Essential role for γ -tubulin in the acentriolar female meiotic spindle of *Drosophila*

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Microtubule nucleation *in vivo* requires γ -tubulin, a highly conserved component of microtubule-organizing centers. In *Drosophila melanogaster* there are two γ -tubulin genes, γ TUB23C and γ TUB37C. Here we report the cytological and molecular characterization of the 37C isoform. By Western blotting, this protein can only be detected in ovaries and embryos. Antibodies against this isoform predominantly label the centrosomes in embryos from early cleavage divisions until cycle 15, but fail to reveal any particular localization of γ -tubulin in the developing egg chambers. The loss of function of this gene results in female sterility and has no effect on viability or male fertility. Early stages of oogenesis are unaffected by mutations in this gene, as judged both by morphological criteria and by localization of reporter genes, but the female meiotic spindle is extremely disrupted. Nuclear proliferation within the eggs laid by mutant females is also impaired. We conclude that the expression of the 37C γ -tubulin isoform of *D.melanogaster* is under strict developmental regulation and that the organization of the female meiotic spindle requires γ -tubulin.

Keywords: *Drosophila*/meiosis/MTOC/mutant/ γ -tubulin

Introduction

The highly ordered microtubular array found in eukaryotic cells is organized by a specialized organelle called the microtubule-organizing center (MTOC). MTOCs organize microtubules by initiating their assembly and anchoring them at their minus ends, thus facilitating microtubule extension at the rapidly growing plus ends. The morphology, subcellular localization and molecular make up of MTOCs vary across different species and different cell types within single species. The major MTOC in proliferating animal cells is the centrosome. Because of its central role in many essential aspects of cell physiology, both in interphase and during cell division, an intense research activity has been carried out to characterize this organelle since it was discovered over 100 years ago (for reviews, see Kirschner and Mitchison, 1986; Bornens *et al.*, 1987; Kalt and Schliwa, 1993; Kellogg *et al.*, 1994).

Although the molecular characterization of the centrosome is still very poor, some centrosomal components which are highly conserved across species and whose

disruption impairs centrosome function are already known (reviewed in Kalt and Schliwa, 1993). One of such gene products is γ -tubulin, a member of the tubulin superfamily of proteins which was first identified as the product of the *mipA* gene in *Aspergillus nidulans* (Weil *et al.*, 1986; Oakley and Oakley, 1989). Since then, γ -tubulin isotypes have been found in a large number of organisms, for example *Plasmodium falciparum* (Maessen *et al.*, 1993), *Schizosaccharomyces pombe* (Horio *et al.*, 1991), *Ustilago violacea* (Luo and Perlin, 1993), *Anemia phyllitidis* (Fuchs *et al.*, 1993), *Zea mays* (Lopez *et al.*, 1995), *Xenopus laevis* (Stearns *et al.*, 1991) and *Homo sapiens* (Zheng *et al.*, 1991).

Immunofluorescence and immunoelectron microscopy studies have revealed the presence of γ -tubulin in every major MTOC that has been looked at, including the spindle pole bodies of *S.pombe* and *A.nidulans* (Oakley *et al.*, 1990; Horio *et al.*, 1991), the centrosomes of *Drosophila*, *Xenopus* and mammalian cells (Stearns *et al.*, 1991; Zheng *et al.*, 1991; Debec *et al.*, 1995; Sunkel *et al.*, 1995), the MTOCs of differentiated cells such as neurons, chicken retinal epithelium and post-mitotic ciliated cells (Baas and Joshi, 1992; Joshi and Besharse, 1993; Muresan *et al.*, 1993) as well as the MTOCs of higher plants (Liu *et al.*, 1993; reviewed in Joshi, 1993). Nevertheless, γ -tubulin is not localized exclusively at the spindle pole; it has also been observed over the entire length of the spindle, and at the midbody in a pattern which is cell cycle dependent in a variety of organisms (Lajoie-Mazenc *et al.*, 1994). Moreover, in mammalian cells, a significant fraction of the total pool of γ -tubulin is found in cytosolic complexes (Moudjou *et al.*, 1996).

There is now a considerable body of evidence based on functional assays showing that, in those species in which it has been examined, γ -tubulin provides an essential MTOC function. This conclusion is based on experimental manipulations which include antibody blocking and over-expression in mammalian cells (Joshi *et al.*, 1992; Julian *et al.*, 1993; Ahmad *et al.*, 1994; Shu and Joshi, 1995); immunodepletion and biochemical manipulation of *in vitro* reconstituted centrosomes from *Xenopus* egg extracts (Felix *et al.*, 1994; Stearns and Kirschner, 1994); automated electron tomography (Moritz *et al.*, 1995); biochemical purification and functional assays (Zheng *et al.*, 1995); and genetic analysis in *S.pombe* (Horio *et al.*, 1991), *A.nidulans* (Oakley *et al.*, 1990) and *Drosophila* (Sunkel *et al.*, 1995). Furthermore, the function performed by γ -tubulin seems to be highly conserved across different species since the expression of human γ -tubulin restores viability to *S.pombe* cells mutant for γ -tubulin (Horio and Oakley, 1994).

