Mutational analyses reveal a novel function of the nucleotide-binding domain of γ-tubulin in the regulation of basal body biogenesis

Yuhua Shang, Che-Chia Tsao, and Martin A. Gorovsky

Department of Biology, University of Rochester, Rochester, NY 14627

We have used in vitro mutagenesis and gene replacement to study the function of the nucleotide-binding domain (NBD) of γ-tubulin in *Tetrahymena thermophila*. In this study, we show that the NBD has an essential function and that point mutations in two conserved residues lead to over-production and mislocalization of basal body (BB) assembly. These results, coupled with previous studies (Dammermann, A., T. Muller-Reichert, L. Pelletier, B. Habermann, A. Desai, and K. Oegema. 2004. *Dev. Cell.* 7:815–829; La Terra, S., C.N. English, P. Hergert, B.F. McEwen, G. Sluder, and A. Khodjakov. 2005. *J. Cell Biol.* 168:713–722), suggest that to achieve the precise temporal and spatial regulation of BB/centriole assembly, the initiation activity of γ-tubulin is normally suppressed by a negative regulatory mechanism that acts through its NBD.

Introduction

The timing and position of the assembly of centrioles and basal bodies (BBs), which are two equivalent microtubule (MT)-organizing centers (MTOCs), are precisely regulated by cytoplasmic factors, and deregulation can lead to centrosome overproduction, genetic instability, and tumorigenesis (Delattre and Gonczy, 2004; Badano et al., 2005). Replication of centriole-containing centrosomes in mammalian cells normally occurs only once per cell cycle, and reduplication is prevented by a change intrinsic to the centrosome (Wong and Stearns, 2003; Delattre and Gonczy, 2004) and probably to the centriole itself (La Terra et al., 2005), suggesting that centriolar/BB components are direct targets of this inhibitory regulatory machinery. Genetically, a centriole/BB-associated molecule that qualifies for this role should meet two criteria: (1) its inactivation should block the assembly process, and (2) increased initiation activity caused by either overexpression or specific neomorphic mutations should lead to the deregulation of BB assembly.

γ-Tubulin, discovered as a suppressor of an *Aspergillus nidulans* β-tubulin mutation (Oakley and Oakley, 1989), is conserved in eukaryotes (Burns, 1995) and is structurally similar to α- and β-tubulins (Inclán and Nogales, 2001; Aldaz et al., 2005). γ-Tubulin is an essential protein involved in MT nucleation and dynamics (Wiese and Zheng, 1999; Oakley, 2000).

Therefore, it is not surprising that it is required for BB assembly and maintenance in ciliated protozoa (Ruiz et al., 1999; Shang et al., 2002a) and that centriole assembly failed to initiate in γ-tubulin–depleted *Caenorhabditis elegans* embryos (Dammermann et al., 2004). When centrioles/BBs are formed, the first visible structure that is assembled is a non-MT scaffold (Dippell, 1968; Anderson and Brenner, 1971). γ-Tubulin localizes to these centriole/BB precursors before centriolar MT assembly occurs (Khodjakov et al., 2002; Suh et al., 2002). However, γ-tubulin is not necessarily the limiting factor for centriole/BB formation, as the overexpression of γ-tubulin in *Tetrahymena thermophila* and in COS cells did not result in centriole/BB overproduction (Shu and Joshi, 1995; Shang et al., 2002a), although ectopic nucleation of MTs not associated with centrosomes was observed in the COS cells (Shu and Joshi, 1995). Thus, it is unclear whether γ-tubulin has a function in regulating the initiation stage of centriole/BB biogenesis that is distinct from its MT nucleation activity.

In *T. thermophila*, γ-tubulin localizes to precisely positioned BBs in highly organized somatic rows in the cell cortex and in the oral apparatus (OA) and is essential for their duplication and long-term maintenance/stability (Shang et al., 2002a). To study the role of γ-tubulin in BB biogenesis in *T. thermophila*, we performed systematic mutagenesis on the single γ-tubulin gene and screened for conditional mutants with defects in BB number or localization. Most point or clustered mutations in the non–nucleotide-binding domain (NBD) regions of *T. thermophila* γ-tubulin were cold sensitive and showed a loss of BB

Correspondence to Martin A. Gorovsky: goro@mail.rochester.edu

Abbreviations used in this paper: BB, basal body; GDP, guanosine diphosphate; KF, kinetodesmal fiber; ML, M loop; MT, microtubule; MTOC, MT-organizing center; NBD, nucleotide-binding domain; OA, oral apparatus.

