

Tetrahymena thermophila contains a conventional γ -tubulin that is differentially required for the maintenance of different microtubule-organizing centers

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The gene (*GTUI*) encoding *Tetrahymena thermophila* γ -tubulin was cloned and analyzed. *GTUI* is a single-copy, essential gene encoding a conventional γ -tubulin. HA-tagged GTUIp localizes to four microtubule-organizing centers (MTOCs) in vegetative cells: basal bodies (BBs), macronuclear envelopes, micronuclear envelopes, and contractile vacuole pores. γ -Tubulin function was studied by placing the *GTUI* gene under control of an inducible-repressible promoter. Overexpression of *GTUI* had no detectable effect on cell growth or morphology. Depletion of γ -tubulin resulted

in marked changes in cell morphology and in MT bundling. MTOCs showed different sensitivities to γ -tubulin depletion, with BBs being the most sensitive. γ -Tubulin was required not only for the formation of new BBs but also for maintenance of mature BBs. BBs disappeared in stages, first losing γ -tubulin and then centrin and glutamylated tubulin. When *GTUI* expression was reintroduced in depleted cells, BBs reformed rapidly, and the normal, highly organized structure of the *Tetrahymena* cell cortex was reestablished, indicating that the precise patterning of the cortex can be formed de novo.

Introduction

Microtubules (MTs)* form essential, structurally and functionally diverse systems in eukaryotic cells. They are nucleated from MT-organizing centers (MTOCs) which control the localization, orientation, and the timing of assembly in vivo.

The discovery of γ -tubulin was a major breakthrough in analyzing MTOCs (Oakley and Oakley, 1989). γ -Tubulins are conserved in eukaryotes (Burns, 1991) and are structurally similar to α - and β -tubulins (Inclán and Nogales, 2001) to which they are $\sim 35\%$ identical. γ -Tubulin functions in MT nucleation. During interphase, it localizes in MTOCs such as the centrosomes of animal cells and the spindle pole bodies of yeast, and it accumulates at spindle poles in most cell types examined (Horio et al., 1991; Stearns et al., 1991; Zheng et al., 1991). γ -Tubulin localizes to basal bodies (BBs), the MTOCs for axonemes (Fuller et al., 1995), and is required for BB duplication in *Paramecium* (Ruiz et al., 1999). γ -Tubulin is present in the cytoplasm

of *Xenopus* and *Drosophila* eggs as open-ring complexes (γ TuRCs) of ~ 25 nm diameter (Moritz et al., 1995; Zheng et al., 1995) that can nucleate MTs and bind to MT minus ends. Immunodepletion of γ -tubulin in vitro (Felix et al., 1994; Moritz et al., 1998) or microinjection of anti- γ -tubulin antibodies in vivo (Joshi et al., 1992) inhibits centrosomal MT nucleation. Disrupting γ -tubulin genes in fungi (Oakley et al., 1990; Spang et al., 1996) or flies (Sunkel et al., 1995) produces phenotypes, suggesting a role for γ -tubulin in MT nucleation in vivo. These studies argue that γ -tubulin is an essential component of MTOCs and has a direct role in MT nucleation. Mutational analyses in fungi (Paluh et al., 2000; Jung et al., 2001; Hendrickson et al., 2001; Prigozhina et al., 2001) indicate that γ -tubulin also plays a role in regulating MT dynamics.

Tetrahymena thermophila cells contain at least 17 differentiated MT systems (Gaertig, 2000), including BBs, axonemes, transverse and longitudinal MTs, contractile vacuole pores (CVPs), and mitotic and meiotic spindles. To study how these systems are organized and regulated, we cloned the *Tetrahymena* γ -tubulin (*GTUI*) gene. It encodes a conventional γ , $\sim 65\%$ identical to the γ -tubulins of multicellular eukaryotes. *GTUI* is essential for growth. An epitope-tagged γ -tubulin that rescues a *GTUI* knockout localized to oral and somatic BBs, to the poles of the mitotic micronucleus (Mic), in envelope-associated particles of macronuclei (Macs) and Mics, and in the CVPs.

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*Abbreviations used in this paper: BB, basal body; *cs*, cold sensitive; CVP, contractile vacuole pore; KF, kinetodesmal fiber; Mac, macronucleus; Mic, micronucleus; MT, microtubule; MTOC, MT-organizing center; OA, oral apparatus; OP, oral primordium; SPP, super proteose peptone; TM, transverse microtubule.

Key words: γ -tubulin; basal body; centrin; MTOC; *Tetrahymena*

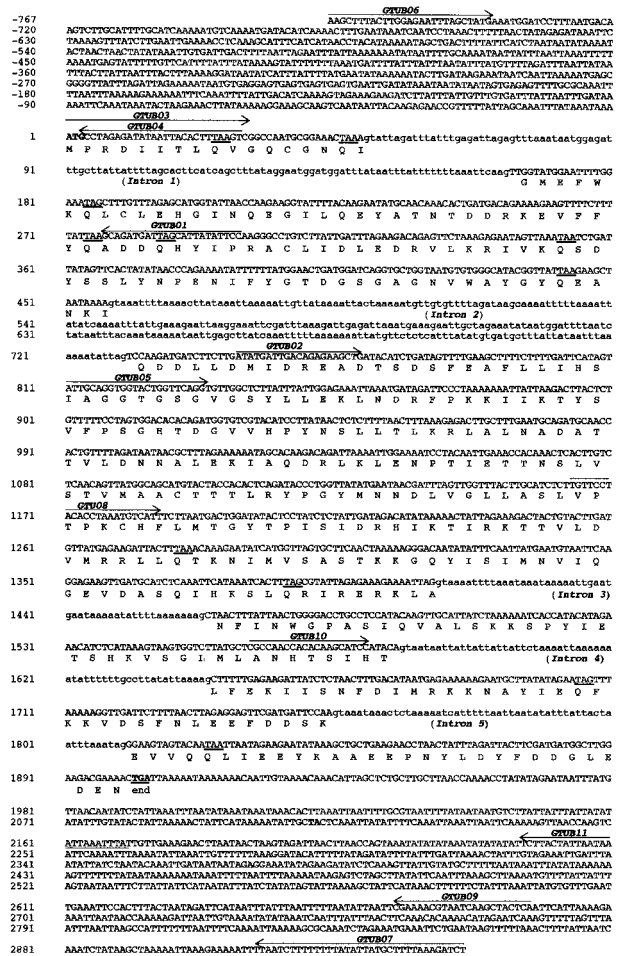


Figure 1. The complete DNA sequence and translation of the *GTU1* gene. TAA and TAG, which encode glutamines in *Tetrahymena*, are underlined. The bold T and A in the 3' flanking sequence indicate the putative sites of transcription termination. The arrows show the positions of primers used for cloning and sequencing. These sequence data are available from EMBL/GenBank/DBJ under accession no. U96076.

We analyzed the effects of depleting γ -tubulin, either by disrupting the *GTU1* gene (Hai and Gorovsky, 1997; Hai et al., 2000) or by placing it under control of the inducible-repressible *MTT1* promoter (Shang et al., 2002). Different MTOCs showed different rates of loss of γ -tubulin. New BBs failed to replicate and already formed BBs disappeared. BBs disappeared in stages, first losing γ -tubulin and then centrin and glutamylated tubulin. These results show that γ -tubulin is required not only for BB duplication, but also plays a role in maintenance of normal BB structure. Basal bodies could also reform rapidly, and normal cell morphology could be reconstituted by reintroducing γ -tubulin expression.