In *Drosophila*, there are two γ -tubulin isoforms, which are encoded by two different genes (Zheng *et al.*, 1991; Oakley, 1994). The percentage of divergence between

these two isoforms is ~20%, much higher than that found between the *Xenopus* and human isoforms, which is only ~2%. The polypeptide encoded by the *Drosophila* 23C gene is expressed in a variety of tissues and developmental stages including larval brains and imaginal discs, and testis (Sunkel *et al.*, 1995). Mutation in this gene has been reported to affect both the number of microtubules, which is decreased dramatically in mutant tissues, and the structure of the MTOCs which, in mitotically active cells, shows a variety of shapes and sizes very different from those found in wild-type cells (Sunkel *et al.*, 1995). Here we report the pattern of expression, subcellular localization and mutant phenotypes of the second isoform, 37C.

Results

There are two γ -tubulin genes in *Drosophila*. The first one was described by Zheng *et al.* (1991) and is located in position 23C of the polytene maps. The phenotypes of mutation in this gene have been reported by Sunkel *et al.* (1995). As a first step towards the characterization of the second isoform (Oakley, 1994), we mapped it by *in situ* hybridization to polytene chromosomes and carried out a molecular analysis of the corresponding genomic region. The gene encoding this isoform maps to position 37C. Therefore, we will refer to it as the 37C γ -tubulin isoform or γ TUB37C. The molecular characterization of the γ TUB37C gene is summarized in Figure 1. Using a genomic DNA library made in λ -dash, we have isolated a series of overlapping clones which, altogether, span ~10 kb at each side of the γ TUB37C cDNA. A 7.2 kb *EcoRI* fragment (P[γ TUB37C⁺ w⁺] in Figure 1), which contains the coding sequence flanked by ~5 and 0.7 kb up- and downstream of the 37C γ -tubulin open reading frame (ORF), was isolated from phage λ B3 and subcloned into the *Drosophila* transformation vector PW8 (Klemenz *et al.*, 1987). This construct was injected into embryos for germ-line transformation in order to generate transgenic flies carrying a wild-type copy of the 37C isoform. These flies were used to identify the complementation group which corresponds to the γ TUB37C gene, as described below. Transgenic flies carrying a construct containing a deletion derivative of P[γ TUB37C⁺ w⁺] from which the γ TUB37C ORF had been removed (P[γ TUB37C⁻ w⁺] in Figure 1) were also obtained as a control. The position and size of the introns were deduced by sequencing both genomic and cDNA from the initial ATG to the stop codon (TAA). There are two introns of 69 and 59 nucleotides each, which are located immediately after nucleotides 49 and 844 of the cDNA. Both our genomic and cDNA sequences differ from the published cDNA in six nucleotides. Conceptual translation of our cDNA sequence renders a protein which differs from the published protein sequence (Oakley, 1994) by two amino acids at positions 345 (K instead of N) and 346 (L instead of V). K and L are part of a conserved domain of the γ -tubulin molecule.

The γ TUB37C polypeptide is only found at significant levels in ovaries and embryos

To study the pattern of expression of the γ TUB37C polypeptide, we raised antibodies against a peptide which contains the last 14 amino acids of the C-terminus of this polypeptide, a highly divergent region of the molecule

which is specific for this isoform. Polyclonal antibodies raised against the full-length molecule cannot be used because they can cross-react extensively with the 23C isoform. Western blots of the major developmental stages and some isolated organs stained with the antipeptide antibody Rb1011 indicated that the γ TUB37C protein is restricted to ovaries and embryos. Therefore, we examined the expression of this polypeptide in these tissues in more detail (Figure 2). The expression of the 37C γ -tubulin protein is up-regulated during oogenesis from undetectable levels during the first eight stages of oogenesis to a maximum at stage 14. This level is maintained in early embryos and decreases as embryogenesis proceeds (Figure 2a). The absence of detectable amounts of this polypeptide at other stages of development is summarized in Figure 2b, which shows negative staining of tissues which contain post-embryonic, mitotically active cells (larval brains), polytene cells (salivary glands) and meiotic spermatocytes (adult testes).

Subcellular localization of the γ TUB37C polypeptide

To determine the subcellular localization of the γ TUB37C protein, we have performed immunofluorescence analysis with the Rb1011 antibody during embryogenesis (Figure 3). During the synchronous cleavage divisions, the γ TUB37C polypeptide is mainly, but not exclusively, localized at the centrosome. The centrosomal localization of this antigen seems to be fairly constant during the entire length of each nuclear cycle, so that no major differences are observed in the signal produced by the Rb1011 antibody between interphase and mitosis. Nevertheless, the localization of the γ TUB37C isoform is clearly not restricted to the centrosome. Firstly, during mitosis, the Rb1011 antibody detects a significant amount of γ TUB37C over the regions which correspond to each hemi-spindle. This occurs even in embryos which have been fixed under conditions which do not preserve microtubules (Figure 3c). The presence of γ -tubulin over the mitotic spindle has been observed previously in *Drosophila* and other organisms (Lajoie-Mazenc *et al.*, 1994; Sunkel *et al.*, 1995). Secondly, there is a low, but substantial amount of immunofluorescence scattered throughout the embryo. At high magnification, this staining appears as a punctuate pattern made up of dots of varying sizes, always <1 μ m in diameter (Figure 3c).