Figure 1. Mutations in the NBD, but not K358AK359A on the inside surface, showed BB overproduction. Log phase *MTT1::GTU1*-HA (WT), *MTT::gtu1*-K358AK359A-HA, and *MTT1::gtu1*-A101G-HA grown at 30°C were shifted to 15°C in MEPPS containing 0.5 μg/ml CdCl₂ for the indicated period and were stained with anticentrin antibodies for BB morphology and DAPI for nuclear morphology. (A, a) Normal BB morphology in (wild type) *MTT1::GTU1*-HA cells; centrin antibody stains BBs in the oral apparatus (OA) and somatic basal body (sBB). (A, b) Failure of mutant *MTT1::gtu1*-K358AK359A-HA cells to duplicate BBs (loss of BB). (A, c) BB overproduction in *MTT1::gtu1*-A101G-HA cells is indicated by the increased density of BBs in some BB rows and OA. (B) Western blot probed with anti-HA antibodies to determine γ-tubulin levels in the three strains as shown.
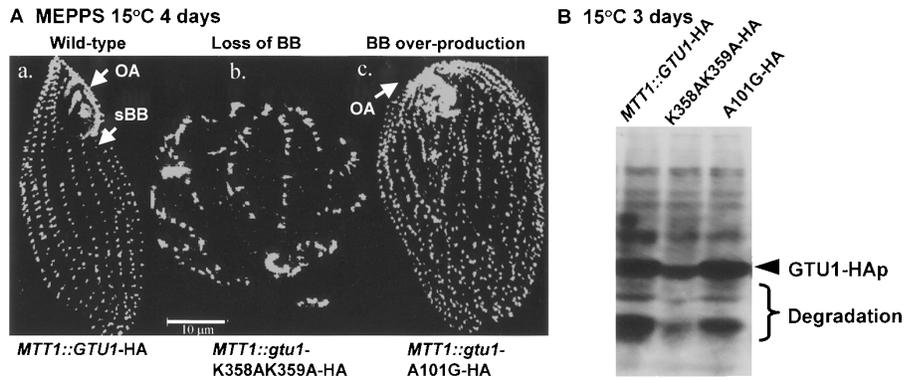
phenotype, similar to γ-tubulin depletion (Shang et al., 2002a). On the contrary, most of the point mutations that mapped to the putative NBD were lethal. However, two point mutations in the NBD glycine-rich loops, A101G in the T3 loop and T146V in the T4 loop, caused cells to overproduce BBs with random orientations outside of the cortical rows and deep in the cytoplasm. This suggests that γ-tubulin can nucleate BB assembly and that its nucleation activity is inhibited by interacting with an unidentified negative regulatory mechanism through its NBD.

Results

Systematic mutagenesis reveals an essential role for the NBD in γ-tubulin

Mutagenesis studies performed in *A. nidulans* and yeast identified five regions, all on the protein's surface (plus end, minus end, H3 surface, M loop [ML] surface, and COOH terminus), that were important for γ-tubulin function (Hendrickson et al., 2001; Jung et al., 2001; Vogel et al., 2001). However, these organisms do not contain centrioles or BBs. Moreover, these studies did not analyze the highly conserved NBD in γ-tubulin. Thus, we undertook a systematic mutagenic analysis of the role of the typical γ-tubulin of the ciliated protozoan *T. thermophila* in BB formation. To facilitate our studies, the γ-tubulin genes of the mutant and wild-type control strains were tagged with HA at the COOH terminus and were under the control of an inducible promoter (*MTT1*).

We first analyzed the phenotypes of mutations in the five regions homologous to those studied previously in fungal γ-tubulins (Table I; Hendrickson et al., 2001; Jung et al., 2001) at permissive (30°C) and nonpermissive (40, 15, or 20°C) temperatures. Normal *T. thermophila* cells contain large number of BBs, including those found in the OA at the anterior end and in rows of somatic BBs oriented in the long axis of the cell (Fig. 1 A, a). Most mutations in the five regions were either lethal or yielded cold-sensitive mutants that showed BB phenotypes similar to those described for γ-tubulin depletion in *T. thermophila* (Table I and Fig. 1 A, b; Shang et al., 2002a), including strong staining of γ-tubulin on the interphase macronuclear envelope and defects in BB duplication and BB stability (Shang et al., 2002a; unpublished data). None showed significant phenotypes

specifically at a high temperature (40°C). Cells with mutations in these regions lost their OA and most somatic BBs at 15°C. Centrin staining of the remaining somatic BBs also showed an abnormal dispersed pattern rather than punctate dots. Thus, mutations in these regions yielded hypomorphic phenotypes, indicating they are important for folding, stability, or other general functions of γ-tubulin in *T. thermophila* (Hendrickson et al., 2001; Jung et al., 2001). These mutants were not further studied.

Although most of the sites that affected γ-tubulin function in fungi also affected γ-tubulin function in *T. thermophila*, the behavior of some mutations in *T. thermophila*, especially in the COOH-terminal tail, differed from similar mutations in yeast. For example, in budding yeast, deletion of the COOH-terminal 44 residues was not lethal (Vogel and Snyder, 2000), but in *T. thermophila*, deletion of even 21 COOH-terminal residues was lethal (Table I). In yeast, Y445 in the DSYL motif in the COOH-terminal tail is phosphorylated, and mutation of this residue to aspartate (Y445D) resulted in a temperature-sensitive mutant with increased numbers of MTs and cell cycle arrest before anaphase (Vogel et al., 2001). However, the equivalent mutation (Y437D) in *T. thermophila* showed no phenotype under any of the conditions we tested, suggesting that the phosphorylation on Y445 observed in *Saccharomyces cerevisiae* γ-tubulin is not conserved. Interestingly, Y429, which also is highly conserved in γ-tubulins, is essential (Table I) in *T. thermophila* but not in yeast.