Results

Sequence of the *T. thermophila* *GTU1* gene

The major products of genomic and RT-PCR reactions using two degenerate primers derived from highly conserved regions found in γ - and β -tubulin were cloned. The complete sequence of these clones identified a putative γ -tubulin

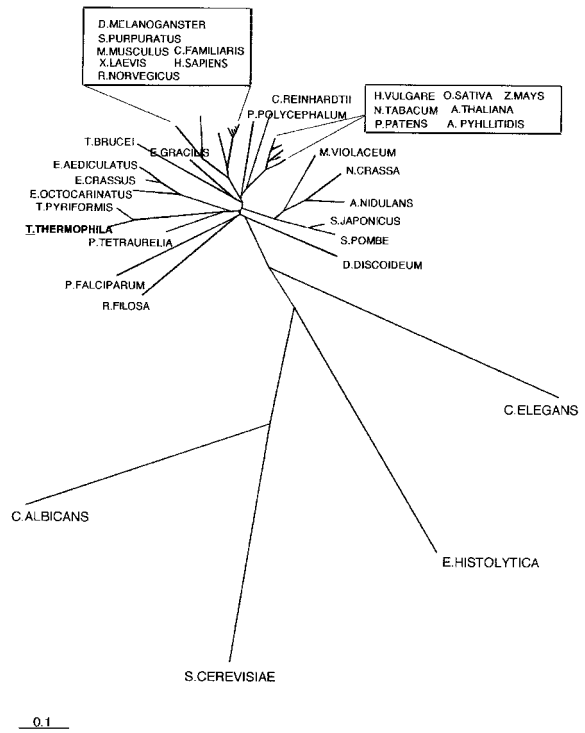


Figure 2. Phylogenetic analysis of γ -tubulins. 39 complete γ -tubulins were analyzed. The unrooted tree was generated using the neighbor-joining method. The scale bar indicates the horizontal branch length that corresponds to 0.1 substitutions per one amino acid.

coding gene (*GTU1*) that shared ~60% identity with the known consensus sequence. Southern blotting showed a single band (Materials and methods), indicating that *GTU1* was a single copy gene in the *Tetrahymena thermophila* genome, which was confirmed by the genetic analyses described below. It encodes a 50.9-kD protein of 449 amino acid residues (Fig. 1). The coding region contains five introns and 11 TAA or TAG glutamine codons. The cDNA sequence indicates that polyadenylation occurs 208 nt downstream of the TGA termination codon. As in other *Tetrahymena* genes, there is no properly positioned, canonical polyadenylation site. *Tetrahymena* γ -tubulin shares ~65% identity with most γ -tubulins. A phylogenetic analysis (Fig. 2) places it with other ciliate tubulins at a position consistent with the phylogeny of ciliates. *Tetrahymena* γ -tubulin is 35–45% identical to the divergent, nonconventional γ -tubulins of *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Entamoeba Histolytica* (Burns, 1995). Thus, *Tetrahymena* contains a conventional γ -tubulin.

GTU1* is essential in *T. thermophila

To study the effect of *GTU1* depletion, we created *GTU1* knockout heterokaryon strains with both copies of the *GTU1* gene disrupted in the diploid Mics but with the 45 WT copies still present in the polyploid Macs (Materials and methods). We then created Δ *GTU1* cells (whose Mics and Macs lacked any *GTU1* genes) by crossing two heterokaryon strains to each other. The progeny of this mating separated (exconjugated) at the proper time, indicating that zygotic expression of the *GTU1* gene is not required for completion of

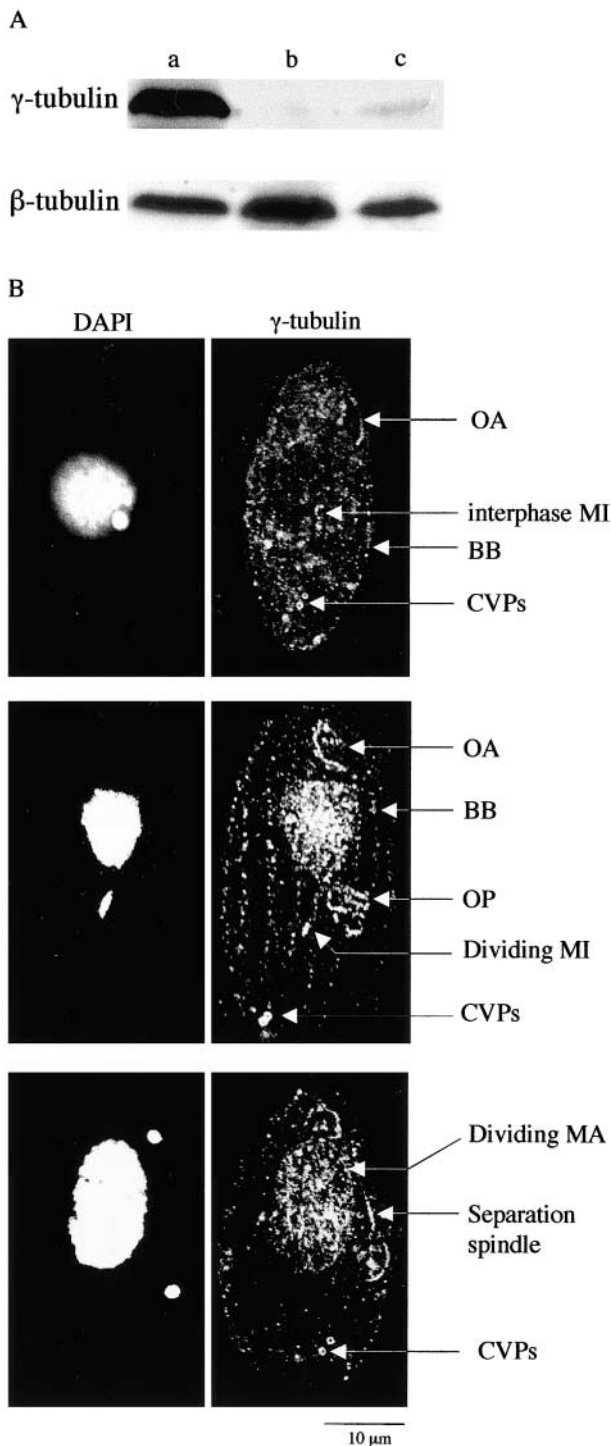


Figure 3. HA-tagged γ -tubulin in *Tetrahymena thermophila*. (A) Western blots of *Tetrahymena* cell extracts probed with anti-HA (γ -tubulin) or DM1B (β -tubulin) antibody showing regulation of γ -tubulin expression by the *MTT1* promoter. Strains used are: (a) cTTMG-HA in which the pTTMG-HA construct was used to rescue the *GTU1* knockout heterokaryon; (b) CU428 WT cells; and (c) tG2N-HA in which the pG2N-HA construct was used to replace the WT *GTU1* gene in Macs of CU428 cells. The cells used for all three lanes were grown in SPP media with 1.0 μ g/ml CdCl₂. (B) Log phase growing cTTMG-HA cells expressing an HA-tagged γ -tubulin stained with an anti-HA antibody show four major locations of γ -tubulin in vegetative cells: (a) BBs in somatic rows (BB), OA, and OP; (b) small patches on the dividing macronuclear envelopes (dividing MA); (c) patches on interphase micronuclear envelopes

conjugation. When refed, Δ *GTU1* cells regenerated oral apparatuses (OAs) (unpublished data) and initiated growth, which slowed \sim 5 h after refeeding, and almost stopped between 7.5 and 12.5 h. The apparent growth of the culture after 12.5 h is likely due to the proliferation of contaminating, unmated parental cells. Progeny of a cross between WT control strains CU428 and B2086 grew steadily over the same time course (unpublished data).

The function of γ -tubulin was also assessed by depleting it from growing cells. *GTU1* heterokaryon progeny were rescued with a COOH-terminal HA-tagged *GTU1* gene (Fig. 3 A) under control of the cadmium-inducible *MTT1* promoter (Shang et al., 2002), producing strain cTTMG-HA. These cells grew normally in the presence of cadmium, indicating this tagged gene functions normally and can be over-expressed without causing an obvious phenotype. Log phase cTTMG-HA cells grown in super proteose peptone (SPP)/CdCl₂ (0.5 μ g/ml) were shifted to CdCl₂-free SPP to repress expression of the *GTU1* gene. Growth slowed at \sim 11 h and stopped 16 to 17 h after depletion, whereas similarly treated WT cells continued to grow to maximal density (unpublished data). These studies demonstrate that the *GTU1* gene is essential for growth.

γ -Tubulin localizes to four major MTOCs in vegetative cells

The only functional *GTU1* gene in both tG2N-HA and cTTMG-HA strains is the COOH-terminal HA-tagged *GTU1* gene (Materials and methods), either under control of the endogenous *GTU1* or *MTT1* promoter, respectively. The growth and mating of the cTTMG-HA strain were indistinguishable from that of WT (unpublished data), indicating that the chimeric gene functions normally.

