) and *mitch*^{1}.

Characterization of DNA in the *mitch* region

To delimit the genomic region containing *mitch*, we first determined the relevant breakpoints of the deletions *Df(3R)ry1608* and *Df(3R)ry614*. This was accomplished by Southern blot analysis as previously described (Williams et al., 2003), and by using a single embryo PCR technique we have detailed elsewhere (Yu et al., 2004). To identify the nucleotides altered in presumptive *mitch* point mutations, genomic DNA fragments representing the exons of the five candidate genes in the region of interest (supplementary material Fig. S3) were PCR amplified in duplicate and sequenced from homozygous *mitch*^{1} and *mitch*^{2} animals.

The extent of the small deletions of *mitch* obtained as imprecise excisants of *CK4A* (see above; supplementary material Fig. S3) was determined by PCR using primers in CG7242 and CG7340. PCR products spanning deletion breakpoints were identified as new bands not found in amplifications of wild type or *CK4A* genomic DNA. These PCR products were then sequenced to localize the deletion breakpoints at the nucleotide level.

To amplify the *mitch* locus from other *Drosophila* species, we compared the sequences of the corresponding genomic regions in *D. melanogaster* and *D. pseudoobscura* to find short, highly conserved regions both internal to *mitch* and also in the flanking genes CG7340 and CG7242 that could be used as PCR primers. Genomic DNA from *D. simulans*, *D. mauritiana*, *D. teissieri*, *D. sechellia*, *D. erecta*, *D. yakuba*, *D. orena*, *D. eugracilis*, and *D. lutescens* were prepared and amplified using these primers and Taq polymerase (Fermentas, Hanover, MD). Additional, species-specific primers were also required in certain PCR amplifications. The DNA sequences of these primers, as well as PCR reaction parameters, are available upon request.

All primers were ordered from Integrated DNA Technologies (Coralville, IA). Amplified bands were extracted and purified from agarose gels (Qiagen, Valencia, CA), and sequenced by the BioResource Center (Cornell University, Ithaca, NY). Each gene was sequenced on both strands and from multiple, independently performed PCR amplifications. Additional *mitch* coding sequences were compiled using Seqman (DNAStar, Madison, WI) from the genomic sequences of *D. pseudoobscura*, *D. mojavensis* and *D. virilis* available at NCBI.

We used PAML (Nielsen and Yang, 1998) to compare models of sequence evolution. This analysis included all the species in the subgenus *Sophophora* shown in supplementary material Fig. S4, but did not include the species in the subgenus *Drosophila* (that is, *D. virilis* and *D. mojavensis*) because of uncertainties in amino acid alignment between more distant species. In each of two comparisons, one model (M8) allows for, but does not require, positive selection on a subset of codons, as indicated by a $d_N/d_S>1$. The second model, the null model, does not allow the ratio d_N/d_S to exceed one. Two null models, M7 and M8A, were used. In order to infer positive selection, two conditions must be met. First, the sequence data must fit M8 significantly better than the null model. Second, M8 must find evidence for a class of codons with $d_N/d_S>1$.

Generation of anti-Mitch antibodies

We obtained a full-length CG7242 cDNA clone (LD37196; Research Genetics, Athens, GA), and PCR-amplified a fragment containing the entire open reading frame. The PCR primers introduced a recognition site for the restriction enzyme *Xba*I at the cDNA's 5′ end and a *Hin*dIII site at the 3′ end. Restriction enzyme-digested PCR product was then cloned into the vector pMAL-C2 (New England Biolabs, Beverly, MA) digested with the same enzymes. Native and denatured

fusion proteins were purified, and antibodies to these proteins were raised in rabbits and guinea pigs as previously described (Williams et al., 2003).

Although all four preparations of anti-Mitch antibodies work well in immunofluorescence experiments (see below), they are at best mediocre reagents as probes for western blotting using standard methodology. The antibodies recognized the Mitch protein when it is overexpressed in *E. coli* or in Kc Cells, and we occasionally saw a very faint band of the predicted size (26 kDa) in blots of wild-type larvae that disappeared in blots of *mitch* mutants. A band of approximately the same size was observed in extracts of *Drosophila* Kc line tissue culture cells that were transfected with constructs that overexpress Mitch under the control of the actin 5C promoter (data not shown). The weakness and inconsistencies in the western blots obtained with anti-Mitch antibodies as probes could be caused either by low levels of native Mitch protein expression, or by the relative inability of this protein to bind to the PVDF membranes (Millipore Corp., Billerica, MA) we employed.

Tissue culture

To express V5-epitope tagged Mitch protein in *Drosophila* tissue culture cells, *mitch* sequences were amplified from cDNA LD37196 with primers corresponding to the 5′UTR (5′-CGGAATTCTCTGGAAAACGGCGCTTAG-3′) and the end of the coding region (5′-AGATCTAGAGGTGTGGCTCATCGGCGA-3′) using Taq polymerase (Fermentas, Hanover MD). pAC5.1/V5-HisB (Invitrogen, Carlsbad CA) was digested with *Eco*RV and T-tailed using Taq polymerase in the presence of dTTP, followed by ligation (T4 DNA ligase; Fermentas) with the *mitch* fragment. The *pAC-mitch-v5-his* construct was verified by DNA sequencing. Plasmid DNA was prepared by alkaline lysis (Qiagen) and transfected into Kc cells. For each transfection, 3 μg of plasmid DNA was mixed with 10 μl of Cellfectin (Invitrogen) in HyCCQ medium (HyClone, Logan UT) and used to transfect 3 ml of log-phase Kc cells, which were harvested for western blotting and immunofluorescence after 48 hours.