Previous mutagenesis studies focused on clustered charged residues, which are rare in the NBD, and, therefore, neither study revealed the function of the NBD in γ-tubulin (Hendrickson et al., 2001; Jung et al., 2001). We performed the first mutagenic analysis of the NBD of any γ-tubulin. This domain, conserved in all γ-tubulins, includes loops T1–T6, helix H1, and the NH₂-terminal end of helix H7 (Nogales et al., 1998; Nogales, 2001). It shares high sequence homology with the NBD of α/β tubulins (Inclán and Nogales, 2001), and the affinities of γ and β for GTP or guanosine diphosphate (GDP) are not significantly different (Aldaz et al., 2005). However, in addition to the phosphate-binding tubulin signature motif (GGGTGSG) found in the T4 loop of α- and β-tubulins, the NH₂-terminal region of the T3 loop in all γ-tubulins contains additional glycine insertions (Fig. 2; Burns, 1995; Inclán and Nogales, 2001; Aldaz et al., 2005) generating a

Table1. **Systematic mutagenesis of γ-tubulin in *T. thermophila***

Region	Mutation[1]	Domain[2]	Phenotype	
			30°C	15 or 20°C
H3 lateral surface	K48AE49A	H1-S2	WT	WT
	D56AD57A	H1-S2	Lethal	Lethal
	H59AR63A	H1-S2 + S2	Lethal	Lethal
	K114A	H3	OK[4]	OK
	D116AD117A	H3	Slow	Cold senstive, loss of BB
	D120A	H3	Lethal	Lethal
	E126K	H3	Lethal	Lethal
	K164AK165A	H4-S5	OK	Weak cold senstive, a few losing OAs
ML lateral surface	I275A	M loop(S7-H9)	Lethal	Lethal
	D278A	M loop(S7-H9)	OK	ND
	R285AD291A	M loop(S7-H9) + H9	OK	Cold sensitive, type ND
	R285E	M loop(S7-H9)	OK	OK
	R285Q	M loop(S7-H9)	OK	ND
	K286E	M loop(S7-H9)	OK	Cold sensitive, loss of BB
	R285DK286D	M loop(S7-H9)	OK	Cold sensitive, loss of BB
	R294AR295A	H9	Lethal	Lethal
	V304A	H9-S8	Slow	OK
Plus end	K211A	H6	OK	Weak cold sensitive, a few losing OAs
	T233A	COOH-terminal half of H7	OK	OK
	K396AK397A	H11–H12, plus end, outside surface, longitudinal	OK	OK
	K405AK406A	H11–H12, plus end, outside surface, longitudinal	Lethal	Lethal
Minus end	R243A	T7 loop(H7–H8)	Slow	Cold sensitive, loss of BB
	D251A	T7 loop(H7–H8)	OK	OK
	R335AR337A	H10 + H10-S9	OK	OK
	E338K	H10, ML lateral surface and minus end	Lethal	Lethal
COOH-terminal tail	Y429Dor Y429A	COOH-terminal tail	Lethal	Lethal
	Y429F	COOH-terminal tail	OK	Cold sensitive, loss of BB
	Y437D	COOH-terminal tail	OK	OK
	CΔ20(Δ430–449)	ΔCOOH-terminal tail	OK	OK
	CΔ21 (Δ429–449)	ΔCOOH-terminal tail	Lethal	Lethal
Outside surface	K192AR193A	H5 + H5-S6	OK	OK in MEPPS
Inside surface	K358AK359A	S9-S10	OK	Cold sensitive, loss of BB
	H367AK368A	S9-S10	OK	OK
Nucleotide-binding pocket	C13A	H1	OK	Weak cold sensitive, loss of BB
	Q24V	H1	OK	OK
	R72A	T2 loop	Lethal	Lethal
	A101G	NH2-terminal half of glycine-rich T3 loop	Slow (3–4 h)[3]	BB deregulation
	V104A	NH2-terminal half of glycine loop T3 loop	Lethal	Lethal
	E111A	COOH-terminal half of T3 loop, highly divergent in GTUp	OK	OK
	T146V	T4 loop	Slow (5–7 h)[3]	BB deregulation
	T146A, T146G	T4 loop	Lethal	Lethal
	D179A	T5 loop	Lethal	Lethal
	D205N	T6 loop	Lethal	Lethal
	I224A	H6–H7, βY224 interacts with nucleotide base	Lethal	Lethal
	L230T	NH2-terminal half of`H7	Lethal	Lethal
Wild type	MTT1:GTU1-HA		OK (2.5 h)	OK

[1]In all of the strains, the γ-tubulin gene is under the control of the MTT1 promoter. The MTT1:GTU1-HA strain grows normally and showed no obvious defects and, therefore, will be refered to as wild type.
[2]The domains are defined in Inclán and Nogales (2001).
[3]Indicates the doubling time.
[4]The growth of culture shows no detectable phenotype compared with wild type.

second, similar (GgGAGNN) region, and the crystal structure of human γ-tubulin revealed that unlike β-tubulin, the T3 and T5 loops are disordered in the NBD of γ-tubulin monomers (Aldaz et al., 2005). Thus, the structural differences in the T3 and T5 loops do not affect nucleotide binding, per se, and the γ-tubulin NBD could have unidentified, γ-specific functions in MTOCs.

