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The *Tomaj* mutant alleles of *αTubulin67C* reveal a requirement for the encoded maternal specific tubulin isoform in the sperm aster, the cleavage spindle apparatus and neurogenesis during embryonic development in *Drosophila*

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SUMMARY

The three dominant *Tomaj^D* and their eleven revertant (*Tomaj^R*) alleles have been localized to the *αTubulin67C* gene of *Drosophila melanogaster*. Although the meiotic divisions are normally completed in eggs laid by *Tomaj^D*/+, *Tomaj^D*/−, *Tomaj^R*/− females, embryogenesis arrests prior to the gonomeric division. The arrest is caused by: (1) the failure of prominent sperm aster formation; and (2) a consequent lack of female pronuclear migration towards the male pronucleus. Concomitant with the sperm aster defect, the four female meiotic products fuse (tetra-fusion), similar to what is seen in eggs of wild-type virgin females. In eggs of females heterozygous for weaker *Tomaj^R* alleles, embryogenesis comes to a cessation before or shortly after cortical migration of cleavage nuclei. The apparent source of embryonic defect is the cleavage spindle apparatus. One of the three *Tomaj^D* alleles is cold-sensitive and its cold-sensitive period coincides with the completion of female meiosis and pronuclear migration. Disorganized central and peripheral nervous systems are also characteristic of embryos derived from the temperature-sensitive *Tomaj^D*/+ females. The *Tomaj* mutant phenotypes indicate an involvement of the normal *αTubulin67C* gene product in: (1) the formation of the sperm aster; (2) cleavage spindle apparatus formation/function; and (3) the differentiation of the embryonic nervous system. The *Tomaj^D* alleles encode a normal-sized αTubulin67C isotype. Sequence analyses of the *Tomaj^D* alleles revealed the replacement in different positions of a single negatively charged or neutral amino acid with a positively charged one. These residues presumably identify important functional sites.

Key words: Sperm aster, α-Tubulin, Microtubule, *Drosophila*, Female sterility

INTRODUCTION

Initiation of embryogenesis in *Drosophila* requires the contribution of both maternal and paternal components, e.g. the female and male pronuclei, the egg cytoplasmic factors produced during oogenesis and the sperm-derived centriole. A set of intricate events, shortly after meiosis I metaphase release and fertilization lead to commencement of embryogenesis (Foe et al., 1993; Callaini and Riparbelli, 1996; Riparbelli and Callaini, 1996; Sagata, 1996). We have previously described a collection of dominant female sterile (*Fs*) mutations that, based on their phenotypes, appear to identify important and/or specific components required for the initiation of embryogenesis in *Drosophila* (Erdélyi and Szabad, 1989; Szabad et al., 1989).

This paper presents the developmental genetic and molecular analysis of three dominant female sterile *Tomaj* (*Tomaj^{D1-3}*) alleles and their eleven revertant (*Tomaj^R*) alleles that identify the *αTub67C* locus, a member of the *Drosophila α-tubulin* gene family. This gene family consists of four genes: *αTub67C, αTub84B, αTub84E* and *αTub84D* (Kalfayan and Wensink, 1981; Fryberg and Goldstein, 1990). The *αTub84B* and the *αTub84D* genes are constitutively expressed during all stages of development and encode isotypes differing at only 2 of 450 amino acids (Kalfayan and Wensink, 1982; Theurkauf et al., 1986; Matthews et al., 1989). The *αTub84E* encoded protein differs from the constitutively expressed isotypes at 21 of 450 amino acids and is expressed only in the testis and peripheral nervous system (Theurkauf et al., 1986; Bo and Wensink, 1989; Matthews et al., 1990). The

αTub67C gene encodes the most divergent α-tubulin sharing only 65% amino acid identity with the constitutively expressed α-tubulins (Theurkauf et al., 1986). The *αTub67C* gene is transcribed only in the female germ line during oogenesis, and the encoded mRNA is present in the ovaries and in early embryos (Matthews et al., 1989). Translation occurs only in the ovaries, and the αTub67C isotype accounts for 20% of the α-tubulin pool in a mature egg (Kalfayan and Wensink, 1982; Natzle and McCarthy, 1984; Matthews et al., 1989, 1990, 1993).

The α- and β-tubulin heterodimer represents the structural unit of microtubules (MTs). In general, MTs are important cytoskeletal proteins with different functions during both the cell cycle and development. Structural and functional heterogeneity of the MTs derives from the α- and β-tubulin heterodimers as well as the MT associated proteins (MAPs) e.g. motor molecules and stabilizing proteins. Alternatively, the MAPs can act as targets for intracellular regulatory signals (Holzbaur and Valle, 1994; Moore and Endow, 1996). Furthermore, different MT functions may originate from the combination of numerous posttranslational modifications like tyrosination-detyrosination, polyglycylation, polyglutamylation, acetylation and phosphorylation-dephosphorylation (Joshi and Cleveland, 1990; Bulinski and Gundersen, 1991; Theurkauf, 1992; Paturle-Lafanechere et al., 1994; Larcher et al., 1996; Mary et al., 1996; Multigner et al., 1996). Additionally, modulation of MT activity during the cell cycle is governed by centrosomes. Centrosomes play key roles in the nucleation of MT polymerization, MT depolymerization, release and capture of MTs as well as the establishment of MT polarity (Kimble and Kuriyama, 1992; Schatten, 1994; Raff, 1996).

During *Drosophila* embryogenesis, the αTub67C isotype seems to have a specialized or restricted functional capacity. Matthews et al. (1993) and Komma and Endow (1997) have proposed, based on phenotypes associated with the $\alpha Tub67C^{1\text{-}4}$ mutant alleles, that the αTub67C isotype is required for the proper structure and function of the MTs during female meiosis and cleavage divisions. Further, the possible involvement of the αTub67C isotype in differentiation of the embryonic nervous system was proposed by Theurkauf (1992).

The $Tomaj^{D}$ and $Tomaj^{R}$ mutant alleles provide new insights into the function of the α*Tub67C* gene during *Drosophila* embryonic development. Our developmental genetic analyses have shown that the function of the *αTub67C* gene is required for: (1) the formation of a prominent sperm aster, which ensures the appropriate positioning of the male and female pronuclei; (2) the normal structure and function of the cleavage spindle apparatus; and (3) the differentiation of the embryonic nervous system, which requires MTs that contain the αTub67C tubulin isotype.