The overall centrosomal signal observed by immunofluorescence with the Rb1011 antibody decreases as embryogenesis proceeds. During the last nuclear cycles before cellularization, the centrosomal signal is significantly lower than that found at earlier cycles. Moreover, during these stages, the concentration of the γ TUB37C isoform is no longer constant through the nuclear cycle; it is considerably higher in mitosis than in interphase. This trend is maintained during cycle 14, immediately after cellularization (Figure 3d), and the following cycle 15, when the γ TUB37C polypeptide is barely detectable during interphase, but is still visible in centrosomes of cells undergoing mitosis.

Immunofluorescence analysis with the Rb1011 antibody consistently failed to reveal any localized focus of the γ TUB37C polypeptide in the developing egg chambers or at the poles of the female meiotic spindle. These experi-

Fig. 1. Genetic and molecular characterization of the genomic region which contains the γ TUB37C gene. *In situ* hybridization with a γ TUB37C probe maps this gene to bands 37C5–D1. The bars on the top depict the extent of the region deleted by some of the deficiencies which are available [for a detailed description of the deficiencies listed see Stathakis *et al.*, (1995), and references quoted therein]. λ B1, λ B2 and λ B3 are overlapping inserts isolated from a *Drosophila* genomic DNA library made in λ -dash (C.Gonzalez, unpublished data). The arrow over the cDNA indicates the direction of transcription with respect to the restriction map shown above. We have not determined the orientation of the molecular map with respect to the polytene maps. P[γ TUB37C⁺ w⁺] is a 7.2 kb *Eco*RI fragment isolated from phage λ B3 which contains the γ TUB37C coding sequences flanked by 5 and 0.7 kb up- and downstream, respectively, cloned into the *Drosophila* transformation vector PW8. P[γ TUB37C⁺ w⁺] is a derivative of P[γ TUB37C⁺ w⁺] generated by PCR, from which the coding region of the γ TUB37C gene has been deleted. The sequence at the bottom corresponds to the genomic sequence from the start (ATG) to the stop (TAA) codon. The two introns are represented by lower case letters.

Fig. 2. Developmental expression of the γ TUB37C polypeptide. Western blot hybridized with the Rb1011 antibody. Each line contains protein extracts from different tissues as indicated. (a) Egg chamber stages are as defined by King (1970). (b) Larval brains and salivary glands were obtained from third instar larvae. Adult testes were isolated from 2- to 10-day-old males. Balanced loading of total protein was controlled by scanning and quantitation following Ponceau staining of the membrane before incubation with the antibody. All the tracks contain similar amounts of protein except for embryos 2–7 h which contain $\sim 1/3$ of the average and the adult testis track which was overloaded.

ments were carried out following the standard formaldehyde fixation protocol described by Theurkauf and Hawley (1992) and the new methanol fixation procedure described in Materials and methods. The absence of localized γ -tubulin staining at these stages has been reported previously (McKim and Hawley, 1995; Matthies *et al.*, 1996). Likewise, and consistently with the results of the immunoblotting experiments shown before, immunofluorescence failed to reveal any positive signal in larval brains and imaginal discs.

The γ TUB37C isoform is the product of the *fs(2)TW1* complementation group

The entire chromosome region containing the *Dopa Decarboxylase* gene cluster, which includes subdivision 37C, has previously been the subject of intensive molecular and genetic characterization (Stathakis *et al.*, 1995). Some of the available deficiencies which uncover 37C are shown in Figure 1. We have performed *in situ* hybridization to polytene chromosomes of larvae heterozygous for these deficiencies and showed that the γ TUB37C gene is uncovered by *Df(2L)VA23*, *Df(2L)VA19* and *Df(2L)TW158* but not by *Df(2L)V18* or *Df(2L)VA6*. The region defined by these deficiencies, which has been mutagenized to near

Fig. 3. Immunofluorescence analysis of the γ TUB37C polypeptide during embryogenesis. (a), (c) and (d) were obtained following 3.7% formaldehyde fixation. The γ TUB37C polypeptide was visualized with the Rb1011 antibody (green/yellow) and DNA was stained with propidium iodide (red). (b) was obtained following methanol fixation. The γ TUB37C polypeptide is shown in orange, tubulin in green and DNA in blue. (a) Interphase nuclei from a syncytial blastoderm embryo. (b) High magnification of a single telophase. The γ TUB37C antigen co-localizes with the two MTOCs at each pole of the mitotic figure. A very prominent mid-body can be observed between the segregating chromatin masses. (c) High magnification of a single metaphase. Most of the γ TUB37C polypeptide is localized at the centrosomes, but significant amounts are also present over the spindle and scattered around the mitotic figure. (d) Area from the surface of an embryo during cell cycle 14, immediately after cellularization. Only mitotic cells show positive staining with the Rb1011 antibody.