γ–tubulin sequence comparison

	T3 loop	T4 loop
Tetrahymena	GsG**A**GNv......	GG**T**GSG
Chlamydomonus	GgG**A**GNn......	GG**T**GSG
Human	GgG**A**GNn......	GG**T**GSG
Drosophila	GgG**A**GNn......	GG**T**GSG
S.pombe	GGG**A**GNn......	GG**T**GSG
S.cerevisiae	GasA**A**GNs......	GG**T**GSG
GTU Consensus	GgG**A**GNn......	GG**T**GSG
BTU Tetrahymena	G--**A**GNn......	GG**T**GSG

Figure 2. **γ-Tubulin sequence comparison.** Sequence comparison of the sequence of the T3 and T4 loops of γ-tubulin of *T. thermophila* and five other species with *T. thermophila* β-tubulin (BTU). GTU, γ-tubuin.

Many mutations in the *T. thermophila* γ-tubulin NBD, including T146G/T146A in the tubulin signature motif (T4 loop), a region shown to contact the nucleotide phosphates in human γ-tubulin, were lethal (Table I). Thus, as expected, the NBD has an essential function in γ-tubulin.

A novel role of the NBD in γ-tubulin in BB functions

To further study the function of the NBD in γ-tubulin, we generated two more mutations: *MTT1:gtu1*-A101G-HA in the unstructured region of the T3 loop and *MTT1:gtu1*-T146V-HA (an essential site likely involved in nucleotide binding) in the T4 loop, both of which are absolutely conserved in γ-tubulins (Fig. 2). These could rescue the knockout γ-tubulin heterokaryons with low efficiency. Both A101GHA and T146VHA mutants grew slower than wild type at 30°C and showed a cold-sensitive phenotype in both SPP (0.5 µg/ml CdCl$_2$) and MEPPS media (0.75 µg/ml CdCl$_2$) at 15 and 20°C, respectively, indicated by slower growth rate and lower maximum cell density (unpublished data).

In *T. thermophila*, in addition to being in BBs, γ-tubulin is located on the micro- and macronuclear envelopes and is required for the division of both nuclei (Shang et al., 2002a). However, neither mutant showed detectable phenotypes associated with abnormal nuclear divisions, including abnormal micronuclear spindle morphology, abnormal macronuclear division, loss of micro- or macronuclei, and multiple micronuclei (≥3/cell) or macronuclei (≥2/cell; unpublished data).

Moreover, instead of losing their BBs like the viable mutants with hypomorphic mutations in other regions, these two cold-sensitive NBD mutants showed increased densities of BBs in some somatic rows and in the OA (Fig. 1 A, c). This BB overproduction phenotype is specific for the NBD mutations rather than for overexpression of γ-tubulin by the *MTT1* promoter because (1) *MTT1:gtu1*-A101G-HA and wild-type *MTT1:GTU1*-HA strains contained similar amounts of γ-tubulin, which is in contrast to reduced levels observed in a typical hypomorphic mutant strain (Fig. 1 B; *MTT1:gtu1*-K358AK359A-HA), and (2) BB overproduction was not observed at 15°C in *MTT1:GTU1*-HA cells, in which wild-type *GTU1*-HA is overexpressed to a similar level as that in the *MTT1:gtu1*-A101G-HA mutant.

Therefore, we conclude that the NBD of γ-tubulin has a specific function in BB assembly or organization. Interestingly, although the two mutated residues both reside in the γ-NBD,

only T146 directly contacts the nucleotide (Aldaz et al., 2005). A101 is in the disordered T3 loop that appears to have little effect on GTP or GDP binding (Aldaz et al., 2005), suggesting that nucleotide binding, by itself, may not be responsible for the mutant phenotypes.

Mutations in the NBD specifically affect the orientation, position, and density of BBs

Because BB duplication is controlled temporally so that it is coordinated with the cell cycle in normal *T. thermophila* cells, the increased density of BBs suggests that these NBD mutants could have defects in the regulation of BB assembly. BB duplication in *T. thermophila* also is highly regulated spatially (Nanney, 1975; Kaczanowski, 1978). New somatic BBs are produced by a typical templated pathway immediately anterior and perpendicular to an old one, followed by a 90° rotation and migration to the surface. Therefore, the mother BB is always posterior to the new BB, and somatic BBs are found in the rows and almost never in between two rows. Thus, new somatic BBs are normally located on the cell surface anterior to and in the same rows as the mother BBs and face the same direction (Allen, 1969). This pattern of formation helps ensure the inheritance of the normal BB row organization during growth and division.

In *T. thermophila*, the orientation and position of individual mature BBs can be detected easily by antibodies against kinetodesmal fibers (KFs), which are nonmicrotubular structures that associate only with mature somatic BBs (Jerka-Dziadosz et al., 1995; Shang et al., 2002a). In wild-type cells, KFs are oriented parallel to the longitudinal axis of the cell, running from posterior to anterior on the same side of all somatic BBs (Fig. 3 A, a and e). Within 1 d of cold treatment, before most of the mutant cells had ceased dividing, BBs were observed with KFs pointing in random directions (Fig. 3 A, f and g; arrows), indicating a failure to orient properly. In addition, some BBs were abnormally positioned outside of rows and were not adjacent to a preexisting BB (Fig. 3 A, f and g; arrowheads). Internal BBs in the cytoplasm beneath the cell surface were also detected (Fig. 3 B; similar results for *MTT1:gtu1*-T146V-HA mutants are not depicted). The existence of internal BBs was confirmed by transmission EM (not depicted). Again, the density of the somatic BBs in some areas of the mutant cells is higher compared with the wild-type strain (Fig. 3 A, bottom), suggesting that these mutant cells generate more BBs than wild-type controls. Therefore, the two mutations in the NBD of γ-tubulin lead to defects in BB orientation, position, and density.

