Interacting genes that affect microtubule function: the *nc2* allele of the *haywire* locus fails to complement mutations in the testis-specific β -tubulin gene of *Drosophila*

Cathy L. Regan and Margaret T. Fuller

Department of Molecular, Cellular and Developmental Biology, University of Colorado at Boulder, Boulder, Colorado 80309

A mutation that fails to complement certain alleles of the testis-specific β 2-tubulin gene (*B2t*) of *Drosophila melanogaster* maps to a separate locus, *haywire*, located at 3–34.4 map units in polytene region 67E3-F3. Second-site non-complementing mutations such as *hay^{nc2}* and *B2t* alleles could identify genes that encode products that participate in the same functions or that interact in the same structure. Consistent with a structural interaction between the *hay* gene product and β 2-tubulin, the genetic interaction between *hay^{nc2}* and *B2t* requires the presence of the mutant *hay* gene product; a deficiency for the *hay* region complements the same alleles of *B2t* that *hay^{nc2}* fails to complement. *hay^{nc2}* is a recessive male sterile mutation in a genetic background that is wild type at the *B2t* locus. Homozygous males have defects in meiosis, flagellar elongation and nuclear shaping, the three major microtubule-based processes in which the testis-specific β 2-tubulin participates. The *hay^{nc2}* allele also has effects outside of spermatogenesis. It is a temperature-sensitive semi-lethal mutation, and homozygous *hay^{nc2}* females have reduced fertility. These phenotypes are consistent with a role for the *haywire* gene product in general microtubule function. Analysis of second-site non-complementing mutations such as *hay^{nc2}* offers a genetic tool for analysis of interacting proteins in complex assemblies.

[Key Words: Microtubules; interacting genes; second-site non-complementing mutations; *Drosophila*; spermatogenesis]

Received September 16, 1987; revised version accepted November 9, 1987.

Complex microtubule arrays mediate several diverse processes necessary for normal cellular function. Many aspects of the regulation and coordination of microtubule function, as well as the structural interactions between microtubules and other cellular organelles, may be mediated by accessory proteins. Microtubule-based structures are composed of two highly conserved subunits, α - and β -tubulin, and a heterogeneous set of proteins known collectively as microtubule-associated proteins (MAPs) (reviewed in Cleveland and Sullivan 1985 and Olmsted 1986). MAPs participate in microtubule functions such as transport of organelles along microtubule tracks (Vale et al. 1985) and the movement of cells by flagella and cilia (Warner and Mitchell 1980; reviewed in Gibbons 1981). MAPs are also likely to be important in the movement of chromosomes by mitotic and meiotic spindles (Izant et al. 1982; Vallee and Bloom 1983). Some MAPs have been found in association with other cellular components such as intermediate filaments and microfilaments, where they could be involved in linking microtubules to other structures in the

cell (Schliwa and van Blerkom 1981; Griffith and Pollard 1982; Leterrier et al. 1982). Microtubules and their associated proteins might also play important roles in cellular morphogenesis during development (Brown and Bouck 1973; Burnside 1973; Lindsley and Tokuyasu 1980). However, in most cases neither the identity of essential MAPs nor their role in microtubule-based processes is well established.

One way to identify important accessory protein components of microtubule-based organelles is to isolate and characterize mutations that affect the function of microtubules but that are not in tubulin genes. Genetic analysis should allow the characterization of the function of these proteins in vivo through analysis of mutant phenotypes. In addition, genetic analysis should allow identification of minor components of microtubule-based structures, as well as identification of molecules that interact with microtubules transiently but are not present in the final structure.

To identify genes involved in microtubule function we have utilized the excellent genetics and cytology of

spermatogenesis in *Drosophila melanogaster*. Over 20 mutations in the gene for the testis-specific β 2-tubulin (*B2t*) have been isolated that perturb the function of complex microtubule arrays during spermatogenesis, causing male sterility (Kemphues et al. 1980; 1982; 1983; Raff and Fuller 1984; Fuller 1986; Fuller et al. 1987). Analysis of the phenotypes of these mutant *B2t* alleles has shown that β 2-tubulin is a major structural component of the meiotic spindle, the flagellar axoneme, and the perinuclear cytoplasmic microtubules that mediate nuclear shaping, and that β 2-tubulin is required for the function of each of these microtubule arrays (Kemphues et al. 1982; Fuller et al. 1987).

In genetic screens designed to isolate additional alleles of *B2t*, recessive mutations were identified that failed to complement recessive mutations in *B2t*, but did not map to the *B2t* locus (Raff and Fuller 1984; Fuller 1986). The heterozygous combination of recessive mutations in two different genes resulted in defects in spermatogenesis. One of these second-site non-complementing (*nc*) mutations has been mapped to the major α -tubulin gene of *Drosophila* (T.S. Hays et al., in prep.). This result confirmed that this unusual genetic interaction, failure to complement between mutations in different genes, can be due to gene products that interact structurally. The other second-site non-complementing mutations do not map to any of the known tubulin genes. If the genetic interactions between these mutations and *B2t* are also due to structural interactions between the *nc* gene products and β 2-tubulin during microtubule assembly and function, the loci identified by the *nc* mutations would be good candidates for genes that encode accessory proteins that are important in microtubule function in vivo.