Fig. 4. Developmental expression of the γ TUB37C polypeptide. Western blot hybridized with the Rb1011 antibody. The lines contain protein extracts from different tissues as indicated. Egg chamber stages are as defined by King (1970). Tissues were obtained from individuals which were wild-type (wt/wt), *fs(2)TWI*¹ homozygous (mut/mut), *fs(2)TWI*¹ hemizygous over *Df(2L)VA23* (Df/mut) and *fs(2)TWI*¹ over the same deficiency plus the P[γ TUB37C⁺ w⁺] transgene that carries one copy of the γ TUB37C gene (P[+]/Df/mut). Balanced loading was controlled by scanning and quantification following Ponceau staining of the membrane before incubation with the antibody. The region of the scanned membrane containing the major bands present in ovarioles and early embryos is shown at the bottom of the figure.

saturation (Schüpbach and Wieschaus, 1989; Stathakis *et al.*, 1995), contains only two known complementation groups. To determine whether any of these complementation groups corresponds to the γ TUB37C gene, we carried out rescue experiments with the P[γ TUB37C⁺ w⁺] transgene described above. Only mutations in complementation group *fs(2)TWI* were rescued by the transgene. P[γ TUB37C⁺ w⁺] complements all the transheterozygous combinations of different mutant alleles of this gene that have been tested, i.e. *fs(2)TWI*¹/*Df(2L)VA23*, *fs(2)TWI*¹/*Df(2L)VA19*, *fs(2)TWI*¹/*fs(2)TWI*^{HL2} and *fs(2)TWI*¹/*fs(2)TWI*^{RU34}. The control P[γ TUB37C⁻ w⁺] transgene does not rescue any complementation group from this region. These results strongly suggest that complementation group *fs(2)TWI* encodes the γ TUB37C isoform of *D.melanogaster*. This conclusion is substantiated further by the perfect agreement between the restriction map of the 37C γ -tubulin genomic region shown in Figure 1 and the map of the genomic region containing the *fs(2)TWI* locus, as described in Stathakis *et al.* (1995).

Additional evidence in this regard is provided by a series of Western blotting experiments using the Rb1011 antibody on three different tissues from individuals carrying four different allelic combinations of the 37C γ -tubulin gene (Figure 4). The tissues studied were isolated egg chambers from the first 13 stages of oogenesis, isolated stage 14 egg chambers and 0–2 h embryos. The flies analyzed carried the two wild-type copies of the γ TUB37C gene (Oregon-R strain), the two mutant copies of the *fs(2)TWI*¹ allele [*fs(2)TWI*¹ homozygous], only one copy of this mutant allele [*fs(2)TWI*¹/*Df(2L)VA23*] and one copy of the mutant allele plus the transgene which carries a wild-type copy of the γ TUB37C gene [*TWI*¹/*Df(2L)VA23*; P(γ TUB37C⁺ w⁺)]. All the tracks containing tissues derived from wild-type individuals show the 51 kDa band corresponding to the γ TUB37C polypeptide. This band is absent in both homo- and hemizygous *fs(2)TWI*¹ mutant

individuals. The band can be seen again in hemizygous individuals which contain the P[γ TUB37C⁺ w⁺] transgene which rescues the mutant phenotype. Identical results are obtained with the M152 antibody raised against a full-length 6 $\times$ His-tagged fusion of the γ TUB37C protein (not shown). Therefore, we are confident that the Rb1011 antibody specifically recognizes the γ TUB37C isoform and that this polypeptide is encoded by complementation group *fs(2)TWI*. Moreover, the absence of the 51 kDa polypeptide in *fs(2)TWI*¹ homozygous tissues indicates that this mutation results in either a lack or a very severe loss of γ TUB37C function.

Mutation in the γ TUB37C gene results in female sterility

The extant collection of mutant alleles of the 37C γ -tubulin gene includes 15 ethyl methanesulfonate (EMS)-induced alleles which were obtained in two independent mutagenic experiments performed by Stathakis *et al.* (1995) and Schüpbach and Wieschaus (1989). The only major phenotype displayed by individuals hemizygous for most of the chromosomes carrying these mutant alleles is female sterility; no alterations in viability or male fertility have been observed, and major cuticle abnormalities are also absent. The same conclusion holds true for individuals carrying every possible heteroallelic combination of these mutant chromosomes, including hemizygosity. The only exception to this rule is the *fs(2)TWI*¹ chromosome which, in addition to female sterility, caused reduced viability and impaired male fertility when homozygous. Nevertheless, viability and male fertility were normal in individuals heteroallelic for *fs(2)TWI*¹ and any other mutant allele of this gene, suggesting that a second mutation present in this chromosome was responsible for the additional phenotypic traits. This point was confirmed by meiotic recombination which showed the presence of a second mutation which mapped in the *c–px* interval and affected viability and male fertility. Different lines of *fs(2)TWI*¹ recombinants free from the second mutation were obtained and all of them showed female sterility as the only obvious phenotypic trait. Female fertility is rescued back to wild-type levels in these flies when the P[γ TUB37C⁺ w⁺] transgene is introduced. On the basis of this genetic evidence, it seems reasonable to conclude that the product of the *fs(2)TWI* gene provides a function which is essential exclusively for female fertility. All the phenotypic analyses involving the *fs(2)TWI*¹ allele described below were carried out with clean recombinant lines.