Anti-HA antibodies detected a protein of the expected size (\sim 60 kD) in tG2N-HA and cTTMG-HA (Fig. 3 A). To study the localization of γ -tubulin, we stained vegetative cells with anti-HA antibodies (Fig. 3 B). In WT cells, no specific staining was detected (unpublished data). In *GTU1*-HA transformants, four organelles, likely to be MTOCs based on their associated MTs, stained strongly. First, all BBs, including those associated with somatic and oral cilia and those associated with the newly forming OA known as the oral primordium (OP) before they became ciliated. Staining intensities changed during the cell cycle. The newly formed OP was stained more strongly than the old OA in dividing cells and the somatic BBs in cells containing an OP were stained more strongly than those in interphase stage. Second, numerous small patches that were closely associated with Mac envelopes were stained and showed changes during the cell cycle. Mics and Macs divide by different mechanisms and at different times in the cell cycle. Mics divide by a closed mitosis before Macs, which divide amitotically, without chromosome condensation or segregation of sister chromatids. Macs lack distinct spindles but contain an elaborate array of MTs (Fujiu and Numata,

(interphase MI), with preferential accumulation at the poles of dividing Mics (dividing MI) and the separation spindle between the two recently divided daughter Mics; and (d) the CVPs.

1999). Strong signals could only be observed on dividing MACs. The stained spots were originally distributed relatively evenly across the surface of Macs (unpublished data) and then lined up, forming short rows. Third, small particles were observed associated with interphase Mic envelopes, the likely MTOCs for Mic spindle MTs. During mitosis, staining occurred along the spindle axis with preferential accumulation at the poles of the dividing Mics. In telophase, we could observe a string of staining between the two daughter Mics, indicating that the separation spindle also stains. And fourth, the two posteriorly located CVPs stained strongly and were the only MTOCs in *Tetrahymena* that showed no obvious cell cycle changes in staining throughout the cell cycle (Fig. 3 B). CVPs are ring structures marking the sites at which *Tetrahymena* cells eliminate liquids (Cameron and Burton, 1969). They contain circular MTs and are sites from which other MTs radiate (Elliott and Bak, 1964). Although not rigorously established as such, we will refer to the (nonspindle) structures containing γ -tubulin as MTOCs for convenience.

MTOCs show different sensitivities to depletion of γ -tubulin

To determine the effects of depletion of γ -tubulin on the function of the four MTOCs, we stained *GTU1* knockout progeny with an antibody to α -tubulin (Fig. 4). Loss of OAs could be observed within 7.5 h after refeeding. Some cells also lacked CVPs, whereas the other MTOCs appeared to be normal (Fig. 4 A, a). Because progeny cell samples also contain \sim 5% unmated parental cells which can grow normally after refeeding, we restricted subsequent analyses to cells without OAs or with abnormal OP development. We classified cells into four categories: cells with normal longitudinal MT structure (LG-MT) (Fig. 4 A, b); cells beginning to lose their shape and round up (Fig. 4 A, c, round); cells with misplaced division furrows (Fig. 4 A, d and e), and cells with abnormal nuclear morphology, including two dividing Mics, elongated dividing Mics, abnormal Macs, or small nuclear

fragments (Fig. 4 A, f–i). These cells showed progressive changes in cytoplasmic and nuclear morphology. From 7.5 to 9.5 h, most contained longitudinal MTs, whereas $>$ 50% of them had started to round up. Some cells had two or more normal dividing Mics and a few cells showed abnormalities in nuclear divisions and cytokinesis. By 12 h, $>$ 80% of the cells were round. By 14.5 h, almost all the cells (\sim 90%) were round and the longitudinal MTs in some cells (\sim 8%) were disorganized. Anti- α -tubulin staining in these cells also showed disturbed cytoplasmic MTs, some of which formed bundles. In addition, more cells (\sim 10%) show abnormalities in cytokinesis and nuclear morphology. The cTTMG-HA strain, containing an *MTT1*-driven *GTU1* coding sequence, showed similar progression of phenotypes when shifted to CdCl₂-free SPP medium (unpublished data).

We conclude from these studies that *Tetrahymena* γ -tubulin is essential for the normal function of both BBs and nuclei, and that BBs are more sensitive to the depletion of γ -tubulin than nuclei. After refeeding, unmated parental cells that contaminate the progeny of a knockout heterokaryon mating, soon outgrow progeny cells. In addition, it is not possible to restore γ -tubulin to these cells to study the recovery of MTOCs and MTs. Therefore, for subsequent studies, we used strain cTTMG-HA to analyze the effect of inactivation of *GTU1* expression after transfer to CdCl₂-free medium and the effects of re-addition of CdCl₂ after depletion.

γ -Tubulin is required for both duplication and maintenance of normal basal bodies

To study the localization of γ -tubulin in BBs, we stained depleted cTTMG-HA cells with anti-HA antibody (Fig. 5). By 17 h, when the culture had stopped growing, HA staining could not be detected on somatic BBs, OAs, or CVPs, but still remained or even increased on both nuclear envelopes. In WT cells, the Mac envelope does not stain strongly before the Mic starts dividing. However, in the depleted cells, we observed many cells with interphase Mics that had small, strongly staining patches on the Mac envelope. Thus, the

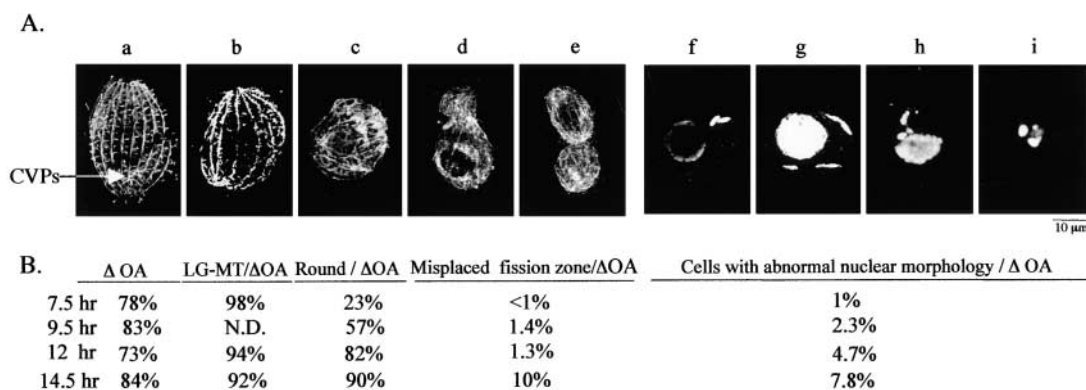


Figure 4. α -Tubulin localization in progeny of knockout heterokaryons. *GTU1*-KO heterokaryons were mated in 10 mM Tris for 24 h, refed with SPP, and fixed at different times (given in hours) after refeeding. (A) Immunofluorescence staining of α -tubulin (DM1A; a–e) and of DNA (DAPI; f–i) in knockout heterokaryon progeny cells. (a) No visible OA with normal cell shape and CVPs. (b) No visible OA and CVPs; with visible longitudinal cortical MT structure (LG-MT). (c) Round cells without visible OA and CVPs. (d and e) No visible OA and CVPs with misplaced division furrow. (f–i) Cells with abnormal nuclear phenotypes. (f) Two dividing Mics (same cell as c). (g) Elongated dividing Mics. (h) Abnormal Mac (same cell as d). (i) Degrading Macs. (B) Summary of appearance of the five categories of cells after cadmium depletion. The percentage of cells without OA or with abnormal OP development (Δ OA) was first determined. These Δ OA cells were then classified into four categories as shown in Fig. 4 B and the percentage of Δ OA cells in each category was calculated.