MATERIALS AND METHODS

Strains and culture

Drosophila cultures were kept at 25°C on standard cornmeal agar medium. Marker mutations, the *Dp(3;3)S2a2* duplication and the *Df(3L)AC1* deficiency as well as the balancer chromosomes were described by Lindsley and Zimm (1992). The three female sterile $Tomaj^{D}$ mutations and their EMS-induced revertants were previously described by Erdélyi and Szabad (1989), and are summarized in Table 1. Additional revertants were induced by P-element and X-ray mutagenesis, according to the method of Erdélyi and Szabad (1989). We also made use of the $\alpha Tub67C^{1\text{-}4}$ mutant alleles that were described by Matthews et al. (1993).

Genotypes analyzed

For the developmental genetic analysis of the mutant phenotypes, egg chambers and eggs derived from females with the following genotypes were analyzed: wild-type (*Canton-S*), $Tomaj^{D}/+$, $Tomaj^{D}/+/+$ (where + stands for the *Dp(3;3)S2a2* duplication), $Tomaj^{D}/-$ (where – stands for the *Df(3L)AC1* deficiency), $Tomaj^{D}/Tomaj^{R}$, $Tomaj^{R}/Tomaj^{R}$, $Tomaj^{R}/\alpha Tub67C^{1\text{-}4}$. Females with the above mentioned genotypes were mated with wild-type males and over a hundred of their eggs were collected and analyzed. In some experiments we also analyzed eggs of virgin $Tomaj^{D}/+$, $Tomaj^{R}/-$ and wild-type females.

Immunostaining, Feulgen-Giemsa staining, light and confocal microscopy

To analyze meiosis I, mature oocytes were isolated and fixed according to the method of Theurkauf and Hawley (1992) and Puro (1991). For analysis of meiosis II and the pronuclear stage of embryogenesis, eggs were squeezed out from the uterus. Whole-mount eggs and embryos were processed and fixed according to Whitfield et al. (1988), Matthews et al. (1990), Karr (1991), Puro (1991) and Maldonado-Codina and Glover (1992). The Feulgen-Giemsa double stainings were carried out according to the method of Puro (1991).

The MTs were detected with YL1/2 rat monoclonal anti-tubulin (Sera Lab) or mouse monoclonal anti-αtubulin (Amersham) antibodies. Centrosomes were detected by either the Rb188 rabbit anti-centrosome (specific for CP190 antigen) or CNN rabbit anti-centrosomin antibodies (Whitfield et al., 1988; Li and Kaufman, 1996). The mouse T47 anti-lamin monoclonal antibody was used to stain the nuclear lamina (Paddy et al., 1990). The sperm tail was detected with the Ax-D5 mouse monoclonal antibody (Karr 1991). The embryonic central and peripheral nervous systems (CNS and PNS) were stained with the 22C10 mouse monoclonal antibody (Fujita et al., 1982; Goodman and Doe, 1993; Jan and Jan, 1993).

Samples were incubated in the primary antibodies overnight at 4°C. The secondary antibodies were obtained from Jackson Immunoresearch Laboratories Inc. (Avondale, PA). Incubations with the secondary antibodies were performed either at room temperature for 2 hours or overnight at 4°C. When appropriate, DNA was stained with propidium iodide (PI). Specimens were mounted in 85% glycerol, containing 2.5% *n*-propyl gallate.

A Zeiss Axiolab light microscope was used to analyze the Feulgen-Giemsa stained specimens. Digital images of optical sections through fixed and immunostained specimen were collected with a Zeiss 410 confocal laser scanning microscope. Confocal images were analyzed by the Carl Zeiss Microscope System LSM version 3.59 software.

Temperature sensitivity of $Tomaj^{D2}$

The $Tomaj^{D2}$ allele is temperature sensitive. At 18°C, $Tomaj^{D2}$ has a fully penetrant dominant female sterile phenotype. However, at 29°C larvae hatch from about 20% of the eggs, most of which develop into adults. The remaining 80% of the eggs show polyphasic lethality. To determine the temperature-sensitive period, $Tomaj^{D2}/+$ females were mated to *Canton-S* males and temperature shift experiments performed on the crosses. In the shift-down experiment, flies were shifted down from 29°C to 18°C, eggs collected at two hour intervals and the hatch rate monitored. In the shift-up experiment, the parent flies were transferred from 18°C to 29°C, eggs collected at one hour intervals and the hatch rate determined. Preparations of the cuticle-producing dead embryos were analyzed to identify their mutant phenotype (Wieschaus and Nüsslein-Volhard, 1986).

Germline autonomy of the *Tomaj^{R}* phenotype

To decide whether the *Tomaj^{R}* mutations affect the female germ line or soma, two types of germ line chimeras were constructed. In the first set of experiments *Tomaj$^{RE1.1}$/Tomaj$^{RE3.1}$*, *Tomaj$^{RE2.1}$/Tomaj$^{RE3.1}$* pole cells (progenitors of germ line cells) were transplanted into *Fs(1)K1237*/+ host females (Table 2). The *Fs(1)K1237* (= *ovo^{D1}*) mutation blocks germ line function without affecting the somatic cells (Komitopoulou et al., 1983). The eclosing *Fs(1)K1237*/+ host females were mated with *mwh e* males and the progeny scored for these markers. In the reciprocal experiment wild-type pole cells marked with *y v f mal* marker mutations were transplanted into *Tomaj$^{RE1.1}$/Tomaj$^{RE3.1}$*, *Tomaj$^{RE2.1}$/Tomaj$^{RE3.1}$* host embryos (Table 3). The eclosing *Tomaj^{R}/Tomaj^{R}* host females were mated with *y v f mal* males and the progeny scored for these markers. The eclosing *Tomaj^{R}/TM3, Tomaj^{R}/TM6 and TM3/TM6* sibling chimeras served as internal controls.

The molecular nature of the *Tomaj^{D}* alleles

The 7 kb *Bam*HI clone containing the wild-type *αTub67C* gene was kindly provided by K. A. Matthews (Kalfayan and Wensink, 1981; Matthews et al., 1989). Since the *αTub67C* gene is included in a *Bam*HI fragment, genomic libraries were generated after partial *Bam*HI digestion from Canton-S, *mwh e* as well as each of the *Tomaj^{D}/Df(3L)AC1* flies. The *αTub67C* clones were identified in two steps: first by an α-tubulin specific 1.7 kb *Xho*I-*EcoR*I probe and by a second 96 bp *Bst*NI-*Ava*II probe specific only for the *αTub67C* gene. The five clones containing the two wild-type and the three *Tomaj^{D}* alleles were subcloned into M13 and then were sequenced on both strands by the dideoxy method using the USB DNA Sequencing Kit (Sequenase Version 2.0).