Mutations in the NBD result in defects in newly formed BBs

γ-Tubulin is required for the long-term maintenance of mature BB structure (Shang et al., 2002a), so it is possible that preexisting BBs became unstable in the cold-treated mutants, which caused delocalization of BBs from the BB rows and led to the dissociation of some BB-associated structures. To test this hypothesis, we first examined the stability of preexisting BBs by analyzing BB organization in starved cultures at the restrictive temperature. After 5 d, most of the cells in both mutant strains
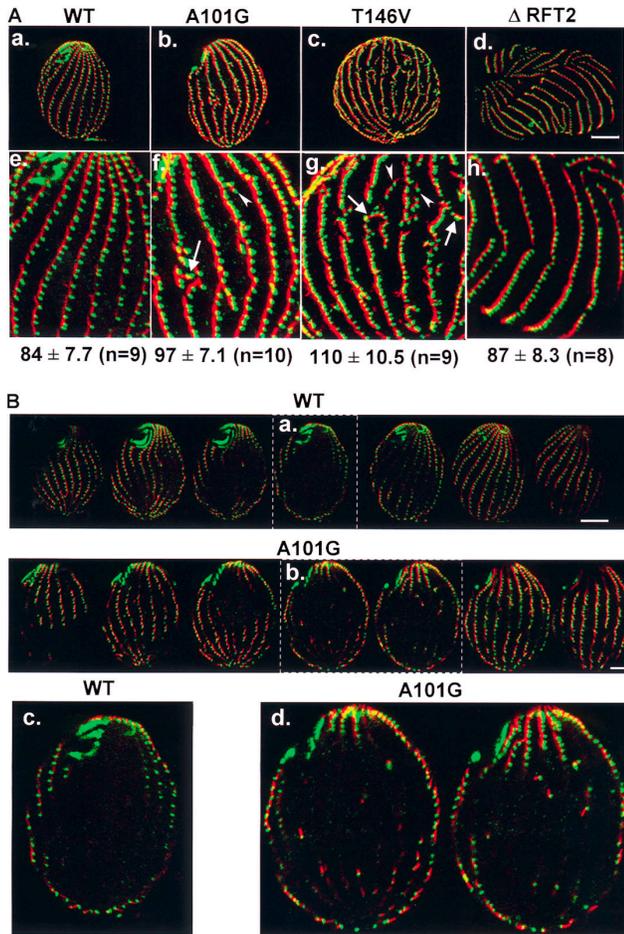
Figure 3. **The abnormal pattern of BBs in NBD mutants.** Wild-type (WT), *MTT1::gtu1*-A101G-HA mutant, and T146V mutant strains (see Materials and methods for details) grown at 15 or 20°C in 0.5 μg/ml CdCl₂ for 1 or 4 d were fixed and stained with anticentrin (green) and anti-KF (red) antibodies. (A, a–d) Stacks of one half of each cell are shown. (e–h) Images at higher magnification. (a and e) Wild-type cell. (b, c, f, and g) Three types of defects in NBD mutants: (1) BBs with different orientations (arrows); (2) BBs not in rows (arrowheads); and (3) increased BB densities. The BB density (the number of BBs/250 μm²) is listed beneath the bottom panels. (d and h) As a control for the effects of cell enlargement, a ΔRFT2 mutant cell grown at 30°C for 3 d (these mutant cells do not grow at lower temperatures) shows incomplete division furrows without severe disruption of BB row organization or abnormal BB density. (B) Internal BBs in the cytoplasm of a wild-type cell and a mutant. A Z-series shows thin sections of a wild-type (a) and an *MTT1::gtu1*-A101G-HA cell (b) after 1 d at 15°C. (c and d) Images from the lettered panels (a and b) at higher magnification. Bars, 10 μm.

showed normal cortical structure that was indistinguishable from that of wild-type cells (unpublished data). Thus, the BB defects are only observed in mutant cells that are able to grow and make new BBs at the restrictive temperature and are not a result of cold-sensitive disruption of preexisting mutant BBs.

We then examined whether the spatial pattern for the formation of new (immature) BBs was affected, which would strongly argue for ectopic BB formation. Antibodies against K antigens, which are proteins of unknown function that associate only with mature BBs (Williams et al., 1990), can be used to distinguish mature from newly formed BBs (Fig. 4 A, e; white arrowheads and pink arrows, respectively). In mutant cells, but not in wild-type cells, we observed immature BBs at abnormal

positions not adjacent to a preexisting BBs (Fig. 4 A, f and g; pink arrows; similar results for *MTT1:gtu1*-T146V-HA are not depicted). Almost half of the BBs found near the cell surface but outside of rows in mutant cells were immature, and the ratio of immature to mature BBs not in rows was almost three times greater than the ratio within rows in the same cell (not depicted). These observations suggest that new BBs are forming ectopically in the cell cortex in mutant cells. We also detected ectopic formation of immature BBs in the interior cytoplasm beneath the cell surface (Fig. 4 B, a–d).