The γ TUB37C polypeptide is required to organize the female meiotic spindle

To characterize further the alterations which bring about the female sterility phenotype displayed by mutations in the γ TUB37C locus, we performed a cytological analysis of the process of oogenesis in these mutants. Four mutant alleles were included in this study, both in *trans*-heteroallelic combinations and hemizygous over *Df(2L)VA23* which uncovers the γ TUB37C gene. None of these combinations showed any abnormalities in any of the main morphological features that can be observed by phase-contrast microscopy and staining of DNA with 4',6'-diamidino-2-phenylindole (DAPI), from germarium up to stage 13. These included the average number of ovarioles

Fig. 5. Meiotic figures in oocytes. (a), (b) and (c) are micrographs obtained from stage 14 oocytes. The panels in the bottom are drawings from oocytes at the same stage. (a) and the two drawings on the left top corner correspond to wild-type females. (b), (c) and the remaining drawings represent meiotic figures found in females which carried different heteroallelic combinations of mutants for the γ TUB37C gene.

per ovary, the number of nurse cells per egg chamber and the position and size of the oocyte nucleus. The localization of cyclin B transcripts at the posterior pole of the presutiv oocyte, which has been shown to require microtubules (Theurkauf *et al.*, 1993), was also indistinguishable from the wild-type (data not shown). Thus, the function provided by the γ TUB37C isoform does not seem to be essential for germ cell proliferation, oocyte differentiation or egg chamber maturation.

The only recognizable phenotype that we have identified in association with mutation in the γ TUB37C isoform occurs in the final stage of oogenesis (stage 14 after King, 1970) and consists of a dramatic alteration of chromosome behavior during meiosis. In wild-type females, meiosis is usually arrested at the metaphase of the first meiotic division (King, 1970; Mahowald and Kambyzellis, 1980), and only after passage through the oviduct is the block released and meiosis proceeds (Doane, 1960). The metaphase-I arrest in wild-type females (Figure 5a) is characterized by a very distinctive chromosome arrangement in which non-recombinant homologs are segregated, one at each side of the spindle, and recombinant bivalents remain in the middle (Theurkauf and Hawley, 1992). Therefore, although these figures can vary depending on which chromosomes undergo recombination, they always show a bilateral symmetry and the central points of all the chromatin figures define a line which corresponds with

Fig. 6. Immunofluorescence analysis of the meiotic spindle in *fs(2)TW1* mutant oocytes. (a) Meiotic figure in a stage 14 oocyte from a wild-type female. (b–f) Meiotic figures found in oocytes from *fs(2)TW1* homozygous females. Tubulin was visualized with the DM1a anti- α -tubulin antibody (green) and DNA was stained with propidium iodide (red). All figures shown are projections made from 10–17 optical sections generated by confocal microscopy.

the pole–pole axis of the meiotic spindle (Theurkauf and Hawley, 1992).

This arrangement is severely disrupted in oocytes mutant for γ TUB37C (Figure 5b and c). Both the bilateral symmetry and the linear arrangement of all the chromatin masses are very often lost. The analysis of a total of 350 meiotic figures in individuals carrying different heteroallelic combinations of mutants for the γ TUB37C locus revealed no consistent pattern of chromosomal arrangement. We believe, therefore, that chromosomes are arranged randomly in these oocytes. The percentages of abnormal meiotic figures observed in oocytes of *fs(2)TW1* mutant females were ~80% for hemizygous *fs(2)TW1* and *fs(2)TW1*³, 30% for *fs(2)TW1*^{HL2} and 15% for *fs(2)TW1*^{RU34}. Therefore, there is a clear difference in the extent to which meiosis is disrupted by each of these alleles, ranging from the very strong phenotype displayed by *fs(2)TW1* to the weak effect produced by *fs(2)TW1*^{RU34}.

To determine whether the abnormal meiotic figures displayed by oocytes mutant for γ TUB37C could be due to alterations of the female meiotic spindle, we performed immunofluorescence analysis of mutant oocytes. We studied oocytes from *fs(2)TW1*¹/*Df(2L)VA23* and *fs(2)TW1*¹ homozygous females, which, as described above, show a very high level of abnormal meiotic figures. Tubulin was visualized with the DM1a anti- α -tubulin antibody (Blöse *et al.*, 1984), which previously has been shown to recognize the *Drosophila* female meiotic spindle (Theurkauf *et al.*, 1992). The results of this study are summarized in Figure 6. A typical wild-type female meiotic spindle is shown in Figure 6a. As reported by

Fig. 7. Immunofluorescence analysis of embryos derived from *fs(2)TWI^l* homozygous females. Distribution of DNA (**a**, **b** and **c**) and co-localization of DNA (red) and tubulin (green) (**d**, **e** and **f**) in 0- to 30-min-old embryos derived from wild-type (**a** and **d**) and *fs(2)TWI^l* homozygous females (**b**, **c**, **e** and **f**). Tubulin was visualized with the DM1a anti- α -tubulin antibody and DNA was stained with DAPI.