Bacterial expression of the *Tomaj^{D}* encoded αTub67C isotypes

For the bacterial expression of wild-type *αTub67C* and the three *Tomaj^{D}* encoded αTub67C isotypes, DNA fragments corresponding to the cDNA region were generated by PCR both from the cloned and the genomic DNAs. Two primers were used: 5′CGTA CAT ATG CGA GAA GTA GTG TCC, and 5′GCTA GGA TCC TTA GAA CTC ATC GAA GTC C. The PCR fragments were cleaved with *Nde*I and *Bam*HI at the primer region and inserted into pET3d expression vectors (Promega), resulting in pETαTub67C^{+}, pETTomaj^{D1}, pETTomaj^{D2} and pETTomaj^{D3} plasmids. The *Escherichia coli* BL21 cells transformed with the expression constructs produced the expected proteins following IPTG induction. The bacterially expressed proteins were identified on western blots probed with a polyclonal antiserum against the αTub67C isotype.

To generate polyclonal antiserum against αTub67C, an oligopeptide was synthesized corresponding to amino acid residues 32-61. This sequence is specific to the αTub67C isotype among the *Drosophila* α-tubulins. The serum was affinity purified and used in western blot analyses. On western blots of bacterially expressed proteins, the antiserum solely identified the wild-type and *Tomaj^{D1-3}* encoded Tub67C isotypes in the same molecular mass range (48 kDa). Unfortunately, the antiserum does not recognize αTub67C in situ (following different fixation procedures), suggesting that the antigenic determinants within the N-terminal domain of αTub67C tubulin are unexposed on the MT surface or were masked by other proteins.

RESULTS

The *Tomaj^{D1}* and *Tomaj ^{D3}* mutant phenotypes

Heterozygous or hemizygous *Tomaj^{D1}* and *Tomaj^{D3}* females are sterile: they lay normal numbers of phenotypically normal eggs that do not hatch. Sperm tail and centrosome antibody stainings clearly show that the majority of the eggs are fertilized. Typically, the first cleavage (gonomeric) division is never established in these eggs (Fig. 1).

Female meiosis

To elucidate whether abnormal female meiosis is the reason for the absence of the gonomeric division, we analyzed stage 13 and 14 oocytes and eggs from the uteri of wild-type and both *Tomaj^{D1}* and *Tomaj^{D3}* heterozygous and hemizygous females. The oogenetic stages 13 and 14 correspond to meiosis I prophase and metaphase, while a high proportion of the eggs squeezed out of the uterus show meiosis II. It was apparent, based on both Feulgen-Giemsa and combined anti-tubulin and DNA stainings, that both meiosis I and II proceeded normally (data not shown), and thus defective female meiosis is unlikely to be the source of the lack of commencement of embryogenesis in eggs of *Tomaj^{D1}* or *Tomaj^{D3}* heterozygous or hemizygous females.

Sperm aster defect

The anti-tubulin, anti-centrosome (Rb-188, CNN), anti-lamin and DNA stainings on the newly deposited eggs by heterozygous and hemizygous *Tomaj^{D1}* and *Tomaj^{D3}* females revealed that the primary developmental defect seen was associated with sperm aster structure and function. In wild-type eggs, a small sperm aster emanates from the reorganized and replicated-separated centrosome after fertilization (Fig. 1A). The small sperm aster is composed of MT arrays and remains small until meiosis II anaphase. It then enlarges and becomes prominent by the end of meiotic II telophase (Callaini and Riparbelli, 1996) (Fig. 1B). In fertilized eggs of heterozygous and hemizygous *Tomaj^{D1}* and *Tomaj^{D3}* females a small sperm aster forms similar to that seen in wild-type fertilized eggs (Fig. 1C). However, the small sperm aster never grew to a prominent sperm aster in over a thousand *Tomaj^{D1}* or *Tomaj^{D3}* derived eggs. Failure of prominent sperm aster formation appears thus to be the primary *Tomaj^{D1}* and *Tomaj^{D3}* associated defect. Additionally, it appears that in the absence of a prominent sperm aster the subsequent migration of the female pronucleus towards the male pronucleus does not take place, and thus embryogenesis is blocked prior to the gonomeric division.

It is also interesting that in wild-type eggs, prominent sperm aster formation occurs after the first embryonic centrosome reorganization, replication and separation. The accumulation of CP190 and centrosomin in the first embryonic centrosome as a consequence of the reorganization of the paternally inherited basal body was evident in eggs of wild-type and both *Tomaj^{D1}* and *Tomaj^{D3}* females (Fig. 1A,C). As a rule, the sperm asters remained small, and the first embryonic centrosome clearly replicated once or twice, but the daughter centrosomes failed to separate in the *Tomaj^{D1}* or *Tomaj^{D3}* derived eggs (Fig. 1C).

Polar bodies

Concomitant with the failure of prominent sperm aster formation, the polar bodies migrate toward each other by means of MTs and fuse: first three and subsequently all four of the polar bodies combine (triplo- and tetra-fusion) (Fig. 2). The triplo- or tetra-fusion suggests that in the absence of female pronucleus specification and migration all four female

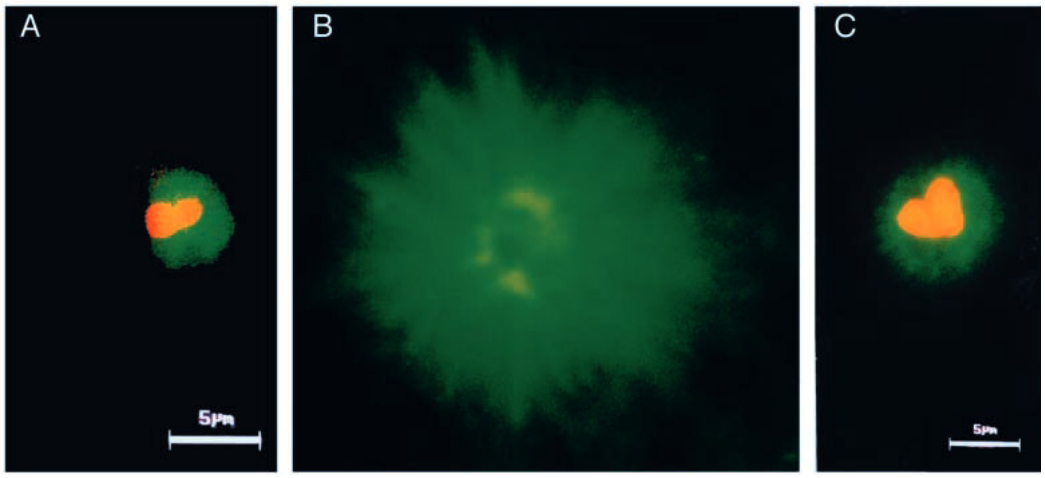

Fig. 1. The onset of embryogenesis in eggs of wild-type and *Tomaj*^{D} females. (A) Small sperm aster in a wild-type egg. (B) Prominent sperm aster in a wild-type egg. (C) Only a small sperm aster forms in the *Tomaj*^{D}-derived egg. Bar is identical for B and C. Tubulin appears in green. The centrosomes, which appear yellow in A and C, were detected using the Cp190 antibody. The replicated first embryonic centrosome is apparent in A. The daughter centrosomes fail to separate in the *Tomaj*^{D}-derived eggs.