RNAi was performed by treating Kc tissue culture cells with dsRNA corresponding to *CID*,*CENP-C* or *mitch* according to standard procedures (Yu et al., 2004). For CENP-C and *mitch*, cells were incubated with dsRNA for 4 days before fixation, whereas CID depletion occurred effectively only after 7 days of exposure to dsRNA, with additional dsRNA added after 3 days. Cells were processed for immunofluorescence as described below for brains, or for chromosome spreads according to (Yu et al., 2004).

Cytology and immunofluorescence

We studied the *mitch* phenotype of the mutant alleles by staining third-instar larval neuroblasts with orcein and by immunolocalization with the anti-Mitch antibodies, using previously described methods (Williams and Goldberg, 1994). Brains were fixed, squashed and immunostained using the following antibodies: mouse anti-alpha-tubulin (Amersham Corp., Arlington Heights, IL), rabbit anti-Zw10 (Williams et al., 1992), rabbit anti-cyclin B and CENP-C (gifts of Christian Lehner, University of Bayreuth, Germany), chicken anti-CID (Blower and Karpen, 2001), anti-Klp10A (Rogers et al., 2004), rabbit anti-dynein heavy chain (Starr et al., 1998), rabbit anti-SCC1 (Warren et al., 2000), rabbit anti-BubR1 and anti-Bub3 (Logarinho et al., 2004), and anti-Mad2 (Logarinho et al., 2004). Antibodies were raised in rabbits against Cenp-meta (Cocalico Biologicals, Reamstown PA) using protein produced in *E. coli* from *pQE-cmet* (Yucel et al., 2000). *Drosophila* Kc tissue culture cells were fixed by a procedure identical to that used for larval brains (Williams and Goldberg, 1994), and stained with mouse anti-V5 antibody (Invitrogen). Secondary antibodies (all from Jackson Laboratories, West Grove, PA) were TRITC (tetramethylrhodamine isothiocyanate)-conjugated anti-rabbit IgG, TRITC anti-chicken IgY, Cy2 anti-guinea pig IgG, and TRITC anti-mouse IgG, all at 0.1 mg/ml final concentration and incubated overnight at 4°C. DNA was stained with Hoechst 33248 dye at a final concentration of 0.5 μg/ml. Samples were examined by epifluorescence, and images were obtained by using a Pentamax CCD camera (Roper Scientific, Tuscon, AZ) attached to an Olympus BX50 microscope.

Homozygous phenotypes were studied by dissecting Tb^+ larvae for all mutant alleles except for $mitch^2$, for which Tb^+ larvae could not be obtained. We assume the chromosome bearing this allele carries an additional lethal mutation from the original EMS mutagenesis. To study the mutant phenotype of $mitch^2$, we instead dissected the brains of $mitch^2/Df(3R)ry614$ larvae.

Time-lapse DIC microscopy of neuroblasts

For analysis of mitotic progression in live *Drosophila* neuroblasts, brains from third instar larva were dissected and processed as described previously (Fleming and Rieder, 2003; Savoian and Rieder, 2002) with the following modifications. The dissected brain was put on a coverslip with a small drop of neuroblast culture medium supplemented with 20% FBS. A small square piece from an agarose layer was gently placed on top of the brain monolayer. This coverslip was then flipped onto a microscope glass slide containing two coverslip fragments that act as spacers. A gradient of flatness was then created by allowing the medium covered by the agar overlay to slip underneath one of the spacers and leaving the other dry. This enables the experimenter to select a degree of flatness ideal for high-resolution LM analyses that does not inhibit progression through mitosis or cytokinesis. The edges of the coverslip were then sealed with vasoline:lanolin:paraffin (VALAP; 1:1:1) to prevent evaporation. All brains used were still viable at the moment the recordings were stopped, in some cases after more than 6 hours. Three brains from the wild-type strain *Oregon R* were used as controls to record six neuroblasts, and six brains from *mitch* mutants were used to record ten neuroblasts. Images of neuroblasts were acquired at room temperature using a Deltavision Restoration Microscopy System mounted on an Olympus IX70 DIC inverted light microscope. Single image planes were acquired every 30 seconds using a Roper CM350 CCD camera.

Purification of protein complexes containing Mitch

The entire coding sequence of *mitch* was cloned into pMK33-NTAP and pMK33-CTAP (Veraksa et al., 2005) and the resulting constructs were stably transfected into *Drosophila* Kc tissue cells using Cellfectin (Invitrogen). TAP-Mitch was assayed on western blots using HRP-conjugated anti-protein A antibody (Rockland, Gilbertsville PA) and by immunofluorescence of fixed cells using goat anti-protein A antibody followed by TRITC-conjugated anti-goat antibody (Jackson Laboratories). Protein complexes from one liter of TAP-Mitch stable line cells were isolated following (Puig et al., 2001), using a lysis buffer for making *Drosophila* extracts (Veraksa et al., 2005). After purification using IgG-Sepharose and Calmodulin-Sepharose beads (Invitrogen), the final eluate was precipitated with trichloroacetic acid (TCA), resolubilized in Laemmli sample buffer (Bio-Rad, Hercules CA) and subjected to SDS-PAGE. Bands were excised, trypsinized and analyzed by MALDI (Cornell Bioresource Center.)

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