During the cell cycle, all old BBs in wild-type *T. thermophila* cells do not give rise to new ones. Instead, BB proliferation shows an antero-posterior gradient (Nanney, 1975; Kaczanowski, 1978). If a wild-type cell is divided into four sectors from posterior (sector I) to anterior (sector IV), the most active region of BB duplication in most cells is localized to sector II, which is just posterior to the midline, where the fission zone will be formed the next time the cell divides (Fig. 4 C; Nanney, 1975; Kaczanowski, 1978). 9/10 control MTT1:GTU1-HA cells showed a spatial pattern of new BBs (Fig. 4 C) similar to that reported previously, whereas the spatial pattern of new BBs either in the BB rows or outside rows in nine A101G mutant cells examined was different and more variable (Fig. 4 C; Nanney, 1975; Kaczanowski, 1978).

Together, the aforementioned observations argue that some new BBs in the NBD mutant cells are being formed in the absence of adjacent old BBs. Therefore, we conclude that the phenotypes in these mutants are caused by ectopic localization of newly formed BBs, suggesting an essential function of the NBD in γ-tubulin in assembly of new BBs, which differs from those of other essential domains in γ-tubulin.

Defects in BB assembly are not caused by cell cycle arrest

Because the NBD mutant cells stopped dividing and large cells accumulated at the restrictive conditions, the accumulation of BBs observed in the NBD mutants could be caused by cell cycle arrest at a stage when BBs are formed. However, despite the variable number of somatic BBs, these mutants contained neither numerous or enlarged micro- or macronuclei (unpublished data), which is suggestive of a cell cycle defect. Moreover, in growing *T. thermophila* cells, the new OA is formed at the same stage in the cell cycle as when most somatic BBs duplicate, so that a cell cycle block at the stage when BBs duplicate might be expected to result in cells with multiple OAs. However, <4% of the mutant cells contained an extra OA (unpublished data) in any of the conditions we tested.

Abnormal BB phenotypes were detected in mutant cells within 1 d after they were shifted to the restrictive temperature, whereas the cells continued dividing for 3 d. Before cell division had ceased at the nonpermissive temperature, in some dividing mutant cells that exhibited normal nuclear division and a normal fission furrow, we observed increased numbers of BBs that were misoriented and were located outside of rows in sector III (Fig. 5; similar results for *MTT1:gtu1*-T146V-HA are not depicted). Also, when mutant strains were released from 15 to 30°C, they resumed growth within 5–7 h (not depicted) without detectable syn-
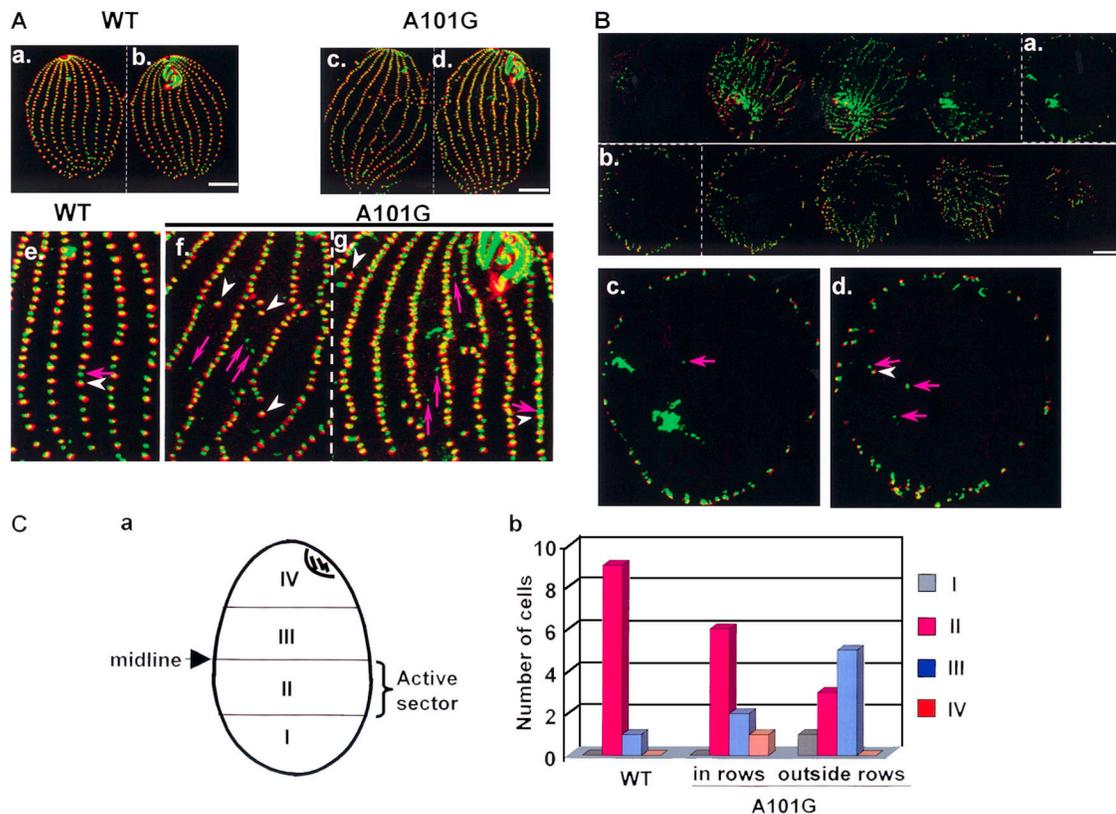
Figure 4. **Abnormal location of new BB formation in the NBD mutants.** Log phase wild-type or *MTT1::gtu1*-A101G-HA cells grown at 15°C in MEPPS (0.5 μg/ml CdCl₂) for 1–2 d were fixed and stained with anticentrin (green) and anti-K (red) antibodies. Pink arrows and white arrowheads indicate new BBs without anti-K staining and mature BBs with anti-K staining, respectively. (A) Stacks of both upper and lower halves of wild-type (a and b) and mutant cells (c and d) are shown. (e–g) Images from a, c, and d at higher magnification, respectively. Bars, 10 μm. (B) A Z-series showing thin sections of a *MTT1::gtu1*-A101G-HA mutant cell with internal BBs. Two focal planes (a and b) through internal sections of the cell are shown at higher magnification in c and d. Internal BBs were rarely seen in wild-type cells. (C) To study the spatial pattern of new BB formation, each cell was divided into four sectors (a), and the percentage of new BBs in each sector in each cell was calculated. Cells were then grouped into four categories based on which sector is the most active region for new BB assembly, and the number of cells in each category was determined. For the *MTT1::gtu1*-A101G-HA mutant, the new BBs inside or outside BB rows were calculated separately.