meiotic products will differentiate into polar bodies. The chromatin decondensation inside the four polar body nuclei and the sperm head/male pronucleus takes place synchronously, indicating the normal functioning of regulatory mechanisms that synchronize pronuclear behaviour. The observation that triplo- and tetra-fusions are the common rule in unfertilized eggs of both wild-type and heterozygous and hemizygous *Tomaj*^{D1} and *Tomaj*^{D3} females (approx. 500 and over a thousand analyzed eggs, respectively), implicates the involvement of the prominent sperm aster in the specification of female pronucleus and its migration towards male pronucleus.

Cold-sensitive period of *Tomaj*^{D2}

Tomaj^{D2}/+ and *Tomaj*^{D2}/− females are sterile at 18°C and the *Tomaj*^{D1} or *Tomaj*^{D3} phenotype noted above is seen in about 10% of their eggs. In the remaining 90%, embryogenesis proceeded to stages 13 to 17, but was not completed. At 29°C, however, about 20% of the eggs hatch and a significant fraction of the larvae develop to adulthood. Thus, the *Tomaj*^{D2} alleles possesses dominant cold-sensitive female sterility, and temperature shift experiments were used to determine the time(s) that the *αTub67C* gene product is required. In the shift-up experiment the *Tomaj*^{D2}/+ females were raised to 18°C and then shifted to 29°C. One hour after the shift up, the 0% hatch rate elevated to 20% and subsequently remained at 20%. In the shift-down experiment the *Tomaj*^{D2}/+ females were raised to 29°C and then shifted to 18°C. The hatch rate dropped from 20% to 1% two hours after the shift down. These results show that the *Tomaj*^{D2} associated temperature-sensitive period covers stage 14 of oogenesis and the pronuclear stage of embryonic development.

Tomaj^{D2} and neurogenesis

At both 18°C and 29°C, 90% and 70%, respectively, of the *Tomaj*^{D2}/+ derived eggs escaped from pronuclear arrest and embryogenesis proceeded to stages 13 to 17, but was not completed. In such embryos the cleavage divisions, blastoderm formation and gastrulation proceeded normally as was inferred by immunostainings with anti-tubulin, anti-lamin antibodies

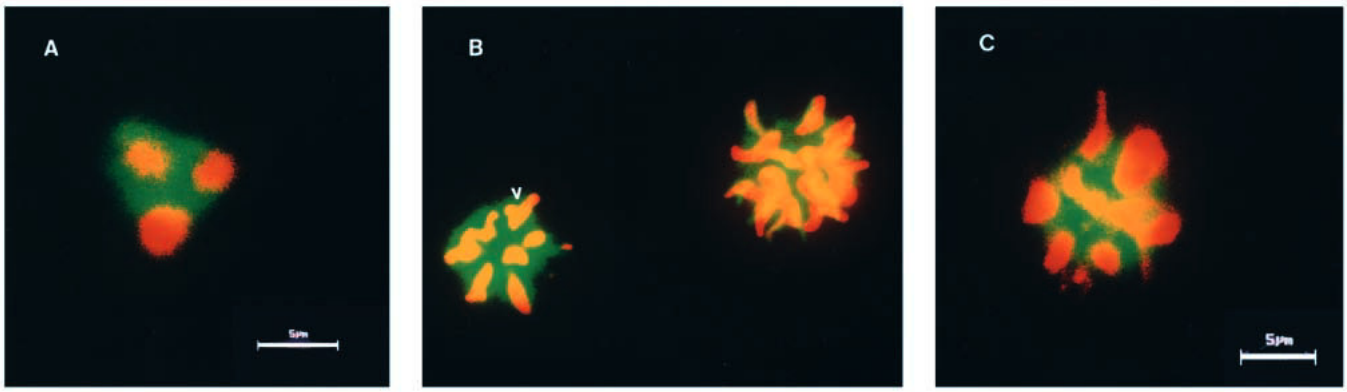

Fig. 2. The identical fate of meiotic products in eggs of unfertilized wild-type and *Tomaj*^{D} females. Merged confocal pictures of polar bodies as stained for tubulin (green) and DNA (red). (A) Three polar body nuclei about to fuse at the time shown in Fig. 1D. Notice intact nuclei, the uniformly decondensed chromatin and the microtubules between the nuclei. (B) Triplo-fusion of polar bodies (right) and a single polar body (left). Notice the typical unipolar metaphase configuration of chromosomes with two sister chromatids (arrowhead), suggesting the occurrence of chromatin replication at the pronuclear stage. Identical configurations develop in unfertilized wild-type and in *Tomaj*^{D} derived eggs whether fertilized or not. Bar is identical for A and B. (C) Tetra-fusion results in the formation of a single polar body with polyploid DNA content in both unfertilized wild-type as well as in *Tomaj*^{D} derived eggs (whether fertilized or not). The fused, overcondensed and fragmenting chromosomes are indicators of chromatin degeneration.