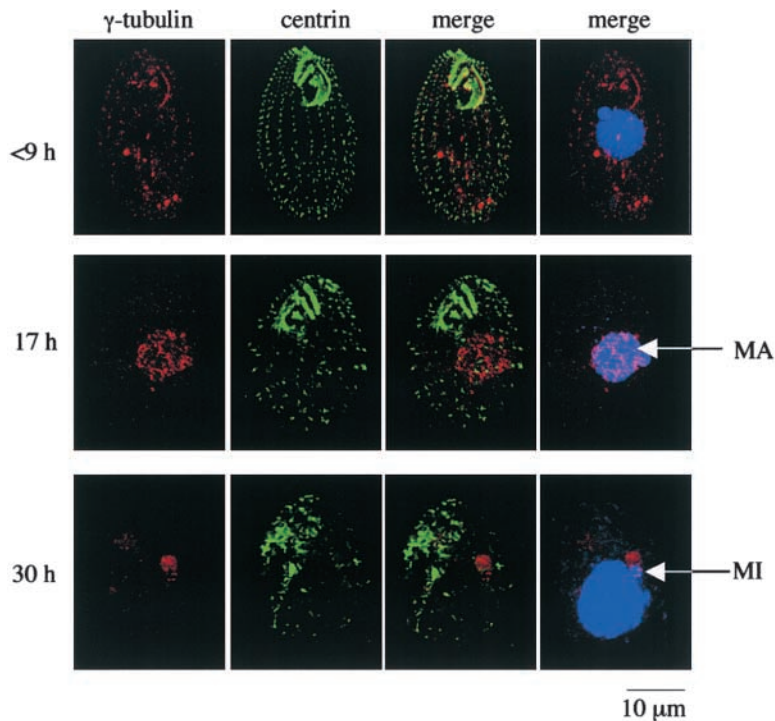


Figure 5. Comparison of γ -tubulin and centrin localization at different times after cadmium depletion.

Cells stained with anti-HA (γ -tubulin) (red) or anti-centrin antibody (green) and with DAPI (blue). After 17 h, Macs (MA) and Mics were the only organelles that were stained by the anti-HA antibody, while anti-centrin antibody still showed strong staining on all the somatic BBs. By 30 h, cells started losing γ -tubulin staining on the macronuclear envelope but still showed punctate structures on the micronuclear envelope (MI).

more rapid effects of γ -tubulin depletion on MT systems organized by BBs than on nuclei can be explained by the fact that γ -tubulin is depleted more rapidly from BBs. Interestingly, Mac staining also disappeared eventually, and by 30 h after depletion, the only detectable MTOCs in most cells were three to five spots on Mic envelopes (Fig. 5). These studies indicate that γ -tubulin is a dynamic component of *Tetrahymena* MTOCs, as it is of centrosomes in mammalian cells (Khodjakov and Rieder, 1999).

Next, we examined the effects of γ -tubulin depletion on BB components and BB associated structures. Antibodies to centrin, an essential component of BBs that is also found in yeast spindle pole bodies (Biggins and Rose, 1994), stained BBs strongly. Surprisingly, centrin staining could not be detected in the other γ -tubulin containing MTOCs (Fig. 5) and could not be detected in isolated nuclei on Western blots (unpublished data).

Surprisingly, anti-centrin staining of somatic BBs and OAs remained almost normal for many hours, even after γ -tubulin could no longer be detected, suggesting that γ -tubulin could be lost without immediate disruption of BBs. We classified centrin staining patterns into four categories (Fig. 6, A and C). 9 h after initiating depletion, most cells are type I, with normal OAs, some of which were dividing cells lacking normal oral primordia. Stage I cells can be subdivided into those with normal centrin staining (Fig. 6 A, stage Ia) and cells whose centrin staining on somatic BBs at the posterior end was partially disrupted (Fig. 6 A, stage Ib). After 11 to 12 h of depletion, the number of Δ OA cells, most with near normal organization of somatic BBs detected by centrin staining (Fig. 6 A, stage II), started to increase. Still later, more cells showed disrupted or near absence of centrin staining at their posterior ends (Fig. 6 A, stage III a), whereas retaining punctate rows of BBs at their anterior ends (Fig. 6 A, stage IIIb). Eventually, most punctate BBs disappeared and

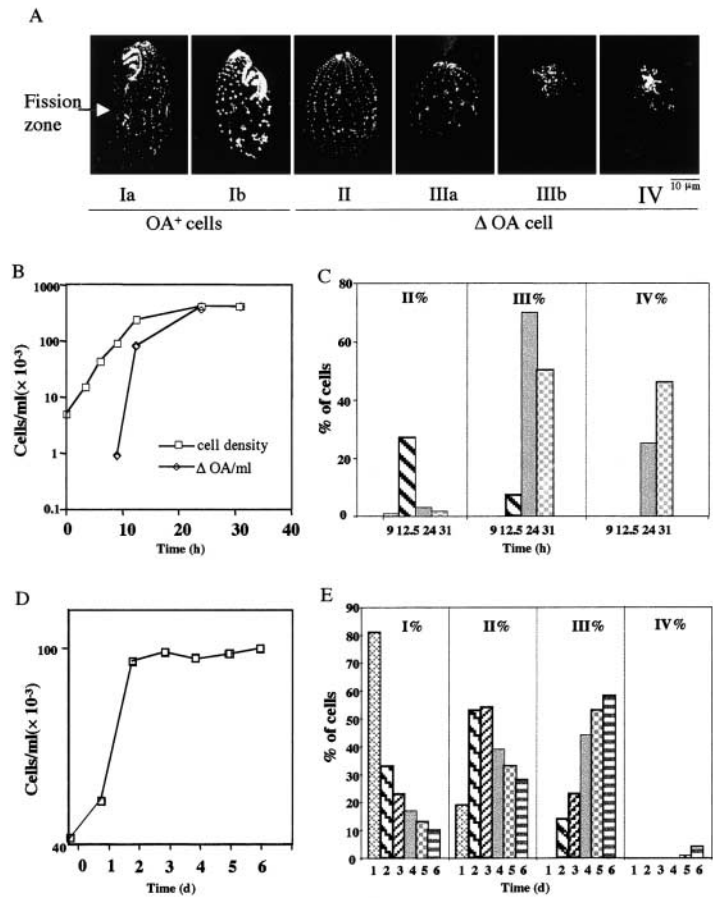
centrin staining accumulated at the anterior end in large aggregates (Fig. 6 A, stage IV).

The changes in centrin distribution (Fig. 6 C) can be analyzed relative to the growth of cells after cadmium depletion (Fig. 6 B). The culture grew actively for \sim 9 h, slowed down at \sim 11 h, and completely stopped by 24 h. As in Fig. 6 B and in three additional, independent experiments, the decrease in the number of cells containing an OA that occurred when culture growth had slowed or stopped always exceeded the increase in cell number. Cells without an oral apparatus cannot grow in SPP medium and we have never observed a dividing cell completely lacking an anterior OA, suggesting that the old OA is never completely resorbed in a dividing cell. Therefore, it is likely that loss of oral BBs is not due simply to their failure to replicate in dividing cells. Loss of punctate, somatic BBs also continues after cells have stopped dividing. From 24 to 31 h after the culture stopped dividing, the number of type IV cells increased dramatically, from 25 to 46%.

To confirm that the appearance of type III cells results from the disassembly of BBs rather than dilution by cell growth, we shifted cells containing a γ -tubulin cold-sensitive (α) mutation (which showed no phenotype in *Aspergillus*; Jung et al., 2001), to 15°C in SPP/CdCl₂ (0.5 μ g/ml) and studied the kinetics of the changes in centrin distribution. These cells stopped dividing completely by 48 h (Fig. 6 D), after which the number of type III cells increased \sim 40% from days 2–6, whereas the cell number remained unchanged (Fig. 6 E).

The progressive changes that occurred at the later stages of depletion are not due to cell death, because \sim 75% of the cells that had been in CdCl₂-free SPP medium for 61 h could regrow when plated as single cells in SPP containing 0.5 μ g/ml CdCl₂. Moreover, $>$ 90% of the α mutant cells which had been maintained at 15°C for 6 d recovered af-

Figure 6. Effects of γ -tubulin depletion and inactivation on centrin localization. (A–C) Log phase cTTMG-HA cells were shifted from SPP medium containing 0.5 $\mu\text{g/ml}$ CdCl_2 to SPP medium without cadmium. Cells were fixed and stained at different time points. (A) Anti-centrin antibody staining was used to analyze the behavior of BBs during γ -tubulin depletion. (Ia and Ib) Cells with an OA (OA^+). (Ia) Dividing cell with a fission zone but missing a normal OP. (Ib) Cell showing disruption of normal BB organization in the posterior region. (II–IV) Cells without an OA (ΔOA). (II) Cell with normal centrin staining of somatic BBs. (IIIa) Cell starting to lose centrin staining in the posterior end. (IIIb) Cell that has lost most centrin staining at the posterior end while still retaining some BB-like structures at its anterior end. (IV) Most punctate BB-like structures have disappeared. Aggregates or fibers containing centrin are observed at the anterior end. (B) The number of total cells and of cells without OA after depletion of cadmium. (C) Summary of appearance of the three categories (type II–IV) of cells in Fig. 6 A after cadmium depletion. (D–E). A *cs* γ -tubulin mutant cK358AK359A-HA strain was shifted to restrictive temperature (15°C). (D) Growth curve of the *cs* mutant after being shifted to 15°C . (E) Summary of appearance of the four categories of the *cs* cells after the shift.



ter they were transferred back to permissive temperature (30°C). In the depletion study, $\sim 80\%$ of the cells started making new BBs after we added CdCl_2 (see below). These analyses indicate that γ -tubulin is not only required for BB replication in *Tetrahymena*, but also for maintenance of BBs in the highly organized OA and the cortical rows.