Theurkauf and Hawley (1992), these spindles do not have asters at the poles and are highly tapered, with the highest concentration of microtubules immediately adjacent to the main chromatin mass. Chiasmate chromosomes are found in the middle of the spindle and achiasmate chromosomes, which move precociously to the poles, are evenly distributed at each side of the spindle.

Most of these features are severely altered in mutant oocytes, although the extent of disruption is rather variable (Figure 6b–f). Firstly, the microtubular structure associated with the chromosomes in these oocytes is less dense than a wild-type spindle and is far less uniform. Instead, tubulin seems to be clumped in some regions and very reduced or absent in others. Secondly, the structure found in mutant oocytes is not spindle shaped; although it still appears somewhat rod like, the meiotic microtubular structure found in mutant females is not focused at the poles and, in contrast to the normal meiotic spindle, is not wider in the middle. Finally, the position of the microtubular structure has no significant correlation to the distribution of the chromatin masses. Although they are always very close, probably in direct contact, it is not possible to predict the position of the microtubular array found in mutant oocytes on the basis of the localization of the chromosomes. These observations led us to conclude that the function provided by the γ TUB37C polypeptide is required to organize the meiotic spindle in *Drosophila* females.

Early cleavage divisions are also severely impaired by mutation in the γ TUB37C isoform. Embryos derived from mutant mothers display very abnormal patterns of chromatin distribution. Some embryos contain many small chromatin masses scattered throughout (Figure 7b) while others contain only a few, very large ones (Figure 7c). Microtubule organization is extremely abnormal as well;

only aster-less microtubular arrays of varying sizes and shapes can be observed associated with the chromatin masses (Figure 7e and f). Development is arrested very early in these embryos, i.e. they never reach the syncytial blastoderm stage. Interestingly, the embryonic phenotype is very severe even in cases like the *fs(2)TWI^{RU34}* allele which displays a very weak meiotic phenotype. Therefore, it seems likely that the embryonic phenotype is due to a real requirement for γ TUB37C function during embryogenesis and is not just a pleiotropic effect brought about by the abnormal meiosis displayed by mutant oocytes. This conclusion is consistent with the results of our immunofluorescence analysis which revealed the presence of substantial amounts of the γ TUB37C polypeptide in the centrosome of very early embryos (Figure 3).

Discussion

There are two main conclusions that can be drawn from the results presented here. Firstly, we have shown that female meiosis in *D.melanogaster* requires γ -tubulin. Secondly, our results provide an instance in which the expression of γ -tubulin is under tight developmental regulation in a metazoan.

The relevance of the first conclusion is based on the fact that female meiosis in *Drosophila* is generally regarded as being driven by a centrosome-less spindle and, indeed, is very often quoted as an example of how spindles can be built in the absence of centrosomes. The *Drosophila* female meiotic spindle is atypical in many ways (reviewed in Sawin and Endow, 1993; McKim and Hawley, 1995): the spindle poles do not display astral microtubules and do not contain centrioles (Sonnenblick, 1950); many of the spindle microtubules terminate before reaching the spindle poles (Theurkauf and Hawley, 1992); and a grow-

ing body of evidence indicates that chromatin plays a major role in spindle assembly (Hatsumi and Endow, 1992; Theurkauf and Hawley, 1992; McKim *et al.*, 1993; Matthies *et al.*, 1996). It seems quite clear, therefore, that canonical centrosomes of the kind found in mitotic cells, i.e. a pair of centrioles surrounded by pericentriolar material from which aster and spindle microtubules nucleate, are absent in the meiotic spindle of *Drosophila* females. These considerations, and the failure to visualize by immunofluorescence some of the polypeptides which are present at the poles of the mitotic spindles in *Drosophila* cells, such as CP190, CP60 (Whitfield *et al.*, 1988; Kellogg *et al.*, 1989) and γ -tubulin itself (Matthies *et al.*, 1996; reference quoted in McKim and Hawley, 1995; and our own results) has led to the hypothesis that the organization of the female meiotic spindle may not require the gene products which are present in the MTOCs of mitotic spindles (McKim and Hawley, 1995; Matthies *et al.*, 1996; Riparbelli and Callaini, 1996).

Our results suggest otherwise, and clearly argue that at least one of the main components of the MTOCs of mitotic cells is required to build the female meiotic spindle in *Drosophila*. The failure to visualize a localized signal of γ -tubulin over these spindles suggests that either this polypeptide gets detached from microtubules before the bipolar spindle is formed or it is evenly scattered over the spindle. This in turn means that the failure to visualize by immunofluorescence other centrosome-associated gene products like CP190 and CP60 on the female meiotic spindle (Matthies *et al.*, 1996) should not be taken as evidence to conclude that their function is not required. Therefore, the difference between the microtubule-organizing materials of mitotic and female meiotic spindles may not affect so much their molecular composition as their structure, shape and subcellular localization, along the lines discussed by Mazia (1984). Hopefully, a definitive answer to this question will be provided when mutant alleles of other MTOC-related genes are identified. This point is particularly relevant in the case of CP60 and CP190 because it has been reported that in embryo extracts γ -tubulin is part of a complex that contains these two proteins, and that a substantial fraction of the γ -tubulin present in this complex is tightly associated with CP60 (Raff *et al.*, 1993). It is not clear, though, which isoform of γ -tubulin is found in this complex.