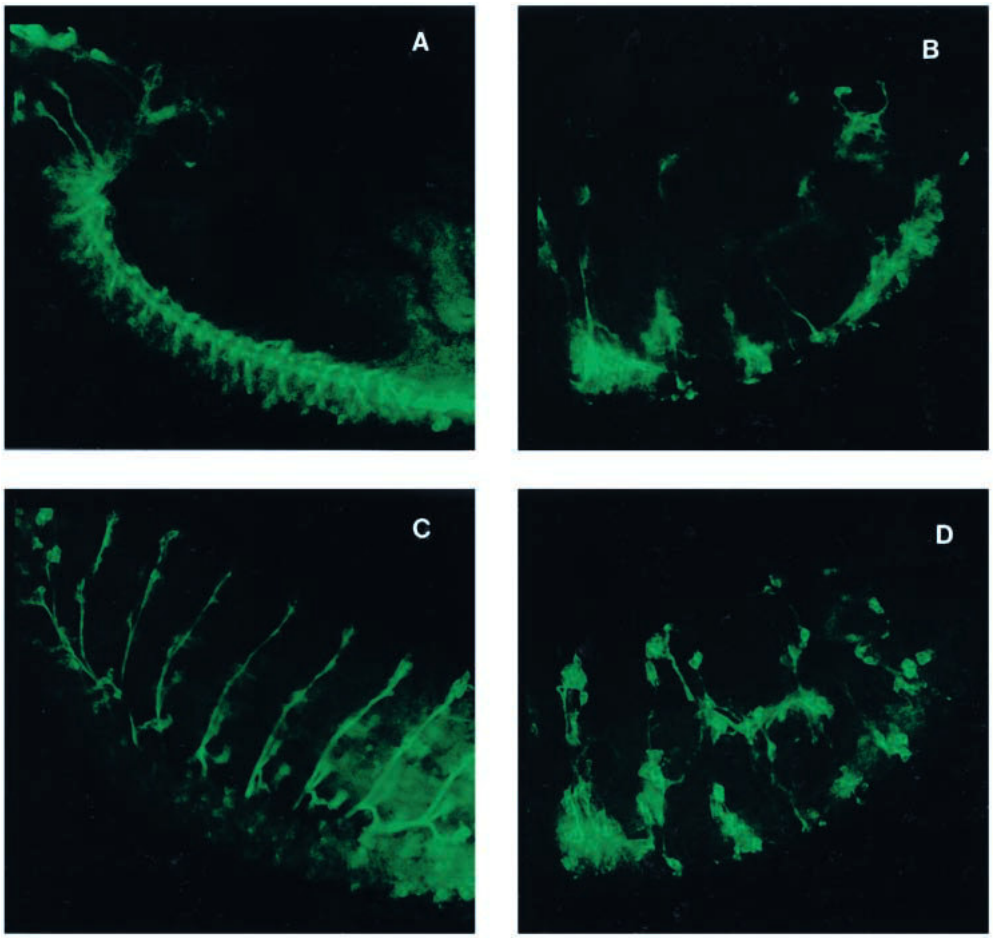

Fig. 3. Abnormal nervous system of the $Tomaj^{D2}$ derived embryos. The ventral nerve cord in a wild-type (A) and a $Tomaj^{D2}$ derived (B) embryo at stage 13-17. Notice the disrupted neuronal structures in the $Tomaj^{D2}$ derived embryo. (C) The PNS in the same wild-type and (D) same $Tomaj^{D2}$ derived embryo.

and DNA staining (data not shown). Although these embryos do not complete embryogenesis, they produce some cuticular structures. Since the cuticle of the dead embryos was reminiscent of the so-called neurogenic phenotype (Campos-Ortega, 1993), we analyzed both CNS and PNS of the $Tomaj^{D2}$ derived embryos by immunostaining (Fig. 3). Observation of the CNS and PNS showed highly disorganized neuronal structures. The brains remained rudimentary, while the commissural and longitudinal processes of the ventral nerve cord were disrupted. Interruptions in the PNS were also apparent, e.g. the sensory neurons and their axons were abnormally oriented. Theurkauf (1992) has shown that the maternally supplied αTub67C isotype perdures into the embryonic nervous system. The phenotype of the escaper animals produced by the $Tomaj^{D2}$ allele now demonstrates a requirement for this isotype in formation of the embryonic CNS and PNS.

The antimorphic nature of the $Tomaj^{D}$ alleles

To determine whether the $Tomaj^{D1-3}$ encoded mutant αTub67C isotypes participate in the same events as the wild-type isotype or interferes with normal processes, we constructed $Tomaj^{D}$/+/+ females, where + stands for the *Dp(3;3)S2a2* duplication which covers the *αTub67C* locus. The $Tomaj^{D}$/+/+ females were fertile: 26%, 5% and 8% of the eggs developed to adults in the case of $Tomaj^{D1}$, $Tomaj^{D2}$ and $Tomaj^{D3}$, respectively. Since an extra copy of the wild-type locus is capable at least partially of ameliorating the defects associated with the dominant alleles, it would appear that the $Tomaj^{D1-3}$ alleles are antimorphic and their products disrupt or poison the same phenomena in which the normal αTub67C isotype functions.

Molecular nature of the $Tomaj^{D}$ alleles

To determine the molecular nature of the gain-of-function $Tomaj^{D}$ mutations, we cloned and sequenced the $Tomaj^{D1-3}$ alleles. Single base pair substitutions in two different positions resulted in replacement of $Glu^{-}\rightarrow Lys^{+}$ in both the $Tomaj^{D1}$ and $Tomaj^{D3}$ encoded αTub67C isotypes, while in the $Tomaj^{D2}$ encoded product a $Gln\rightarrow Arg^{+}$ change has occurred (Table 4). We also expressed the wild-type as well as the $Tomaj^{D1-3}$ alleles in bacteria and established using western blots probed with a polyclonal serum against αTub67C, that the $Tomaj^{D1-3}$ alleles encode a normal-sized peptide (data not shown). On the basis of these data we can conclude that the gain-of-function $Tomaj^{D1-3}$ mutations lead to single amino acid changes that may identify important functional sites in αTub67C.

The loss-of-function $Tomaj^{R}$ alleles

To determine the loss-of-function *Tomaj* phenotype, we reverted the $Tomaj^{D1-3}$ alleles by EMS, X-ray and P-element mutagenesis (Table 1). In situ hybridizations to the polytene chromosomes of the P-induced revertants identify a common

Table 1. The *Tomaj* mutant alleles

Tomaj^{D} alleles	Founder chromosome	*Tomaj*^{R} alleles, induced by		
		EMS	X-rays	P-element
Tomaj^{D1}	*Canton-S*	RE1.1	RX1.1	RP1.1
Tomaj^{D2}	*mwh e*	RE2.1	RX2.1, RX2.2	–
Tomaj^{D3}	*mwh e*	RE3.1-RE3.3	RX3.1	RP3.1

Tomaj^{D1}, *Tomaj*^{D2} and *Tomaj*^{D3} were originally described as *Tomaj*^{4091}, *Tomaj*^{3a} and *Tomaj*^{16d}, respectively (Erdélyi and Szabad, 1989). *Tomaj*^{D2} possesses dominant cold-sensitive sterility. The *Tomaj*^{RP} alleles are weak hypomorphs.

insertion point in 67C4-5, where the *αTub67C*$^{1-4}$ mutant alleles were previously mapped (Matthews et al., 1993). The *αTub67C*$^{1-4}$ mutant alleles fail to complement the *Tomaj*^{R} alleles, and therefore the two groups of mutations identify the same locus.