We also examined the localization of glutamylated tubulin, another marker for BB composition. In *Tetrahymena*, tubulin is glutamylated in a number of MT systems (Gaertig, 2000), but BBs stain particularly strongly with an antibody (R-polyE) raised against polyglutamic acid which reacts mainly with tubulins on Western blots of *Tetrahymena* whole cell protein (unpublished data). In the early stages of γ -tubulin depletion, glutamylated tubulin in BBs colocalizes with centrin staining (Fig. 7 A) in punctate BBs. By 17 h, some cells showed disperse centrin staining at their posterior ends which does not colocalize with the more punctate polyE staining. At later stages (30 h after depletion), staining of both centrin and glutamylated tubulin is no longer punctate and does not colocalize in most areas of the cell. The complete loss of organized oral and somatic BBs, coupled with the distinct patterns of disproportionation of the three BB components, indicate that BBs are dynamic structures whose components turn over at different rates.

A possible explanation for the apparent BB disassembly observed with centrin and polyglutamylation staining is that many BBs were not mature when γ -tubulin was either depleted or inactivated and that immature BBs are unstable. To test this, we analyzed the effects of depletion on trans-

verse microtubules (TMs) a marker for the MTOC activity of BBs. TMs appear late during BB formation, so are markers for relatively mature BBs (Allen, 1969). We visualized TMs with anti- α -tubulin antibodies (Fig. 7 B). By 17 h, as the number of BBs dropped, many cells started losing their TMs. By 30 h, many cells lacked detectable TMs. Interestingly, TMs disappear after γ -tubulin, at about the time that the punctate distribution of centrin and glutamylated tubulin are lost, suggesting that components of BBs other than γ -tubulin are sufficient to maintain TMs after they are formed.

To determine whether loss of γ -tubulin specifically affected the MTOC-related function of BBs or whether non-MT-associated functions were also affected, we analyzed kinetodesmal fibers (KFs, also known as striated rootlets). KFs are nonmicrotubular components associated with BBs in mature ciliary units that can be detected by antibody 5D8 (Jerka-Dzidosz et al., 1995). KFs also are disrupted by γ -tubulin depletion. In undepleted cells, the 5D8 antibody stained regions associated with the somatic BBs, but not the OA. In depleted cells, 5D8 staining showed progressive changes similar to those detected by anti-centrin antibody. Staining adjacent to somatic BBs started disappearing by 17 h and accumulated at the anterior end of cells by 30 h (Fig. 7 C). However, the distribution of centrin and the KF fiber antigen did not overlap completely (Fig. 7 C). Thus, the disappearance of both transverse MTs and KFs is more likely a secondary effect due to loss of BB structure rather than a direct effect of γ -tubulin depletion.

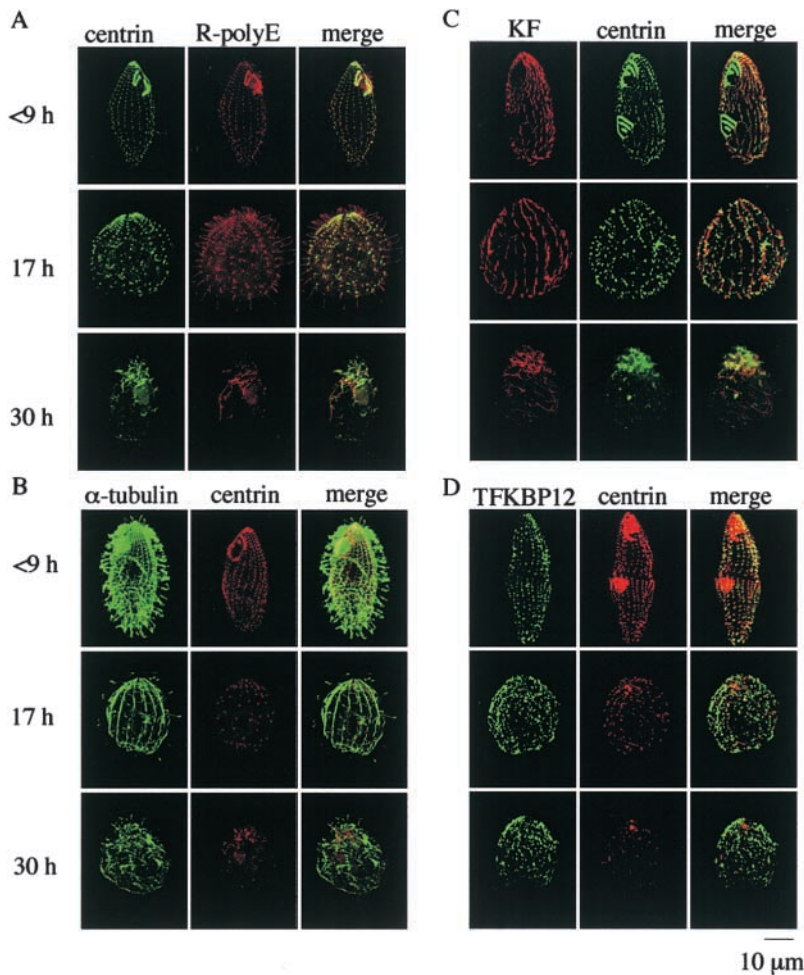


Figure 7. Comparison of BB components (centrin and glutamylated tubulin), transverse MTs, kinetodesmal fibers, and TFKBP12 staining at different times after cadmium depletion.

(A) Comparison of anti-centrin and anti-R-polyE (glutamylated tubulin) staining. (B) Comparison of anti-centrin and anti- α -tubulin staining. (C) Comparison of anti-centrin and anti-KF (kinetodesmal fiber) staining. (D) Comparison of anti-centrin and anti-TFKBP12 (cortical, non-BB structure) staining.

To determine whether γ -tubulin depletion disrupted cortical structures not directly associated with BBs, we examined the distribution of TFKBP12 (12 kD *Tetrahymena* FK506-binding protein), which normally surrounds the somatic BBs (Dr. Osamu Numata, personal communication). Anti-TFKBP12 staining (Fig. 7 D) was affected by the depletion of γ -tubulin only after punctate BBs and other cortical MT structures had largely been disrupted or had disappeared. In undepleted cells, TFKBP12 localized to rings around the somatic BBs. After depletion, this ring structure was retained but the rings became randomly distributed near the cell surface. These observations suggest that the changes in BB components and BB associated structures reflect specific effects of γ -tubulin depletion on the BBs, and not a more general affect on all cortical structures.

The effects of γ -tubulin depletion are reversible

To reinduce *GTU1* expression, we shifted cTTMG-HA cells that had been in cadmium-free SPP medium for either 28 or 42 h to SPP medium containing 1.0 μ g/ml CdCl₂. At various times after reinduction, cells were fixed and stained with anti-centrin, anti-R-polyE, and anti-KF (5D8) antibodies (Fig. 8). The BB components rapidly relocalized to newly formed somatic BBs. By 1 h after induction, we observed punctate areas throughout the cell, including the posterior end of the cell where there were almost no such structures after depletion, which could be stained by all three antibod-

ies (Fig. 8, a and b). Initially, newly formed BBs were arranged randomly. However, by 5 h, \sim 79% of the cells had short, randomly oriented rows of BB of different lengths. In some of these rows, most of the BBs were not ciliated (Fig. 8, c). After 12 h in CdCl₂, the BBs of most cells were organized into the correct pattern, cells regained WT shape, became motile, and started to divide (unpublished data).