In this paper we report the genetic and phenotypic characterization of the second-site non-complementing mutation *haywire^{nc2}*. As a homozygote in a genetic background that is wild type at *B2t*, *hay^{nc2}* is male sterile and causes defects in all aspects of microtubule function during spermatogenesis that require β 2-tubulin. This phenotype indicates that the *hay^{nc2}* gene product could act in the assembly and function of several kinds of microtubule arrays. The *hay^{nc2}* gene product also appears to act outside of spermatogenesis; *hay^{nc2}* homozygotes exhibit reduced female fertility, as well as reduced viability at elevated temperature.

Results

hay^{nc2} fails to complement certain β 2-tubulin alleles, but does not map to the *B2t* locus

The *hay^{nc2}* mutation was isolated in a screen for new mutant alleles of *B2t*, the gene that encodes the testis-specific β 2-tubulin of *Drosophila melanogaster*, because it failed to complement the recessive allele *B2t³*. The failure to complement between *B2t³* and the *hay^{nc2}* mutation was first roughly localized by recombination mapping to the interval between *h* and *st* on the left arm of the third chromosome. It was then mapped more precisely using the closely flanking markers *cur*, *rs²*, *vi*, and

Ly. To determine if a recombinant chromosome carried the non-complementing mutation, flies bearing recombinant third chromosomes were crossed to *B2t³* and the appropriate class of progeny was tested for male fertility. The failure to complement *B2t³* mapped between *cur* and *rs* at 34.4 ± 0.4 map units, based on the segregation of male sterility in the 66 recombinants between *cur* and *rs*. Although *hay^{nc2}* fails to complement *B2t³*, it clearly identifies a separate gene. The *B2t* locus is at 48.5 map units on the right arm of the third chromosome (Kemphues et al. 1980). *hay^{nc2}* and *B2t³* fail to complement in *cis* as well as in *trans*. We constructed a recombinant with *hay^{nc2}* and *B2t³* on the same chromosome. Male flies bearing one copy of this chromosome over a balancer chromosome are sterile.

As discussed below, *hay^{nc2}* acts as a recessive male sterile mutation in a genetic background carrying no mutations at the *B2t* locus. To determine whether the failure to complement *B2t* alleles and the recessive male sterility associated with *hay^{nc2}* were due to the same mutation, recombinant lines from the mapping experiment above were chosen at random and their progeny were backcrossed to *hay^{nc2} st p⁺ cu sr Bsb* to score homozygous *hay^{nc2}* male sterility. In all 96 cases tested, 63 of which were in the *cur*–*rs* interval, recombinant chromosomes that were sterile in combination with *B2t³* were sterile with *hay^{nc2}*, and those that were fertile with *B2t³* were fertile with *hay^{nc2}*. Thus, the homozygous male sterility always segregated with the failure to complement *B2t³*, indicating that the two effects are closely linked and probably due to the same mutation.

To determine the physical location of *hay* on the chromosome, a series of overlapping deletions in the *rs* region were tested for their ability to complement *hay^{nc2}* for male fertility (Fig. 1). Two of these deficiencies, *Df(3L)lxd6* and *Df(3L)lxd15*, were male sterile in combination with *hay^{nc2}*, while all other deletions shown in Figure 1 were fertile. Our results place *hay* in the 67E3–4;67F3 interval of the salivary chromosome map, as delimited by the distal breakpoints of *Df(3L)lxd6* or *Df(3L)lxd15* on the left and *Df(3L)vin2* on the right. The locations of the four α - and the four β -tubulin genes in *Drosophila* have been determined by in situ hybridization with the cloned genes (Kalfayan and Wensink 1981; Natze and McCarthy 1984). None of the known tubulin genes of *Drosophila* falls into the interval containing *hay^{nc2}*.

The interaction between hay^{nc2} and B2t is allele specific

To determine if the genetic interaction between *hay^{nc2}* and *B2t* mutations is allele specific, heterozygous combinations between *hay^{nc2}* and several different *B2t* alleles (*hay^{nc2} +/+B2t*) were tested for male fertility (Table 1). *hay^{nc2}* fails to complement a null allele of *B2t* (*B2tⁿ*) that makes no protein product. *hay^{nc2}* also fails to complement both of the class I β 2-tubulin alleles tested. The class I *B2t* alleles encode unstable β 2-tubulin subunits that do not accumulate in the testis. In class I homozygotes, most of the α -tubulin in the testis is also

Figure 1. Summary of genetic and cytogenetic analysis of the *hay^{nc2}* locus. The positions of *hay^{nc2}* and other nearby loci on a schematic of salivary gland polytene chromosome 3L, with the corresponding recombination map above. Deficiencies are indicated by open boxes with the ambiguity of breakpoint location indicated by cross-hatching. Deficiency breakpoints according to the literature or personal communications as stated in Methods. The relative size of the chromosome intervals are taken from Bridges' original maps as reproduced in Lefevre (1976). *Df(3L)AC1* removes the α -tubulin in 67C (K. Matthews, pers. comm.).

degraded, so that the total tubulin pool is greatly reduced (Kemphues et al. 1982, 1983). Thus, males heterozygous for *B2tⁿ* or class I *B2t* alleles and wild type probably have partially reduced amounts of α - and β -tubulin in the testes. The similar complementation behavior of *B2tⁿ* and the class I alleles with *hay^{nc2}* is presumably due to the common biochemical defect of reduced testis tubulin pools.