Female sterility

The *Tomaj*^{R}/–, heterozygous *Tomaj*^{R}/*Tomaj*^{R}, *Tomaj*^{D}/*Tomaj*^{R} and *Tomaj*^{R}/*αTub67C*$^{1-4}$ combinations are all female sterile. The female sterility of *αTub67C*$^{1-4}$/– hemizygous combinations has already been described by Matthews et al. (1993). Based on the extent of embryogenesis in eggs of the *Tomaj*^{R}/– females, the *Tomaj*^{R} alleles can be ranked from the strongest to the weakest alleles as follows: *RE3.1=RE3.2=RE3.3>RE2.1=RX2.2=RX2.1=RE1.1=RX3.1>RX1.1=RP3.1=RP1.1*.

Female meiosis

Meiosis I and II proceeded normally in oocytes and eggs of *Tomaj*^{R}/–, *Tomaj*^{R}/*Tomaj*^{R}, *Tomaj*^{D}/*Tomaj*^{R} and *Tomaj*^{R}/*αTub67C*$^{1-4}$ combinations (where *R* stands for *RE3.1, RE3.2, RE2.1, RE1.1, RP1.1)* as was shown by anti-tubulin and DNA stainings and Feulgen-Giemsa staining (approx. 500 eggs/combination were analyzed).

Sperm aster defect

Only a small sperm aster formed in variable frequencies (20-70%), in the fertilized eggs of *Tomaj*^{R}/– females (where R stands for *RE3.1, RE3.2, RE3.3, RE2.1, RE1.1, RX2.1, RX2.2)*. There is no apparent female pronuclear specification nor is there evidence of migration toward the male pronucleus. Additionally, triplo- or tetra-fusion of polar bodies is common. Thus, the gain-of-function and loss-of-function *Tomaj* phenotypes are almost identical, implying that in eggs of *Tomaj*$^{D1-3}$ females the encoded mutant αTub67C molecules block the function(s) of the normal αTub67C ones.

Table 2. Chimeras with normal (*K1237/+*) soma and *Tomaj*^{R}/*Tomaj*^{R} germ line

Host genotype	Genotype of implanted germ line cells	Number of chimeras	Phenotype
K1237/+	*Tomaj*$^{RE1.1}$/*Tomaj*$^{RE3.1}$	7	STERILE
	Tomaj$^{RE1.1}$/*TM3*	10	Fertile
	Tomaj$^{RE3.1}$/*TM6*	12	Fertile
	TM3/TM6	8	Fertile
	Tomaj$^{RE2.1}$/*Tomaj*$^{RE3.1}$	8	STERILE
	Tomaj$^{RE2.1}$/*TM3*	11	Fertile
	Tomaj$^{RE3.1}$/*TM6*	9	Fertile
	TM3/TM6	8	Fertile

Cleavage spindle apparatus defects

The cleavage divisions commenced and proceeded through a variable number of cycles in 20-60% of the eggs laid by females hemizygous and heterozygous for these combinations of the *Tomaj*^{R} alleles. The gonomeric division is not completed in eggs of hemizygous and heterozygous females for stronger *Tomaj*^{R} alleles e.g. *RE3.1, RE3.2, RE3.3, RE2.1, RE1.1*. Embryogenesis comes to a cessation before or shortly after cortical migration of cleavage nuclei in eggs of females heterozygous for weaker (leaky) *Tomaj*^{R} alleles e.g. *RE1.1, RX2.1, RX2.2, RP1.1, RP3.1*. The *αTub67C*$^{1-4}$ alleles are weak hypomorphs (Matthews et al., 1993). Consistent with this fact the cleavage divisions progressed up to blastoderm formation in eggs of the *Tomaj*$^{RE1.1}$/*αTub67C*$^{1-4}$ females.

The most commonly observed cleavage spindle apparatus defect is related to the gonomeric spindle (Fig. 4A,B). In eggs showing an abnormal gonomeric spindle, the female and male pronuclei approach each other presumably using a functional sperm aster, but they never fuse. In wild-type eggs the gonomeric spindle has well defined poles marked by centrosomes and the convergence of aster MTs. In eggs of *Tomaj*^{R}/– and heterozygous *Tomaj*^{R}/*Tomaj*^{R} females, a barrel-shaped nonfunctional gonomeric spindle appears with apparently greater than normal accumulation of centrosomin at the presumptive poles. Another characteristic feature of the defective cleavage spindle apparatus was the gradual appearance of abnormal ectopic cleavage spindles. This abnormality becomes more severe with time (Fig. 4D-H). Barrel-shaped, undersized, mono- and multipolar spindles as well as fused spindles and patches of MTs developed among the wild-type looking cleavage spindles, often in 'clonal' fashion. Replicated, but poorly separated and free centrosomes were seen in close vicinity of MT patches. In degenerating embryos MT patches without centrosomes and chromosomes are rather common.

The germ-line requirement of the αTub67C gene

To determine whether the *αTub67C* gene is required in the female germ line and/or in soma, we constructed two types of germ line chimeras. First, *Tomaj*$^{RE1.1}$/*Tomaj*$^{RE3.1}$ and *Tomaj*$^{RE2.1}$/*Tomaj*$^{RE3.1}$ germ line cells were transplanted into Fs(1)K1237/+ hosts that have a normal soma and nonfunctional germ line (Table 2). All 15 female chimeras were sterile and possessed the same mutant phenotype as the donor females. In the second experiment normal (*y v f mal*) pole cells were transplanted into *Tomaj*$^{RE1.1}$/*Tomaj*$^{RE3.1}$ and *Tomaj*$^{RE2.1}$/*Tomaj*$^{RE3.1}$ hosts (Table 3). The 14 chimeric *Tomaj*^{R}/*Tomaj*^{R} host females in addition to their own non-hatching eggs, produced *y v f mal* offspring. Thus, it would appear that the *αTub67C* gene functions only in the female germ line.