What the precise role of the γ TUB37C polypeptide in organizing the female meiotic spindle may be, we do not know. The process of meiotic spindle assembly in *Drosophila* has been divided into four phases (Theurkauf and Hawley, 1992). During the second phase, microtubule association with the chromatin mass takes place. Whether these microtubules are captured or nucleated from the chromatin mass itself is not known. During the third phase, microtubules are bundled into a bipolar spindle in a process which requires the function of kinesin-like proteins such as *ncd* (Hatsumi and Endow, 1992; Matthies *et al.*, 1996). Thus, the simplest hypothesis is that the γ TUB37C polypeptide is required to polymerize the microtubules which will later on be organized into a bipolar spindle, although a more direct involvement in spindle assembly, kinetics or stability cannot be ruled out at this stage. Whether γ TUB37C may also play any role in the establishment of spindle bipolarity is not clear. On the

one hand, in the most extreme cases, the meiotic figures displayed by mutants in the γ TUB37C gene do not contain a bipolar spindle, but this may well be due to the fact that the microtubules associated with these nuclei are so short and so few that they are not good substrates for bundling. On the other hand, some γ TUB37C mutant nuclei display reasonably populated microtubular structures which are largely bipolar, and yet are not spindle shaped. This phenotypic variability is itself rather intriguing since it is found in alleles like *fs(2)TW1¹* in which the γ TUB37C polypeptide cannot be detected by Western blot, suggesting that the *fs(2)TW1¹* mutation results in either a lack or a very severe loss of γ TUB37C function.

There is an interesting parallelism between the expression patterns and mutant phenotypes of the 37C γ -tubulin described here and those of the α TUB67C described by Matthews *et al.* (1993). Although we are unable to establish any functional link at present, this fact cannot be disregarded. The 67C isoform of α -tubulin is a structurally divergent α -tubulin whose expression is exclusively maternal. Genetic analysis of this locus shows that this isoform is required for meiosis and syncytial blastoderm mitosis (Matthews *et al.*, 1993). These authors showed that both loss of function and overexpression of this isoform are detrimental for spindle function during early embryogenesis, and hypothesized that this isoform could allow the unusually rapid assembly and disassembly that is characteristic of the *Drosophila* cleavage spindles (Rabinowitz, 1941; Sonnenblick, 1950; Zalokar and Erk, 1976; Foe and Alberts, 1983). In the absence of more direct evidence in this regard, the same argument can be made for the 37C γ -tubulin isoform. We currently are testing for possible interactions between the mutant alleles of these two loci.

Drosophila is not the only organism in which the female meiotic spindle lacks aster microtubules and centrioles. Microtubules are nucleated in the absence of centrioles in mammalian oocytes and early embryos. In unfertilized mouse oocytes, meiosis is arrested in the second meiotic metaphase. At this stage, γ -tubulin is concentrated in the broad spindle poles of the meiotic spindle and at the distinct foci which form the centers of the cytoplasmic microtubule asters (Palacios *et al.*, 1993). The integrity of these γ -tubulin foci and their cytoplasmic location is maintained during drug- or cold-induced depolymerization of microtubules (Gueth-Hallonet *et al.*, 1993).

The second major conclusion that can be drawn from this work is that the expression of the γ TUB37C isoform is under tight developmental regulation. One of the most challenging questions raised by the characterization of γ -tubulin in *Drosophila* is why are there two different isoforms in this organism. The two γ -tubulins found in *D.melanogaster* show slightly more than 80% homology, which is much less than that shown between the human and *Xenopus* γ -tubulins (98%), and about as much as between any of them and their *Xenopus* homolog. Whilst this relatively large divergence between the two *Drosophila* isoforms may be indicative of functional divergence (Oakley, 1994), even more distant γ -tubulins seem to have a wide range of functional compatibility, as shown by Horio and Oakley (1994) who reported that human γ -tubulin can restore the growth rate to nearly normal viability in γ -tubulin-deficient *S.pombe*. Nevertheless, the

different expression patterns shown by the two γ -tubulin isoforms found in *Drosophila* suggest that, superimposed on their basic role in microtubule polymerization, they may have acquired functions which are specific for the microtubule organization requirements of the developmental stages at which they are expressed. This point is substantiated further by the very different phenotypes displayed by mutant alleles of these two genes. The experiments presented here show that the expression of the 37C isoform is restricted to ovaries and early embryos, and mutation in this gene has no major consequence other than female sterility. In contrast, the 23C isoform is ubiquitously expressed and mutation in this gene impairs viability (Sunkel *et al.*, 1995).