In contrast, *hay^{nc2}* fails to complement some, but not all of the class II *B2t* alleles. The class II *B2t* alleles produce stable, partially functional β 2-tubulin subunits that exhibit a complex pattern of interallelic complementation. The class II mutations examined to date appear to affect different aspects of tubulin function during

spermatogenesis (Raff and Fuller 1984; Fuller 1986; Fuller et al. 1987, 1988). The allele specificity of the failure to complement between *hay^{nc2}* and the class II mutations probably reflects the functional differences among the mutant proteins encoded by the class II *B2t* alleles.

The genetic interaction between hay^{nc2} and B2t requires the presence of the mutant hay^{nc2} gene product

A deletion of the *hay* locus does not act like the *hay^{nc2}* mutation with respect to its genetic interaction with *B2t*, which indicates that the failure to complement between *hay^{nc2}* and *B2t* requires the presence of the mu-

Table 1. The failure to complement between *hay^{nc2}* and *B2t* is allele specific and requires the presence of the mutant *hay^{nc2}* gene product

	<i>hay^{nc2}</i>	<i>B2tⁿ</i>	Class I		Class II				
			<i>B2t^{D+R1}</i>	<i>B2t³</i>	<i>B2t⁶</i>	<i>B2t⁷</i>	<i>B2t⁹</i>	<i>B2t¹⁰</i>	<i>B2t¹¹</i>
<i>hay^{nc2}</i>	— ^a	—	—	—	+	—	+	—	— ^b
<i>Df(3L)lxd6</i>	—	+	+	+	+	+	+	+	+

^a In each case, 100 males were tested by mating and 20 males were tested by dissection as described in Methods. (—) Sterile; no progeny were produced and no motile sperm were present in the seminal vesicles. (+) Fertile; progeny were produced and motile sperm were present in the seminal vesicles.

^b *hay^{nc2}/B2t¹¹* males were sterile by mating, but a small amount of motile sperm was present in the seminal vesicle upon dissection.

tant *hay* gene product. The smallest deletion that removes the *hay* locus, *Df(3L)lxd6*, was tested for its ability to complement mutant *B2t* alleles for male fertility. Males flies doubly heterozygous for *Df(3L)lxd6* and for all *B2t* alleles tested (*Df(3L)lxd6* +/+ *B2t*) are fertile (Table 1). Although *hay^{nc2}* fails to complement many *B2t* alleles, the deficiency of *hay* complements all of these alleles. This result indicates that *hay^{nc2}* cannot be a null mutation. The *nc2* allele appears to encode a defective product that can interfere with the normal process of spermatogenesis when in a background of reduced tubulin levels in the testis, or in combination with certain *B2t* alleles that encode partially functional products.

hay^{nc2} homozygous males have defects in several microtubule-based functions during spermatogenesis

hay^{nc2} homozygous males are sterile in a genetic background that is wild type at the *B2t* locus. The homozygous males have a variety of defects in spermatogenesis. The earliest noticeable defects arise in meiosis. In wild-type cysts of cells in meiosis (Fig. 2a), the spindle region is seen as a clear area outlined by mitochondria which line up along the spindle periphery. The cells are regular in appearance. In contrast, the meiotic spindles

of *hay^{nc2}* homozygotes are disorganized. Figure 2b is an example of a meiotic cyst in which the spindles were better organized than most cysts of meiotic cells in *hay^{nc2}* homozygotes. More typically, cells in meiosis were barely recognizable (Fig. 2c), and it was not often possible to determine whether the cells were in meiosis I or II.

Following normal meiosis in wild-type flies, all cells in a cyst of spermatids have roughly equal amounts of nuclear material and therefore have the same sized nuclei (Gonzalez et al. 1987) (Fig. 2d, arrowhead). In addition, if spindle alignment and cytokinesis proceed normally, the mitochondria are distributed equally to the daughter cells during meiosis so that when the mitochondria coalesce, all daughter cells in a cyst have the same sized mitochondrial derivative (Fig. 2d, arrow).

Post-meiotic cysts of early spermatids in *hay^{nc2}* homozygous males exhibited the results of defects in the segregation of both chromosomes and mitochondria during meiosis. Spermatid nuclei and mitochondrial derivatives varied in size, with some smaller and others larger than normal (Fig. 2e). The differences in nuclear sizes suggest that the distribution of chromosomes during meiosis was defective. Abnormally sized mitochondrial derivatives were sometimes associated with normally sized nuclei (Fig. 2e) and vice versa. Occasion-

Figure 2. *hay^{nc2}* causes defects in meiosis that result in abnormalities in the distribution of nuclear material and mitochondria in post-meiotic spermatid cysts. Live testis squashes showing cysts of meiotic and post-meiotic cells. (a) Wild-type: complete cyst showing 16 cells in early stages of meiosis I. (b) *hay^{nc2}* homozygote: several cells in meiosis. (Arrow) Apparent tripolar spindle. (c) *hay^{nc2}* homozygote: more typical group of *hay^{nc2}* cells in meiosis. (Arrow) Disorganized spindle. (d) Wild type: post-meiotic early spermatid cells showing the normal, uniform sizes of the nuclei (arrowhead) and mitochondrial derivatives (arrow). (e) *hay^{nc2}* homozygote: post-meiotic spermatids. (Arrowheads) Abnormally small nuclei; (arrows) large mitochondrial derivatives. Bar, 10 μ m.

ally, a single mitochondrial derivative appeared to be associated with more than one nucleus. Although some post-meiotic cysts contained many cells with nuclei and mitochondrial derivatives that were the correct size and were paired up normally, every cyst of post-meiotic cells contained some daughter cells that did not have the correct nuclear or mitochondrial derivative size and distribution. This variability in the extent of the defects visible after meiosis was typical of *hay^{nc2}* homozygotes.