DISCUSSION

The *Tomaj* mutants map to the *αTub67C* gene. In *Drosophila*, early embryogenesis is governed by the maternal dowry, that

Table 3. Chimeras with *Tomaj^{R}/Tomaj^{R}* soma and normal (*y v f mal*) germ line

Host genotype	Genotype of implanted germ line cells	Number of chimeras	Phenotype
Tomaj$^{RE1.1}$/Tomaj$^{RE3.1}$		6	FERTILE*
Tomaj$^{RE1.1}$/TM3		8	Fertile†
Tomaj$^{RE3.1}$/TM6	*y v f mal*	10	Fertile†
TM3/TM6		7	Fertile†
Tomaj$^{RE2.1}$/Tomaj$^{RE3.1}$		8	FERTILE*
Tomaj$^{RE2.1}$/TM3	*y v f mal*	9	Fertile†
Tomaj$^{RE3.1}$/TM6		7	Fertile†
TM3/TM6		7	Fertile†

*Only *y v f mal* offspring derived besides the self non-hatching eggs.
†Offspring derived from both their own and implanted germ line cells.

is, females provide a supply of factors in the egg cytoplasm, including the α-tubulins. The function(s) of the *αTub67C* gene appear(s) to be restricted to late oogenesis, early embryogenesis and the development of the embryonic nervous system (Komma and Endow, 1995, 1997; Matthews et al., 1990, 1993; Theurkauf et al., 1992; Theurkauf, 1994). As we report in the present paper, the gain-of-function *Tomaj^{D1-3}* and the loss-of-function *Tomaj^{R}* revertant alleles provide additional information about the functions of the *αTub67C* gene.

Genetic dissection of normal gene function is usually based on the phenotype caused by null and/or hypomorphic (leaky) alleles. As we have shown, the *Tomaj^{D}* alleles are antimorphic and this fact could obscure any interpretation of αTub67C function based on the phenotype associated with these dominant alleles. However, the phenotype of the strong *Tomaj^{R}/−* combinations is almost identical with the one seen in *Tomaj^{D1}* and *Tomaj^{D3}*. Thus, it would appear that the *Tomaj^{D}* encoded mutant peptides participate in the same processes as the wild type, and consequently the corresponding mutant phenotypes reveal the normal *αTub67C* function(s). Moreover, since the *Tomaj^{D}/+* and *Tomaj^{D}/−* phenotypes are essentially identical, it is clear that the *Tomaj^{D}* encoded protein nullifies the wild-type function of the αTub67C isotype and in this respect the dominant alleles act like nulls. This conclusion is supported by the fact that the 'poison' effect can be partially titrated out by additional copies of the wild-type *αTub67C* locus. The apparent nullifying activity of the *Tomaj^{D1-3}* alleles may be explained by their associated molecular lesions. In both *Tomaj^{D1}* and *Tomaj^{D3}* (in different positions) there is a Glu^{-}→Lys^{+} missense change, while in the *Tomaj^{D2}* encoded tubulin a Gln→Arg^{+} alteration is found (Table 4). The α-tubulin molecules form heterodimers with β-tubulins and comprise the major constituents of MTs. It would appear therefore that the *Tomaj^{D1-3}* encoded mutant *αTub67C* molecules form heterodimers with the available β-tubulins and these are incorporated into MTs. Once incorporated into MTs, elongation is apparently blocked, e.g. there is no transition from the small to the prominent sperm aster (see below). In this model the failure of MT elongation is the consequence of tubulin heterodimers that are defective due to the Glu^{-}→Lys^{+} and Gln→Arg^{+} changes in the mutant αTub67C isotypes. Several possibilities could account for the observed defects e.g. an alteration in charge affecting the conformation of peptide, a defective MAP association site or aberrant posttranslational modification sites. Determining which if any of these may be correct will require further analysis.

αTub67C is required for sperm aster formation and function. Since early embryogenesis does not commence in eggs of the *Tomaj^{D1}*, *Tomaj^{D3}* and in females hemizygous for stronger *Tomaj^{R}* alleles (*RE3.1, RE3.2, RE3.3, RE2.1, RE1.1)*, it appears that the *Tomaj^{D1}, Tomaj^{D3}* and the stronger *Tomaj^{R}* alleles identify an αTub67C function, which is required at or just subsequent to fertilization. However, the αTub67C function is required during female meiosis as was proposed by Matthews et al. (1993), Matthies et al. (1996) and Komma and Endow (1997), based on the *αTub67C$^{1-4}$* alleles and the use of genetic assays for the detection of nondisjunction and/or chromosome loss. We did not detect abnormal meiotic figures using immunocytological or Feulgen-Giemsa staining procedures in oocytes of *Tomaj^{D1-3}* and different combinations of *Tomaj^{R}* females. The difference between the former and present results can be rationalized by assuming that: (1) *αTub67C$^{1-4}$* and *Tomaj^{D}* (and their revertants) identify different αTub67C functions. (2) The cytological methods are much less sensitive to detect abnormal chromosome segregation than the genetic approaches (Szabad, 1986). The *Tomaj^{D}* alleles being sterile, cannot be used in genetic tests for analysis of chromosome segregation.

The first observable *Tomaj^{D1}, Tomaj^{D3}* and *Tomaj^{R}* associated defects appear after fertilization when only a small sperm aster is developed (Fig. 1). This compares to wild-type eggs in which the small sperm aster grows rapidly into the prominent sperm aster. We propose, based on the *Tomaj* mutant phenotype, that the αTub67C isotype is required for formation and function of the prominent sperm aster. Furthermore because the size of the small sperm aster is basically identical in all the mutant genotypes studied and persists for about two hours, we conclude that the αTub67C isotype is not required for small sperm aster formation.

Failure of prominent sperm aster formation leads to the absence of female pronucleus specification and migration toward the male pronucleus. The four meiotic products of female meiosis appear to be equivalent and frequently all four fuse (tetra-fusion). Similarly, Komma and Endow (1997) and Williams et al. (1997) also reported defective female pronuclear formation and fusion of the four polar body nuclei in eggs of the *ncd* and *klp3a* mutant females, suggesting that the NCD and KLP3A kinesin-like motor molecules are also required for female pronuclear migration. We propose that the NCD and KLP3A ensure female pronuclear migration along the prominent sperm aster MTs.