Discussion

The *Tetrahymena thermophila* *GTU1* gene was shown to be a single-copy gene encoding a conventional γ -tubulin that localized to four major sites that agree well with γ -tubulin localization in other ciliates (Liang et al., 1996; Ruiz et al., 2000). The first is BBs, including those associated with somatic and oral cilia and with the OP before it becomes ciliated. Interestingly, the BBs are stained more strongly in dividing than in interphase cells, suggesting either that the amounts of γ -tubulin associated with BBs increases, or that a structural change occurs making it more accessible to antibody. The second is small patches associated with Mac envelopes in dividing cells. The third is patches associated with interphase Mic envelopes and the mitotic spindle, with preferential accumulation at the poles of dividing Mics. In *Tetrahymena*, γ -tubulin staining associated with the nuclear envelopes also increased in dividing cells. In vertebrate somatic cell lines, γ -tubulin staining associated with the centrosome

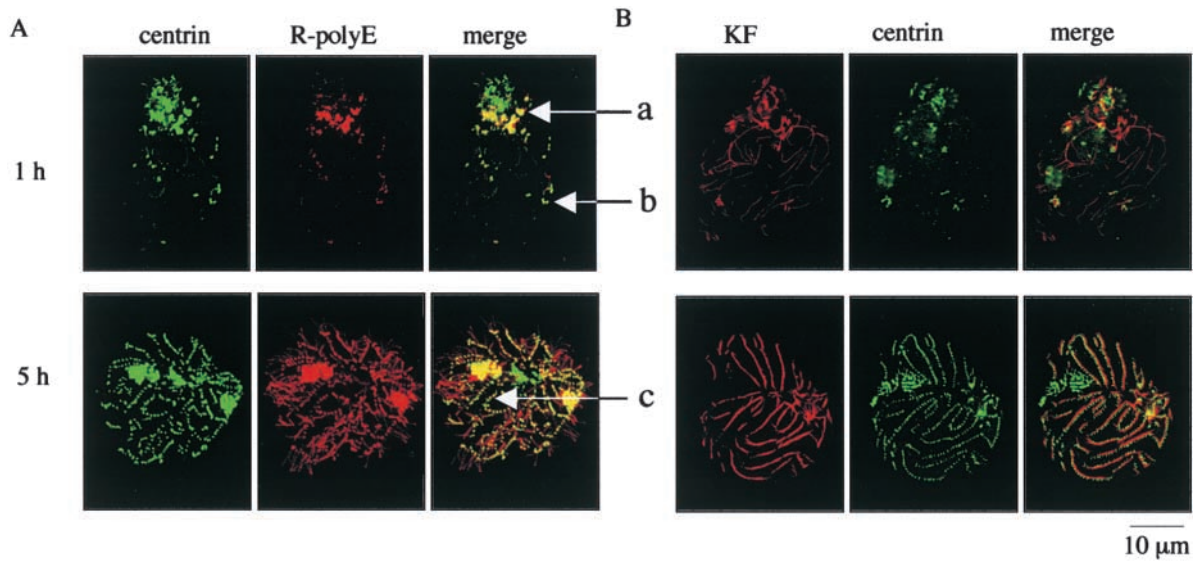


Figure 8. **Analysis of BB reformation after induction of γ -tubulin expression.** cTTMG-HA cells which had been in cadmium-free SPP for 28 h were shifted to SPP containing 1.0 $\mu\text{g/ml}$ CdCl_2 and were then fixed at 1 or 5 h and stained with (A) monoclonal anti-centrin antibody and anti-R-polyE. (a) Small specific areas which were stained by both antibodies. (b) BB-like structures at the posterior. (c) Rows of unciliated BBs. (B) Anti-centrin and anti-KF (kinetodesmal fiber).

remains relatively constant throughout interphase and increases markedly at the onset of mitosis (Khodjakov and Rieder, 1999). Thus, MTOCs associated with nuclear divisions in diverse organisms show similar dynamic changes during the cell cycle, likely reflecting the recruitment of γ -tubulin during MTOC duplication and/or activation. The fourth is the CVPs.

We analyzed γ -tubulin function by knocking out the *GTU1* gene or expressing it under control of the inducible-repressible *MTT1* promoter and repressing its expression. As in other organisms, the gene encoding γ -tubulin is essential in *Tetrahymena*. In the absence of γ -tubulin, cells stop growing and show progressive changes in cell and nuclear morphology (Fig. 9). The first visible phenotypes are the loss of the OA and CVPs. The cells then began to lose their shape and round up. The cortical MT structure is also affected. We also observed bundles of cytoplasmic MTs in some cells, consistent with previous studies in fungi showing that depletion of γ -tubulin led to the bundling of cytoplasmic MTs (Sobel and Snyder, 1995; Jung et al., 2001). Thus, in *Tetrahymena* as in fungi, one function of γ -tubulin may be to regulate the dynamics of cytoplasmic MTs. A novel finding of this study is that different MTOCs have different sensitivities to γ -tubulin depletion: CVPs and BBs are more sensitive than the nucleus-associated MTOCs. Abnormally dividing Macs and Mics were not observed until after many cells had lost their OAs and much of their somatic ciliature, had lost normal shape, and had badly mis-localized division furrows. Consistent with the resistance of nuclei to γ -tubulin depletion, immunofluorescence staining of HA-tagged γ -tubulin was retained, and may even have increased, on nuclear envelopes, especially those of Mics, after staining of BBs and CVPs was lost, suggesting that γ -tubulin in nuclear MTOCs turns over more slowly than γ -tubulin in BBs and CVPs. Thus, in addition to producing MT arrays that have different sensitivities to microtubule depolymerizing drugs

(Morrisette and Sibley, 2002), MTOCs themselves have different stabilities.

Two pathways for BB duplication have been identified (Marshall et al., 2001). In the more common, templated pathway, new BBs form in association with preexisting BBs. In the de novo pathway, BBs form in the absence of preexisting BBs. In *Tetrahymena*, most somatic BBs form once per cell cycle by a templated mechanism, (Allen, 1969) doubling the total number of somatic BBs each cell cycle. In contrast, to generate a new OA, a large number of BBs in the OP region is produced during a small portion of the cell cycle, either by rapid, repeated templating or, more likely, by a de novo pathway. A requirement for γ -tubulin in this rapid synthesis of new BBs likely explains why dividing cells without normal OP development appeared before other phenotypes were observed. We conclude that, as in *Paramecium* (Ruiz et al., 1999), γ -tubulin is required for BB duplication. Two lines of evidence support another novel finding of our study, that γ -tubulin is required for maintenance of mature BBs. First, the increase in cells lacking OAs was greater than the increase in cell number as growth in depleted cultures slowed and eventually stopped. Thus, some cells without OAs cannot be derived from cells that divided after failing to form a new OA. Second, cells continued to lose somatic BBs after they stopped growing. In both the depletion study and α mutant, the number of type IV or type III cells increased dramatically after the culture had stopped dividing. Loss of function of mature somatic BBs was also indicated by the loss of transverse MTs, which are nucleated from nearly fully formed BBs (Allen, 1969), after the culture stopped dividing. Similarly, kinetodesmal fibers, a nonmicrotubular element associated with mature ciliary units, was disrupted after the culture stopped dividing.