Flagellar elongation begins in early spermatid cysts soon after coalescence of the mitochondrial derivatives. During the normal sequence of flagellar elongation a flagellar axoneme grows out from the basal body embedded in each nucleus. The 64 products of meiosis in a cyst remain lined up parallel to one another, with the nuclei clustered at one end of the bundle (Fig. 3a). As spermatid differentiation proceeds, the cyst becomes elongated and compact. As the flagellum grows, the mitochondrial derivative unfolds, splits into two parts, and elongates along the length of the axoneme, passing through a transient phase during which inclusions of cytoplasm can be seen within the mitochondrial derivative. These inclusions normally disappear as the mitochondrial derivative elongates. The two parts of the mitochondrial derivative lie alongside the elongating axoneme and maintain a precise angle with it (Fig. 3c) (Tokuyasu 1974a).

In *hay^{nc2}* homozygotes, bundles of developing spermatids are disorganized and do not elongate to the extent of wild-type flagella (Fig. 3b). The most salient defect in testes from *hay^{nc2}* homozygotes is the distorted shape of the flagella, associated with the aberrant persistence of inclusions of cytoplasm in the mitochondrial derivatives (Fig. 3b,d,e). The disorganization of the developing cyst is also reflected in the scattering of the nuclei along the bundles of elongating spermatids (Fig. 3b). Although axonemes were present in cross-sections through elongating spermatid cysts from *hay^{nc2}* homozygotes examined by electron microscopy, the number of complete axonemes was greatly reduced from that of wild-type, and fragments of axonemes were common (Fig. 3d, arrowheads). Often more than one axoneme was associated with a mitochondrial derivative (Fig. 3e), an arrangement likely to be a secondary effect of the aberrant meiotic segregation of nuclei and mitochondrial derivatives. The observation that the cytoplasmic microtubules that normally surround the mitochondrial derivative during elongation in wild type (Fig. 3c) were present in *hay^{nc2}* (Fig. 3e) indicates that, at least in *hay^{nc2}*, these cytoplasmic microtubules are not sufficient for proper elongation of the mitochondrial derivative.

In the later stages of wild-type spermatid differentiation the nucleus changes shape, starting from a sphere and elongating to become thin and slightly hook shaped (Fig. 4a). This shape change is at least partially mediated by a bundle of microtubules that lie adjacent to the nucleus (Tokuyasu 1974b; Wilkinson et al. 1974; Fuller et al. 1987). During nuclear shaping the chromosomes become very condensed and the nucleus loses volume. In *hay^{nc2}* homozygous males the chromatin appeared to

condense but the nucleus usually remained round (Fig. 4b,b'). In a small fraction of the cells, the nuclei began to elongate but stopped well before reaching the wild-type shape. The variation in nuclear size in late spermatids reflects the abnormal distribution of nuclear size seen in early post-meiotic spermatids (Fig. 2, arrowheads) and indicates that the nuclei received different amounts of chromatin due to the defects in meiosis (Hardy 1975). Both the large and the small nuclei failed to elongate. Nuclei were occasionally observed in cross-sections of elongating cysts examined by electron microscopy. Of the eight nuclei observed in late spermatids from several different *hay^{nc2}* homozygous males, no microtubules were observed at the periphery of the nucleus, even though the cytoplasmic microtubules surrounding the adjacent mitochondrial derivatives were visible in cross-section in the same sections (data not shown).

As discussed above, some of the post-meiotic defects observed in *hay^{nc2}* homozygotes are probably secondary effects of the defects in meiosis, instead of direct effects of the *hay^{nc2}* lesion. It is possible that the presence of very large mitochondrial derivatives in young spermatids could interfere with subsequent flagellar elongation, or that the presence of abnormally sized nuclei could affect the ability of the nuclei to elongate. However, the phenotype of heterozygotes between *hay^{nc2}* and the β 2-tubulin allele *B2t¹⁰* indicates that the aberrant flagellar elongation and the defects in nuclear shaping in *hay^{nc2}* are probably *not* secondary consequences of the defects in meiosis. *B2t¹⁰* encodes a partially functional β 2-tubulin subunit. Meiosis and nuclear elongation occur normally in *B2t¹⁰* homozygous males, but the males exhibit defects in flagellar elongation similar to, but not as severe as, those observed in *hay^{nc2}* homozygotes. *hay^{nc2} +/+ B2t¹⁰* males are sterile, but do not exhibit the meiotic defects characteristic of *hay^{nc2}/hay^{nc2}*. At the completion of meiosis II in *hay^{nc2} +/+ B2t¹⁰* both the nuclei and mitochondrial derivatives are the same size in all cells, which indicates that at least at the gross level, meiosis occurred normally. However, *hay^{nc2} +/+ B2t¹⁰* males do have defects in flagellar elongation and nuclear shaping similar to those observed in *hay^{nc2}/hay^{nc2}* males. Flagellar elongation in *hay^{nc2} +/+ B2t¹⁰* testes is poor, and the characteristic inclusions of cytoplasm in the mitochondrial derivatives persist. Although they are the normal size, the nuclei still fail to elongate (Fig. 4c,c').