chrony in micronuclear division (not depicted), indicating that the mutant cells were not blocked at a specific stage of the cell cycle.

As a control, we also examined another mutant that blocks growth and accumulates enlarged cells. *T. thermophila* mutants with cilia assembly defects tend to arrest at cytokinesis, becoming large cells with extra BBs and nuclei (Brown et al., 1999, 2003; unpublished data). However, the monster cells produced by a mutant defective in intraflagellar transport do not show increased BB densities (Fig. 3 A, d and h), and their BB rows remain mostly well organized over much of their lengths (the distortions reflect the fact that incompletely divided cells tend to reintegrate into monsters whose global architecture is disrupted).

Thus, the BB defects we observed are unlikely to be caused by cell cycle arrest, slow growth, or a block to cytokinesis, indicating a more direct role of the NBD in BB assembly.

Newly formed mutant BBs are functional

To determine whether mutant BBs formed at the restrictive temperature have structural or functional defects that prevent them from maturing and forming rows, we performed depletion–reinduction experiments. Because the γ-tubulin genes in both the two mutants and the wild-type strain described in this study are under

control of the *MTT1* promoter, their expression requires cadmium (Shang et al., 2002a,b). Removal of cadmium at the permissive temperature resulted in the depletion of γ-tubulin and the disassembly of most preexisting BBs in all three strains. When the expression of γ-tubulin was restored by adding cadmium at the restrictive temperature, wild-type cells recovered normal cortical structure. Although mutants failed to fully recover, most of the new BBs formed at 15°C could be assembled into short BB rows at a similar rate as wild-type cells (Fig. 6 A) and were associated with the two mature BB-associated structures, KFs (Fig. 6 B) and K antigens (not depicted). By 29 d, many mutant cells had assembled more BBs than the control strain (Fig. 6 C) and showed increased BB densities in some rows, suggesting that in the mutant cells, the rate of BB formation is uncoupled from (and exceeds) the rate of cell growth. Staining with an antipoly-glutamic acid antibody (Shang et al., 2002a) showed that most of the mutant BBs that assembled at 15°C contained polyglutam-ylated tubulins and were associated with cilia (Fig. 6 D), which require functional BBs for their formation and maintenance.

Thus, new BBs that formed in the NBD mutant cells at restrictive temperature appear to mature normally; they have normal associated structures, can be organized into rows, and
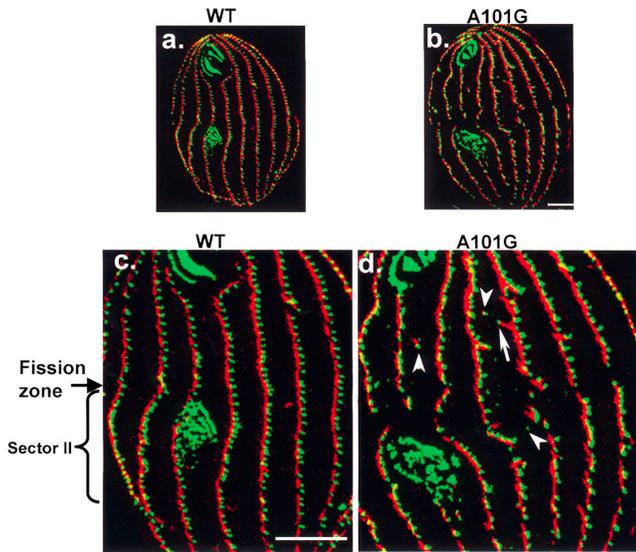
Figure 5. **Abnormal formation of new BBs in sector III of dividing mutant cells.** Both *MTT1::GTU1*-HA (a) and *MTT1::gtu1*-A101G-HA strains (b) were shifted from 30 to 15°C and stained with anticentrin (green) and anti-KF (red) antibodies. A dividing cell with a fission zone from each strain is shown. (c and d) Images at higher magnification. Sector II, the active zone for BB duplication, is normally posterior to the fission zone. In *MTT1::gtu1*-A101G-HA strains (d), the arrow shows BBs with abnormal orientation, and arrowheads show BBs in abnormal positions. Bars, 10 μm.