Surprisingly, the components of the BBs did not disappear in concerted fashion. First, γ -tubulin disappeared from almost all the BBs and from the cytoplasm, followed by the de-

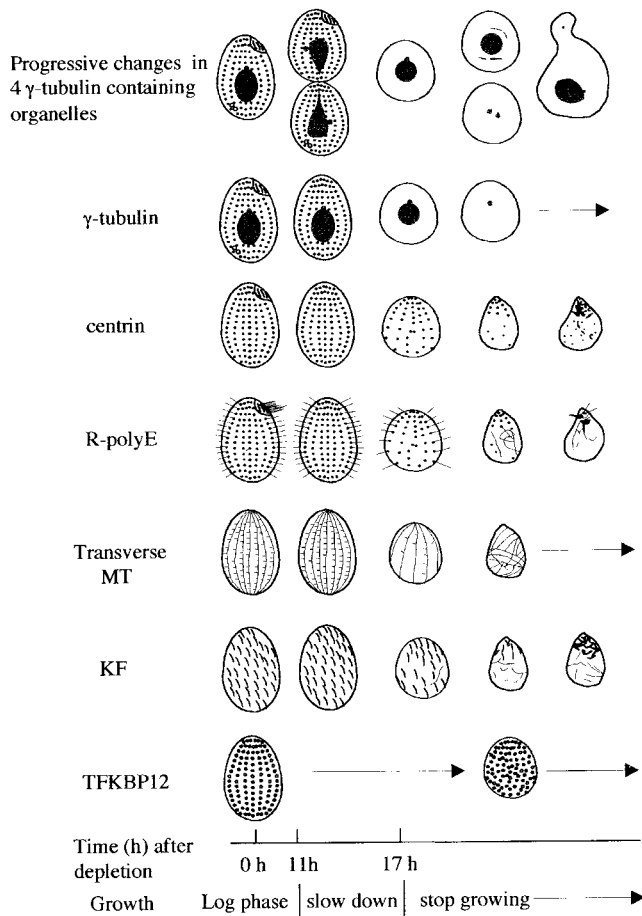


Figure 9. Summary diagram of the changes observed in *Tetrahymena* cells after depletion of γ -tubulin.

localization of centrin and glutamylated tubulin, key components of BBs. At late stages of depletion, few punctate structures remained in the posterior ends of the cells that could be stained by both antibodies. Most of the centrin and glutamylated tubulin staining localized to the anterior of cells, but, for the most part, did not colocalize. This gradual loss of different BB components after γ -tubulin depletion differs from the scattering of BB components that occurred after microinjection of anti-polyE antibody into tissue culture cells (Bobinnec et al., 1998). Asynchronous loss of components suggests that the functions of γ -tubulin in organizing and maintaining BBs are distinct. Consistent with this, the BBs of *Xenopus* sperm lack γ -tubulin, but acquire it in the egg cytoplasm before functioning in centrosomes (Stearns and Kirschner, 1994). In mammalian cells, γ -tubulin is a component of the core of the centriole (Fuller et al., 1995) whose dimensions are similar to those reported for the relatively stable microtubules (γ -tubules) produced by overexpression of γ -tubulin (Shu and Joshi, 1995). Thus, it is likely that γ -tubulin self-assembles into tubules, which organize the centriole and fill the centriolar lumen. In *Tetrahymena*, the loss of γ -tubulin from BBs during depletion indicates that, whatever its structure, the γ -tubulin of BBs is dynamic and is required for long-term retention of other BB components. In addition, the increase in nuclear staining during depletion suggests BBs and other MTOCs likely compete for γ -tubulin pool in the cell.

During recovery, γ -tubulin synthesis precedes the appearance of both centrin and glutamylated tubulin in newly formed BBs, consistent with a nucleating function for γ -tubulin. We first observed punctate, BB-like structures labeled by centrin, R-polyE, or KF antibodies, suggesting that the individualized new BB structures are relatively mature. Later, short rows of BBs appear. Compared with WT cells, there are two major differences in the morphology of these newly formed BB rows. First, the way they are arranged is different. Instead of being organized in continuous rows of relatively uniform length that are positioned roughly parallel to the long axis of the cell, the newly formed BB rows are randomly oriented and vary in length. This random pattern of the BB rows suggests that the first newly formed BBs were probably formed de novo at multiple sites, instead of by a templated pathway from some undetectable BB precursors that had retained their position in the cell. However, the fact that the precise cortical pattern was eventually reestablished suggests the existence of unknown mechanisms that serve to position already formed rows of BBs. The random distribution of TFKBP12 staining after depletion argues against the existence of a persistent, precise cortical pattern as an explanation for this reorganization.

The ability of ciliates to form BBs de novo was demonstrated by Hammersmith and Grimes (1981) who showed that encystment of *Oxytricha fallax* cells led to a loss of all the visible ciliary structures and BBs, which could be reformed upon excystment. However, in *Oxytricha*, all the cortical structures reformed in a correct pattern, indicating that the hypothetical determinative factors/structures for the cortical patterns were retained precisely and dictated the positions in which new BBs formed. The basis for these differences is not known. However, it is possible that, in our study, the depletion of γ -tubulin might have led to the disruption of the hypothetical determinative factors/structures. It is not known whether γ -tubulin is retained and remains localized in *Oxytricha* cysts.

Another observation consistent with a de novo pathway of BB formation in recovering cells is that the newly formed BB rows in these cells are not always ciliated as they are in WT cells. In *Tetrahymena*, new somatic BBs are normally generated immediately anterior and perpendicular to an old one, followed by a 90° rotation and migration to the surface; cilia form when the new BB contacts the cell surface (Allen, 1969). Therefore, in WT cells, only the newly formed, immature BBs are not ciliated. Thus, most somatic BBs duplicate once per cell cycle by a templated pathway, and adjacent unciliated BBs are rare. However, in the recovering cells, we observed BB rows with four or more adjacent unciliated BBs, suggesting either that BBs in these rows are forming more rapidly or that ciliogenesis is slower than in normal cells.

In conclusion, γ -tubulin is found in four MTOCs in *Tetrahymena* that show different sensitivities to γ -tubulin depletion, suggesting that the concentration of γ -tubulin in the cell could regulate the function of different MTOCs. γ -Tubulin is not only required for the formation of BBs but also for the retention of both centrin and glutamylated tubulin in mature BBs, and is, therefore, essential for maintaining the normal structure of BBs after they are formed. Fi-

Table I. Strains used in this study

Strain	Mic genotype	Mac genotype	Mac phenotype ^a
B2086 ^b	WT	WT	II
CU427 ^b	chx1-1/chx1-1	WT	cy-s, VI
CU428 ^b	mpr1-1/mpr1-1	WT	mp-s.VII
B*VI	nonfunctional mic (star)	WT	VI
B*VII	nonfunctional mic (star)	WT	VII
GTUKO5	gtu1::neo2/gtu1::neo2	WT	pm-s
GTUKO6	gtu1::neo2/gtu1::neo2	WT	pm-s
tG2NHA	WT	GTU1HA::neo2/GTU1HA::neo2	pm-r
cTTMG	gtu1::neo2/gtu1::neo2	MTT1/GTU1, gtu1::neo2/gtu1::neo2	pm-r
cTTMGHA	gtu1::neo2/gtu1::neo2	MTT1/GTU1HA, gtu1::neo2/gtu1::neo2	pm-r
cK358AK359A-HA	gtu1::neo2/gtu1::neo2	MTT1/gtu1HA, gtu1::neo2/gtu1::neo2	pm-r

^aIncluding drug resistance and mating type.

^bProvided by Dr. P. Bruns (Cornell University, Ithaca, NY).

nally, *Tetrahymena* cells can reform BBs, probably by a de novo pathway, and can reestablish the normal cortical structure from randomly arranged rows of BBs in the absence of obvious, preexisting organization of BBs.

Materials and methods

Strains, culture growth, and conjugation

Strains are described in Table I. *Tetrahymena* cells were grown in SPP (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA × ferrous sodium salt) at 30°C (Gorovsky et al., 1975). For conjugation, mid-logarithmic phase cultures of two different mating types were washed two times and resuspended in starvation medium (10 mM Tris-Cl, pH 7.5). After 16–20 h, equal numbers of each mating type were mixed at 2×10^5 cells/ml and incubated at 30°C without shaking.

Gene cloning and sequence analysis

Two degenerate primers were designed from regions conserved among available γ -tubulin sequences. First, strand cDNA was prepared by reverse transcription of RNA isolated from growing cells (White et al., 1988) and used as a template for RT-PCR. PCR products were cloned into Bluescript vectors (Stratagene) and sequenced.

For inverse PCR, *Tetrahymena* genomic DNA isolated (Gaertig et al., 1994b) from WT cells was digested with *HindIII* or *BglII*, self ligated, and amplified by inverse PCR with internal primers (GTUB01/02 and 04/05; Fig. 1) based on the partial genomic sequence of *GTU1*. Two primers (GTUB06/07) designed from the known end sequences of the cloned *GTU1* genomic fragment were then used for genomic PCR to amplify the complete genomic *GTU1* gene, which was cloned into pCRII (Invitrogen).