The phenotype of hay^{nc2}/Df(3L)lxd6 is the same as that of hay^{nc2}/hay^{nc2}

For mutations that are functionally null or amorphic alleles, the phenotype of mutant/deficiency and mutant/mutant should be the same (Muller 1932). The defects in spermatogenesis in *hay^{nc2}/Df(3L)lxd6* are indistinguishable from those in *hay^{nc2}/hay^{nc2}* as observed by light microscopy: meiosis is defective, which results in the abnormal distribution of nuclear material and mitochondrial derivatives; flagellar elongation is poor and the characteristic inclusions of cytoplasm in the mitochon-

drial derivatives persist and the different sized spermatid nuclei remain spherical. However, although *hay^{nc2}* behaves as an amorphic mutation with respect to the homozygous male sterile phenotype, *hay^{nc2}* cannot be a null allele because it does not act like the deficiency in its interaction with *B2t* alleles (Table 1).

The male sterility associated with hay^{nc2} is completely recessive

To determine if the male sterility associated with *hay^{nc2}* is recessive, testes from *hay^{nc2}/+* males were examined for defects in spermatogenesis by light microscopy, both as live squashes and in fixed, orcein-stained prepara-

Figure 3. *hay^{nc2}* causes defects in flagellar elongation and organization. (a, b) Light micrographs of live testis squashes. Bars, 10 μ m. (a) Wild type: bundles of elongating spermatids. (Arrowhead) Nuclei clustered at end of bundle have begun to elongate so are not visible. Flagella are roughly parallel to one another and cyst is fairly compact. (b) *hay^{nc2}* homozygote: cyst of elongating spermatids. (Arrows) Persistent inclusions of cytoplasm. (Arrowheads) Different sized nuclei scattered throughout cyst. (c–e) Electron micrographs of cross-sections of elongating spermatid cysts. Bars, 1 μ m. (c) Wild type: (ax) axonemes decorated with accessory tubules and other structures typical of this stage; (md) major and minor mitochondrial derivatives associated with axonemes in regular array; (ct) cytoplasmic microtubules next to mitochondrial derivatives. (d) *hay^{nc2}* homozygote at a comparable stage to (c). (ax1) Complete axoneme; (ax2) axoneme missing central pair; (arrowheads) scattered fragments of axonemes. (e) *hay^{nc2}* homozygote: higher magnification view of earlier stage spermatid showing mitochondrial derivative associated with three axonemes (arrowheads), two of which are incomplete. (ct) Cytoplasmic microtubules both inside and outside of the mitochondrial derivative.

Figure 4. Elongation of spermatid nuclei is defective in *nc2* homozygotes. Spermatid nuclei fixed and stained with aceto-orcein to reveal condensed chromatin. (a) Wildtype elongated and condensed nuclei clustered at ends of cysts. (b, b') *nc2* homozygotes: nuclei are round and of different sizes; [large arrowheads] large nuclei; [small arrowheads] small nuclei. (c, c') Nuclei from two different cysts of *nc2/B2t¹⁰*. Most nuclei round and of uniform size. (Arrow) A few partially elongated nuclei. Bars, 10 μ m.

tions. Meiosis, flagellar elongation, and nuclear elongation all appeared to be normal. The number of progeny produced by *hay^{nc2}/+* and wild-type males was compared by brooding tests. There was no statistical difference between the fertility of *hay^{nc2}/+* and the wild-type control (Table 2). We conclude that the *hay^{nc2}* mutation

Table 2. The *hay^{nc2}* mutation is recessive with respect to male fertility

Genotype	Average progeny per male ^a
<i>hay^{nc2}/Ore R</i>	257.5 $\pm$ 95.0
<i>Ore R/Ore R</i>	284.4 $\pm$ 60.3

^a Eleven males of each genotype were mated individually in brooding tests as described in methods. Results expressed as the average of the total number of progeny produced per individual male.

is completely recessive to wild type with respect to male fertility when in a background wild type for tubulin.

Homozygous hay^{nc2} flies exhibit defects outside of spermatogenesis

To determine if the *hay^{nc2}* mutation causes defects in other tissues or functions, we tested *hay^{nc2}* homozygotes for female fertility and for viability. One hundred forty three *hay^{nc2}* homozygous females were individually mated to wild-type males to test for fertility. One hundred forty were sterile and did not lay eggs. The other females laid few eggs and produced only a small number of progeny. *hay^{nc2}/Df(3L)lxd6* females also exhibited low fertility and few flies laid eggs. Seventy five females of this genotype were mated to wild-type males in batches of five females per vial. Of the 15 vials, 2 had no progeny or eggs and the other 13 each contained fewer than 10 progeny. Thus, the reduced female fertility appears to be due to *hay^{nc2}* and not another mutation on the chromosome.