With the exception of *Arabidopsis*, in which two γ -tubulin isoforms differing in nine amino acid residues have been reported (Liu *et al.*, 1994), only one γ -tubulin isotype has been found in each of the other species which have been looked at. Since this is clearly not the case in *Drosophila*, it would be interesting to re-examine this point and determine how many γ -tubulins are expressed in other species. We currently are isolating several γ -tubulin genes from different species of arthropods, including insects, crustaceans and arachnids, in the hope that obtaining evolutionary information about the divergence between the two isoforms may provide us with some additional insights into their function.

Materials and methods

Drosophila cultures and stocks

Cultures were maintained on standard *Drosophila* medium supplemented with active dry yeast and were raised at 25°C unless stated otherwise. *fs(2)RU34* and *fs(2)HL2* were isolated by Schüpbach and Wieschaus (1984); *fs(2)TW1¹*, *Df(2L)VA6*, *Df(2L)VA16*, *Df(2L)VA17*, *Df(2L)VA18*, *Df(2L)VA19*, *Df(2L)VA23* and *Df(2L)TW158* are described in Lindsley and Zimm (1992). A description of the other mutants and chromosomes that were used can be found in Lindsley and Zimm (1992). The *fs(2)TW1¹* chromosome was recombined with a multiple marked chromosome carrying *al dp b pr c px sp*. Recombinants on each interval were isolated and tested for male and female sterility.

Cloning of the 37C isoform of γ -tubulin

A PCR fragment of 592 bp was amplified from genomic DNA using the 22mer oligos 5' GTT GTA CAA CCA GGA GAA TGT G and 5' CCT CGC AGT CGG ACA TTA GCG G. This fragment was used to screen a *Drosophila* genomic DNA library constructed in λ -dash (C.Gonzalez, unpublished data). The screening was carried out using the non-radioactive ECL random prime labeling and detection system from Amersham. Phages spanning a total of ~20 kb around the γ -tubulin gene were isolated and mapped by restriction analysis using standard methods (Sambrook *et al.*, 1989).

P element-mediated germ-line transformation

A genomic *EcoRI* fragment of 7.2 kb was subcloned into pW8 (Klemenz *et al.*, 1987) and the obtained plasmid P[γ TUB37C⁺ w⁺] was used for germ-line transformation as in Spradling (1986). Single transformants carrying P[γ TUB37C⁺ w⁺] either in the X or in the third chromosome were selected and used to rescue mutants in the 37C region.

Generation of antibodies

The Rb1011 antibody was raised in rabbits against a synthetic peptide specific for the γ TUB37C isoform (PQWSPAVEASKAGK) conjugated to keyhole limpet hemocyanin (KLH). The M152 antibody was raised in mice against bacterially expressed full-length γ TUB37C cDNA isolated from a *Drosophila* embryonic cDNA library (Brown and Kafatos, 1988). The cDNA was subcloned into the pQE30 vector which carries a 6×His tag (Qiagen). The expressed protein was purified according to the

protocol provided by Qiagen. The Rb1011 and M152 antibodies were affinity purified against the synthetic peptide and the full-length protein, respectively.

Western blots

Protein extracts from embryos, testis, ovaries and larval brains were prepared by homogenizing in SDS-PAGE sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, 0.062 M Tris-HCl pH 6.8), boiling them for 3 min and spinning them at top speed to clear the lysate. Even loading of the gels was assessed by Coomassie staining. Total serum anti- γ -tubulin antibodies were used in a 1:500 dilution; affinity-purified antibodies were diluted 1:10; horseradish peroxidase (HRP)-coupled secondary antibodies (Jacksons) were diluted 1:2000. Blocking and incubation with the antibodies were carried out in a solution of 5% powder milk in TNT (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween). Detection of the antibody was done either with the ECL Western blot detection system (Amersham), or using VIP substrate (Vector laboratories), according to the manufacturer's instructions.

Cytology

In situ hybridization to polytene chromosomes and immunostaining of embryos was performed as described in Gonzalez and Glover (1993). Two fixation conditions were used. When microtubule preservation was not needed, we used 3.7% formaldehyde, which gives the best results with our anti- γ -tubulin antibodies. When microtubule preservation was required, we used methanol fixation. Taxol was never included.

DAPI staining of ovaries and embryos was carried out as described by Ruohola *et al.* (1991). *In situ* hybridization to whole-mount ovarioles was performed according to the method of Tautz and Pfeifle (1989) following the modifications described in Dalby and Glover (1992).

The frequency of abnormal meiotic figures was quantified as the percentage of oocytes in which the linear arrangement of the chromatin masses or the bilateral symmetry of the meiotic figure was lost.

Immunostaining of stage 14 oocytes was performed as follows. Ovaries were dissected in absolute methanol and transferred to a 10 ml plastic tube containing ~2 ml of fresh methanol. About 10–20 single ovaries prepared in this way were sonicated with a Sonifier B-12 from Branson Sonic Power Company fitted with a cone-shaped probe of ~3–4 mm in diameter at the bottom. Sonication was applied in five cycles of 1 s each. Oocytes without chorion and vitelline membrane were transferred to fresh methanol and kept at room temperature for a further 2 h. Immunostaining was carried out as described in Gonzalez and Glover (1993).

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