αTub67C is required for cleavage spindle apparatus. Cleavage spindle apparatus defects appeared in 20-60% of the eggs derived from different combinations of weaker *Tomaj^{R}* alleles (*RE1.1, RX2.1, RX2.2, RP1.1, RP3.1),* (Fig. 4). The

Table 4. Molecular nature of *Tomaj^{D}* alleles

Tomaj^{D} allele	Missense mutations	Amino acid change
Tomaj^{D1}	*G*AA→*A*AA	Glu^{-} 244→Lys^{+}
Tomaj^{D2}	C*A*A→C*G*A	Gln 305→Arg^{+}
Tomaj^{D3}	*G*AG→*A*AG	Glu^{-} 79→Lys^{+}

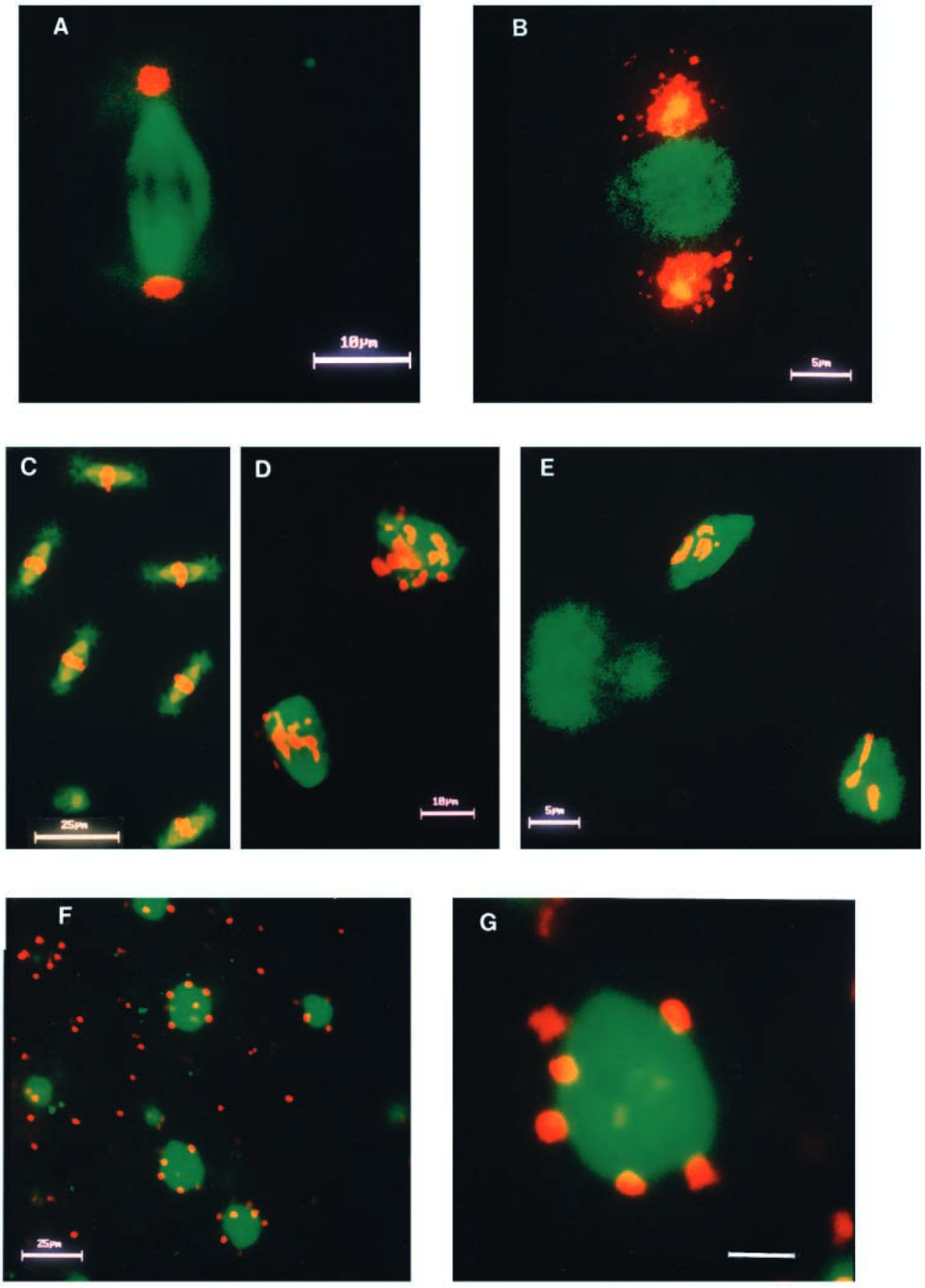

Fig. 4. Features of the cleavage defects in embryos of *Tomaj*^{R} females. Merged confocal pictures of cleavage spindles. (A) The gonomeric spindle in wild-type and (B) in *Tomaj*^{R}/*Tomaj*^{R} derived eggs stained for centrosome (red) and tubulin (green). (C) Typical metaphase wild-type cleavage spindles. (D) Abnormal spindles with fragmented chromosomes in embryos derived from *Tomaj*^{R}/*αTub67C*$^{1-4}$ females. (E) The abnormally organized cleavage spindles do not allow proper alignment and segregation of chromosomes. (C-E) Tubulin and DNA appear in green and red, respectively. (F) Following elimination of chromosomes, MT patches with centrosomes and free centrosomes remain in embryos derived from *Tomaj*^{R}/*αTub67C*$^{1-4}$ females. (G) Enlarged MT patch with several centrosomes. Bar, 8.5 μm. In all the confocal micrographs tubulin is green and the centrosomes are red as detected by the CNN antibody.

most obvious cleavage spindle defect is the nonfunctional, barrel-shaped gonomeric spindle, which presents excessive centrosomin protein accumulation at the presumptive poles. In embryos that pass the gonomeric division, the embryogenesis ceases at the time of cortical migration, showing highly aberrant cleavage spindles with abnormal centrosome and chromosome displacement. Based on the *Tomaj*^{R} associated phenotypes we conclude that the normal αTub67C is specifically needed for the proper structure and function of cleavage spindle apparatus. Whether the αTub67C allows the unusually rapid assembly and disassembly of *Drosophila* cleavage spindles as was proposed by Matthews et al. (1993) or influences the nucleation of MTs by centrosomes remains to be answered.

αTub67C is required in the embryonic nervous system. At permissive temperature embryogenesis proceeds up to gastrulation in eggs of the *Tomaj*^{D2}/+ females. However, at this point most of the embryos die presumably due to the 'poisonous' maternal dowry. The dead embryos show abnormal development of both CNS and PNS (Fig. 3). Based

on perdurance of the maternally loaded αTub67C isotype in the CNS, Theurkauf (1992) proposed the protein showed CNS-specific developmental stability. Although the nature of developmental stability of αTub67C is not known, the neural defects observed in *Tomaj*^{D2}/+ derived embryos indicate that its presence must have functional consequences in the developing embryonic nervous system that may lead to formation of MT bundles in cell division or other neuronal processes.

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