Although homozygous *hay^{nc2}* flies are viable at 25°C, the mutation appears to cause reduced viability at elevated temperature. The proportion of homozygous flies that survived to adulthood at 25°C was close to the expected percent. However, at 28°C viability of *hay^{nc2}* males and females was greatly reduced (Table 3). *hay^{nc2}* in heterozygous combination with a deficiency for the *hay* region also exhibited reduced viability at 28°C (Table 3). Therefore, *hay^{nc2}* is a temperature-sensitive semi-lethal mutation.

hay^{nc2} also appears to cause a visible defect in bristle morphology. The post-scutellar bristles of *hay^{nc2}* homozygous males and females raised at 25°C stand out from the body at different angles, rather than lie flat on the

Table 3. Reduced viability of *hay^{nc2}/hay^{nc2}* and *hay^{nc2}/Df(3L)lxd6* at elevated temperature

F1 genotype	Temperature (°C)	% of total progeny ^c	Total number of progeny
<i>hay^{nc2}/hay^{nc2}</i> ^a	25	25.5	963
	28	5.9	1113
<i>hay^{nc2}/Df(3L)lxd6</i> ^b	25	28.5	1479
	28	6.6	910

^a Progeny of *ru h hay^{nc2} vi/TM3* $\times$ *hay^{nc2} st p^p cu sr Bsb/TM3*.

^b Progeny of *ru h hay^{nc2} vi/TM3* $\times$ *Df(3L)lxd6/TM3*.

^c Expected frequency of the test class is 33% of the total progeny.

thorax and cross over each other. This defect was also seen in *hay^{nc2}/Df(3L)lxd6* males and females. A thoracic bristle defect has also been noted in α -tubulin mutants (Matthews and Kaufman 1987). These effects of the *hay^{nc2}* mutation indicate that the expression *hay^{nc2}* is not limited to spermatogenesis.

Discussion

We have identified a recessive mutation that falls into the same complementation group as mutations in the β 2-tubulin gene, but that maps to a separate locus. *hay^{nc2} +/+ B2t* heterozygotes are male sterile, even in the presence of a wild-type copy of each of the genes. Based on this unusual genetic behavior, the *hay^{nc2}* and *B2t* gene products appear to be involved in the same function or structure. The *hay* gene product is thus a candidate for an accessory protein component of microtubule-based organelles.

The phenotype of hay^{nc2} homozygotes suggests that the hay gene product is required for microtubule function

The meiotic, flagellar elongation, and nuclear shaping defects of homozygous *hay^{nc2}* males suggest a role for the *hay* gene product in the function of several morphologically different microtubule arrays during spermatogenesis. Defects in the same processes arise in flies carrying mutations in a known microtubule component, β 2-tubulin (Kemphues et al. 1980, 1982; Fuller et al. 1987). In combination with the genetic interaction between *hay^{nc2}* and *B2t*, these observations suggest that the *hay* gene product might participate in the same functions as β 2-tubulin.

The unequal distribution of nuclear material and mitochondria to early spermatid cells in *hay^{nc2}* homozygotes is probably due to defects in the structure or function of the meiotic spindle, which often appears disorganized in homozygous *hay^{nc2}* males. Defects in the meiotic distribution of both nuclear material and mitochondrial derivatives can be due to defects in microtubule function. Such defects are common in the *B2t* mutants, as well as in other mutants that affect spindle function but do not map to tubulin genes (Kemphues et al. 1980, 1982; Ripoll et al. 1985; Fuller et al. 1987, 1988). Failure of cytokinesis occurs in the class I *B2t* alleles, which lack a functional spindle (Kemphues et al. 1982), as well as in *B2t⁸*, where spindle microtubules are abnormal (Fuller et al. 1987). In both cases, large mitochondrial derivatives and fragments of mitochondrial derivatives result. Thus the large mitochondrial derivatives observed in early spermatids from *hay^{nc2}* homozygotes could result from failure of cytokinesis. The normal distribution of mitochondrial derivatives might be partly dependent upon association of the mitochondria with the spindle. It is possible that *hay^{nc2}* could cause defects in the distribution of mitochondrial derivatives by affecting the alignment of the mitochondria on the spindle through a mechanism that involves the tubulin binding sites proposed to be on mitochondrial membranes (Bernier-Valentin et al. 1983).

The most striking aspect of the *hay^{nc2}* phenotype during flagellar elongation is the amorphous shape of elongating mitochondrial derivatives associated with abnormal persistence of inclusions of cytoplasm. The defects in the elongating mitochondrial derivatives can be phenocopied in wild-type flies by the microtubule inhibitor colcemid (Wilkinson et al. 1974). Although the mitochondrial derivatives are not microtubule-based structures, this result suggests that the poor elongation and aberrant inclusions of cytoplasm could be a consequence of microtubule defects. Incomplete elongation of both mitochondrial derivatives and flagella in *hay^{nc2}* could result from poor axoneme elongation. Only low numbers of axonemes are present in testes from homozygous *hay^{nc2}* flies, and axoneme fragments are common. Cross-sections through spermatid cysts from *B2t⁷* homozygotes also show many axoneme fragments and reduced numbers of intact axonemes (Fuller et al. 1988). In *B2t⁷* males, this phenotype appears to result from a combination of abnormally short axonemes that fray apart, and scattering of the nuclei (and therefore, the axoneme initiation sites) along the spermatid bundle. Alternatively, poor elongation of the mitochondrial derivatives might be due to their inability to maintain the normal association with the axoneme.

The failure of nuclei to elongate in *hay^{nc2}* homozygotes could be due to the absence of perinuclear microtubules. Analyses of *B2t* alleles, other male sterile mutants, and colcemid-treated wild-type testes indicate that these microtubules are essential for nuclear shaping (Wilkinson et al. 1974; Fuller et al. 1987). In our limited examination of late spermatid nuclei in *hay^{nc2}* males, perinuclear microtubules were never observed.