GTU1 coding regions were sequenced in independent clones three times in coding regions and twice in flanking regions (EMBL/GenBank/DBJ under accession no. U96076; Fig. 1). γ -Tubulin sequences were aligned using CLUSTAL software W (v. 1.6) (Thompson et al., 1994) on the DBJ web site. Trees were produced by neighbor-joining (Saitou and Nei, 1987). Bootstrap values were calculated (Felsenstein, 1985) by resampling 1,000 data sets.

Germline gene knockouts

For the *GTU1* knockout construct, pΔGN, the *neo2* gene cassette which confers paromomycin (pm) resistance when expressed in *Tetrahymena* was inserted between a 1.9-kb fragment of the *GTU1* 5' and a 1.0-kb fragment of the *GTU1* 3' flanking sequence (Gaertig et al., 1994a). The Mic *GTU1* gene was disrupted using biolistic particle bombardment (Cassidy-Hanley et al., 1997) to transform conjugating CU428 and B2086 cells 2.5 h after mixing with a 4.3-kb *KpnI*-*SacI* fragment released from pΔGN. Knockout heterokaryon strains with disrupted *GTU1* genes in their Mics and WT *GTU1* genes in their Macs (GTUKO5 and GTUKO6) were created as described (Hai and Gorovsky, 1997; Hai et al., 2000). When these strains conjugate, the old, pm-sensitive Macs are replaced by new ones produced from the cells' Mics. Consequently, the *neo2* gene disrupting the *GTU1* gene is expressed, allowing drug selection for successful mating. However, if *GTU1* is essential, the mating progeny will die unless they are transformed with a *GTU1* gene that supports growth.

Tagged and rescuing plasmids

To construct plasmid pG2N-HA for the *GTU1*-HA fusion gene under control of the *GTU1* promoter, two *neo2* gene cassettes were inserted into the *EcoRV* site of the WT *GTU1* 3' flanking sequence in pBL-GTU4. A HA epitope sequence (CYPYDVPDYASL) was inserted before the *GTU1* stop codon using the MORPH™ mutagenesis Kit (5 Prime - 3 Prime, Inc.). The insert of pG2N-HA was cut out with *NsiI* and *NotI* and transformed into starved *Tetrahymena* strain CU428 cells. Transformants were initially selected in 60–80 μ g/ml pm and then transferred every 3 to 4 d into media containing increasing drug concentrations until cells failed to grow (3 mg/ml). To construct a rescuing plasmid containing either the WT *GTU1* coding region (pTTMG) or the *GTU1*-HA fusion gene (pTTMG-HA) under control of the *MTT1* promoter, the *GTU1* or *GTU1*-HA coding region was inserted between the *MTT1* 5' and 3' flanking regions. The mutated *cs gtu1* gene (pK358AK359A-HA) was produced by PCR mutagenesis. Knockout heterokaryon strains GTUKO5 and GTUKO6 were mated and transformed with the *KpnI* and *SacI* fragments 24 h after mixing. Rescued progeny were selected with 60 μ g/ml pm in the presence of 1.0 μ g/ml CdCl₂.

Southern blot analysis and genomic PCR

To determine if the *GTU1* gene is a single copy gene in *Tetrahymena*, genomic DNA was digested with *EcoRV*, *HindIII*, or *BglII*, electrophoresed, blotted (Magnagraph membrane; Osmonics), and hybridized at 65°C with an [α -³²P] dATP-labeled PCR product from coding region (Ausubel et al., 1988). To confirm the genotype of the *GTU1*-HA transformants, the *GTU1*-HA fusion region of strain tG2N-HA, cTTMG-HA, or cK358AK359A-HA was PCR amplified and sequenced.

Western blot analysis

To demonstrate expression of HA-tagged γ -tubulin, CU428, tG2N-HA, and cTTMG-HA strains were grown to 2×10^5 cells/ml in SPP/CdCl₂ (1.0 μ g/ml) at 30°C, harvested and washed in 10 mM Tris-Cl, pH 7.5, containing protease inhibitors (Complete Mini; Roche). The cells were pelleted and dissolved in SDS sample buffer containing 5% β -mercaptoethanol (BME), 10% glycerol, 2% SDS, 60 mM Tris-Cl, pH 6.8. Proteins from 0.5 – 1×10^5 cells per lane were separated on 10% SDS-PAGE and transferred onto an Immobilon-P membrane (Millipore) by a semidry electrophoretic transfer unit (Bio-Rad Laboratories). The membrane was blocked in 5% dry milk in TBST (100 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated overnight at 4°C with monoclonal anti-HA antibody (16B12; Covance) or anti- β -tubulin (DM1B; Sigma-Aldrich) at 1:20,000 dilution. The membrane was washed in TBST at room temperature, incubated in rabbit anti-mouse IgG HRP-conjugated secondary antibody (Zymed Laboratories, Inc.) at 1:20,000 dilution for 1 h at room temperature, and washed five times for 5 min each in TBST, and then once in TBS. Proteins were detected using Western Blot Chemiluminescence Reagent (NEN).

Depletion/Inactivation of γ -tubulin

To bring the knockout phenotype to expression, heterokaryons GTUKO5 and GTUKO6 were mated. At 4 to 5 h after mixing, paired cells, which swim poorly and settle to the bottom of the dishes, were collected into fresh starvation medium. This was repeated three times, enriching pairs to ~95% of the population. Mating cells were refed with SPP medium 24 h after mixing. In the depletion studies, log phase cTTMG or cTTMG-HA cells, whose *GTU1* gene is under the control of the *MTT1* promoter requir-

ing CdCl_2 for expression, were shifted from SPP medium with to medium without CdCl_2 (0.5 $\mu\text{g/ml}$). To study the phenotype of the *cs* mutant, log phase cK358AK359A-HA cells were shifted from 30 to 15°C. Growth rates were measured by counting cells using a ZB1 Coulter counter and plotted using Cricket Graph III (Computer Associates).

Immunocytochemistry

To localize γ -tubulin *in vivo*, log phase cells from strain tG2N-HA or cTTMG-HA were processed for immunofluorescent labeling as described (Gaertig et al., 1995). HA antibody (16B12) was used at a 1:1,000 dilution. Secondary antibodies were goat anti-mouse FITC (Sigma-Aldrich) at a 1:200 dilution or Alexa fluor 568 goat anti-mouse IgG (Molecular Probes) at a 1:500 dilution. DAPI (4', 6'-diamidino-2-phenylindole) was used for DNA staining at 100 ng/ml. To study the effects of γ -tubulin depletion on BBs, *GTU1* knockout heterokaryon progeny or cTTMG-HA cells in CdCl_2 -free SPP medium were stained with anti-centrin monoclonal or polyclonal antibodies, provided by Dr. Jeffrey L. Salisbury (Mayo Clinic Foundation, Rochester, NY). Anti-glutamic acid polyclonal antibody (R-polyE), a rabbit antibody raised commercially (Alpha Diagnostic International) against a Cys (Glu)₉ peptide coupled to keyhole limpet hemocyanin, was used to stain BBs and cilia. MT structures were stained with anti- α -tubulin antibodies (DM1A; Sigma-Aldrich). All antibodies were used at a 1:1,000 dilution. Anti-TFKBP12 polyclonal antibody, provided by Dr. Osamu Numata (University of Tsukuba, Ibaraki, Japan) was used to stain the TFKBP12 (*Tetrahymena thermophila* FK506 binding protein of 12 kD) at a 1:250 dilution. Secondary antibodies were goat anti-mouse FITC (Zymed Laboratories, Inc.), goat anti-rabbit rhodamine (Zymed), and goat anti-rabbit FITC (Sigma) at 1:200 dilutions. To stain kinetodesmal fibers (KF, also known as striated rootlets), $2\text{--}5 \times 10^5$ depleted cells were placed on ice for 5 min, fixed in 35% EtOH/0.12% Triton X-100 on ice for 30 min, washed with cold TBS twice (Nelsen et al., 1994), and stained with mAb FI-5D8 (Jerka-Dziodosz et al., 1995) provided by Dr. Joseph Frankel (University of Iowa, Iowa City, IA). Secondary antibodies, Alexa fluor 568 goat anti-mouse IgG (Molecular Probes) and goat anti-rabbit FITC (Sigma-Aldrich), were used at a 1:500 or 1:250 dilution, respectively. Four to eight stacked images of cell were obtained using a Leica TCS SP confocal microscope (PL Apo 100 \times lens, N.A. 1.4, and 1.8 \times zoom).

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