The defects in events that utilize several structurally different kinds of microtubule arrays in *hay^{nc2}* homozygotes indicate that the *hay* gene product is involved in general microtubule function. The greatly reduced female fertility of homozygous *hay^{nc2}* females and the reduced viability of *hay^{nc2}* at elevated temperatures indicate that *hay* is expressed outside of spermatogenesis. The *hay^{nc2}* mutation thus appears to be a non-null allele of an essential gene. At 25°C *hay^{nc2}* appears to cause defects only in *hay* function during gametogenesis. It is feasible that the defects in spermatogenesis are the most salient aspect of the *hay^{nc2}* phenotype because spermatogenesis is the process most sensitive to microtubule defects. In a series of six mutant alleles in the major α -tubulin gene of *Drosophila* leading to defects of increasing severity (Matthews and Kaufman 1987), the least severe allele caused male sterility while the most severe alleles were lethal. Gametogenesis, and spermatogenesis in particular, thus appears to be more sensitive than other processes to defects in microtubule components. Analysis of additional alleles of *hay* should allow us to further elucidate the role of the wild-type *hay* gene product.

The genetic behavior of hay^{nc2} suggests that the hay gene product interacts with microtubules

hay^{nc2}/+ can cause defects in spermatogenesis when

present in a strain that has decreased or defective β 2-tubulin in the testis. Thus, although *hay^{nc2}* is a recessive male sterile mutation, its genetic interaction with the *B2t* locus is dominant. Formally, *hay^{nc2}* acts as a dominant enhancer of β 2-tubulin mutants. Most male sterile mutations are not dominant in the background of reduced β 2-tubulin. Of over 100 random male sterile mutations on the third chromosome tested for complementation with *B2t^{D+R1}*, only one, a *B2t* allele, failed to complement (E.C. Raff, pers. comm.).

The *hay^{nc2}* mutation is not a null allele and instead appears to encode an aberrant gene product that acts to disrupt microtubule assembly or function in certain genetic backgrounds. Mutant *hay^{nc2}* product is required for the genetic interaction between *hay^{nc2}* and *B2t*; a deficiency for *hay^{nc2}* does not fail to complement *B2t* alleles. However, this interaction does not require the presence of mutant β 2-tubulin; *hay^{nc2}* fails to complement *B2t^{null}*, an allele that produces no β 2-tubulin.

The genetic interaction between *hay^{nc2}* and *B2t* can be explained by a model based on structural interactions between the products of the two genes. In *B2t*/+ heterozygotes, the level of wild-type tubulin in the testis is probably reduced (Kemphues et al. 1982, 1983; Fuller et al. 1988). If a fly is also heterozygous for the second-site non-complementing mutation *hay^{nc2}*, a portion of the remaining wild-type tubulin could be complexed with mutant *hay^{nc2}* product and rendered non-functional, thus reducing the level of functional microtubule components below a threshold required for completion of spermatogenesis. In a background that is wild type for *hay*, heterozygous *B2t^{null}* flies may already be near the threshold of tubulin function required for male fertility. Under the conditions of our complementation tests, 25°C, *B2t^{null}*/+ males are fertile. However, if raised at 18°C, *B2t^{null}*/+ males are sterile (M.T. Fuller, unpubl.).

The above structural model can account for the allele specificity of the unusual genetic interaction between *hay* and *B2t*. Any mutation that leads to the production of an accessory protein still able to bind microtubules, but defective in a subsequent process, could potentially act as a second-site non-complementing mutation. The *hay^{nc2}* allele could encode a partially functional protein that binds to microtubules competitively with wild-type *hay* gene product, but is incapable of performing some cross-linking or enzymatic function. *nc* alleles of interacting genes could thus identify protein domains that are important for microtubule function but are not involved in the tubulin-*nc* product binding site. This model does not require a direct physical interaction between the *hay^{nc2}* gene product and tubulin. The *hay* gene product and tubulin could interact through an intermediary protein that has binding sites for both.

Second-site non-complementing mutations have also been identified in other systems. In *S. cerevisiae*, T. Stearns and D. Botstein (manuscript in prep.) have isolated mutations in α -tubulin by screening for mutations that fail to complement mutant β -tubulin alleles and vice versa. A structural interaction between two gene products has been implicated in other examples of ge-

netic interactions between recessive alleles of separate genes. Kusch and Edgar (1986) identified mutations in separate genes that affect the body shape of *C. elegans* which fail to complement one another. They speculated that these mutations might identify genes that encode structural proteins of the cuticle. Mutations in different SIR genes in *S. cerevisiae* exhibit allele specific failure to complement (Rine and Herskowitz 1987). Both structural (Hartley et al. 1987) and regulatory (Vassin et al. 1985) mechanisms have been proposed to explain the failure to complement between mutations in separate genes involved in the development of the nervous system in *Drosophila*. However, this type of genetic interaction does not always imply a structural interaction between the gene products. Atkinson (1985 and pers. comm.) has described failure to complement between recessive alleles in two genes involved in different steps of the sphingolipid biosynthesis in *S. cerevisiae*.

The analysis of second-site non-complementing mutations offers a promising strategy for identification of genes that encode products that interact to build complex protein arrays. Identification of one of the second-site non-complementing mutations of *B2t* as an allele of α -tubulin (T.S. Hays et al., in prep.) demonstrates that the genetic interaction can be due to a structural interaction between the gene products. We have now characterized a second-site non-complementing mutation, *hay^{nc2}*, that is not in α - or β -tubulin. The product of the *haywire* gene might be an accessory protein important for microtubule function in several kinds of microtubule arrays.

Methods

Fly strains, culture and mutant isolation

Flies were raised at 25°C, unless otherwise indicated, on standard corn-meal agar media. Visible mutations and balancers are described in Lindsley and Grell (1968), except for *vi* (Anxolebere and Periquet 1973) and *Bsb* (Lindsley and Zimm 1985). Cytology of deletions are based on: *vi* series (Lindsley and Zimm 1987); *lxd* series (Crosby and Meyerowitz 1986; Schott et al. 1986); *BK* series (B. Leicht and J. Bonner, pers. comm.); and *AC1* (A.T.C. Carpenter, pers. comm.). *B2t* alleles are described in Kemphues et al. 1982, 1983; Raff and Fuller 1984; Fuller 1986; Fuller et al. 1987, 1988.

hay^{nc2} was isolated in a screen of the third chromosome for new alleles of the *B2t* locus. Homozygous *red e* males were mutagenized with 25 mM ethyl methane sulfonate (Lewis and Bacher 1968) and mated to CXD/TM3 virgin females. The *red e* / balancer progeny were individually mated to *st p^p B2t³cu*/TM3 and the resulting *red e/st p^p B2t³ cu* male F2 progeny were tested for fertility by mating to virgin females. In cases where the mutagenized *red e* chromosome was sterile in combination with *B2t³*, the mutation was recovered through the F2 *red e**/TM3 siblings and retested for failure to complement *B2t³* for male fertility in subsequent crosses.

Unless otherwise stated, all figures are of testes from flies bearing a homozygous viable *ru h hay^{nc2} vi* chromosome constructed from a recombinant line. The same phenotypic analyses have also been performed with heterozygotes between the recombinant lines *ru h hay^{nc2} red e* and *hay^{nc2} st p^p cu sr Bsb*, a combination homozygous only in the *hay* region. The *hay^{nc2}* phenotypes of any combination of these chromosomes were in-

distinguishable. All three of these *hay^{nc2}* lines behaved identically genetically and cytologically in complementation tests with the deficiencies and with *B2t* alleles.

Recombination and deletion mapping, complementation tests and fertility tests

Both the failure to complement *B2t*³ and the homozygous male sterility of the *hay^{nc2}* mutation were mapped by meiotic recombination, using the visible markers *cur* (3–30.0), *rs²*(3–35.0), *vi* (3–36.2), and *Ly* (3–40.5). One hundred twenty-six recombinants from crossovers between *cur* and *Ly* were scored. In the mapping study, fertility was scored by the presence of motile sperm in seminal vesicles dissected from males held away from females for at least 5 days. Complementation tests were scored both by dissection as described above and by mating. Mating tests were done in batches of 5 test males and at least 10 virgin females per vial. The presence or absence of progeny was scored after at least 7 days. For brooding tests to assay the level of fertility of *hay^{nc2}/+* males, individual males were mated in yeasted vials to three Ore R or *ry⁵⁰⁶* virgin females. On the third and sixth days, the males were moved to new vials and given three new virgin females. On the ninth day the males were removed. The females were left in the vials to continue to lay eggs until the 10th day after mating. The progeny were allowed to eclose through the 18th day after mating and the total number of progeny were counted.

Temperature-sensitive lethality

Crosses were set up to generate *hay^{nc2}/hay^{nc2}* or *hay^{nc2}/Df(3L)lxd6* at either 25 or 28°C. The bottles remained at the initial temperature throughout the course of the experiment. The number of progeny in each class was counted through the 18th day for those crosses at 25°C and through the 16th day for crosses at 28°C.

Cytology and ultrastructure

Squashes of unfixed testes from young males were prepared for phase contrast microscopy as described in Kemphues et al. 1980. Orcein-stained squashes of fixed testis for examination of nuclei containing condensed chromatin followed the procedure of Lifschytz and Hareven (1977). Testes were prepared for electron microscopy as described in Fuller et al. 1987.

Acknowledgments

We thank Nurit Wolf for her ever-cheerful assistance and excellent electron microscopy, Jeff Hutchens for devoted technical assistance in the isolation and initial mapping of *hay^{nc2}*, and Dr. Elizabeth Raff for support and helpful discussions during the initial stages of this work. We are grateful to Drew Smith, and Drs. S. Dutcher, B. Robertson, P. Ripoll, and K. Kirkegaard for useful comments on the manuscript and our colleagues in the lab for helpful discussions. This work was supported by NIH grant 1-RO1-HD-18127 to M.T.F. who gratefully acknowledges the additional support of a Junior Faculty Research Award from the American Cancer Society and a Searle Scholars award. C.L.R. was supported by NIH Predoctoral Training Grant NRSA 07135-11. We also acknowledge the contribution of BRSG Grant PR07013-18 from NIH towards microscope components.

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C L Regan and M T Fuller

Genes Dev. 1988, **2**:

Access the most recent version at doi:[10.1101/gad.2.1.82](https://doi.org/10.1101/gad.2.1.82)

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