can nucleate cilia. These observations argue that the phenotypes observed in the NBD mutants are not caused by structural or functional defects in centriolar MTs in individual mutant BBs but rather to a defect in the temporal and spatial regulation of new BB formation that leads to BB overproduction and ectopic formation unassociated with preexisting BBs.

Discussion

We performed systematic mutagenesis of γ-tubulin and found that five regions on the protein's surface (plus end, minus end, H3 surface, ML surface, and COOH terminus) are important for γ-tubulin function in *T. thermophila*, which is consistent with the studies performed in *A. nidulans* and *Schizosaccharomyces pombe* (Hendrickson et al., 2001; Jung et al., 2001; Vogel et al., 2001). Many conditional mutants in these areas show defects in BB assembly, mimicking γ-tubulin depletion. Unlike the previous analyses of γ-tubulin mutations (Hendrickson et al., 2001; Jung et al., 2001; Vogel et al., 2001), we also analyzed the NBD, showing that most mutations in the nucleotide-binding pocket are lethal, including T146G in the T4 loop (tubulin signature domain), suggesting an essential role for the NBD in γ-tubulin function in *T. thermophila*.

In a further analysis of the function of the NBD, we studied the phenotype of two cold-sensitive mutant strains with a mutation (A101G) in the T3 loop (the second glycine-rich loop, mimicking the T4 loop) or in the T4 loop (T146V). Surprisingly, both mutations in the γ-tubulin gene conferred a novel phenotype with defects in the formation and organization of newly formed BBs but not in BB maturation, stability, or in γ-tubulin function in nuclear division.

One issue that arises is whether these mutations reflect a dominant gain of function or a recessive loss of function. Unfortunately, this is not easily determined in *T. thermophila* because it is not possible to create a stable diploid in which the mutant allele being tested is expressed in roughly the same amount as the wild-type allele. Although it is possible to create stable diploids in the germline micronucleus, the micronucleus is transcriptionally silent. It is impossible to create a stable diploid situation in the macronucleus, where genes are expressed, because it is polyploid and divides amitotically. Thus, chromosomes in the macronucleus do not segregate; they are distributed randomly to daughter cells in approximately (but not exactly) equal numbers. Therefore, even if one starts out with a heterozygote in the germline micronucleus, as soon as the macronucleus is formed from the micronucleus during the sexual process of conjugation, different cells have different numbers of each allele, and within ~50–100 fissions, all cells are completely homozygous in their macronuclei for one or the other allele (although they are still heterozygous in their micronucleus). Therefore, the conditions necessary to test for dominance cannot be achieved in *T. thermophila*.

We considered three explanations for the ectopic locations of new BBs: (1) deregulation of BB formation; (2) indirect effects of cell cycle arrest, as observed in some mammalian cells (Khodjakov et al., 2002; Wong and Stearns, 2003); and (3) indirect effects of structural/functional alterations in the individual BBs. Several lines of evidence suggest that the disruption of BB rows is caused by the deregulation of BB biogenesis.

First, we showed that BBs with random orientations could be detected at abnormal positions and that in some areas in mutant cells, the density of BBs is higher than that of wild-type cells, suggesting that new BB biogenesis is decoupled from the cell cycle. More importantly, we could detect newly formed BBs in abnormal positions, including deep in the cytoplasm, with random orientations, some of which were not adjacent to a mature BB. The anterior–posterior gradient of BB proliferation in mutant cells was also disrupted. In wild-type cells, the most active region of BB duplication is almost always localized to sector II, just posterior to the midline where the fission zone will be formed in the next division (Nanney, 1975; Kaczanowski, 1978), whereas this spatial pattern is disrupted in mutants. These data strongly suggest that new BBs were ectopically generated in these mutants.

Second, the appearance of BBs with different orientations at abnormal positions is growth dependent, suggesting that the stability of preexisting BBs was not severely affected. Therefore, the defects observed in the mutant cells were not results of the disassembly and delocalization of preexisting BBs from their original positions in BB rows caused by the mutant γ-tubulin protein, but instead resulted from the defects in BBs produced under restrictive conditions.

Third, we have presented evidence that the overproduction of BBs observed in the NBD mutants was not caused by cell cycle arrest at a stage when BBs are formed. Before cell division had ceased at the nonpermissive temperature, we observed an increased number of BBs with different orientations and outside of rows in dividing mutant cells exhibiting normal

Figure 6. **NBD mutant cells can regenerate BBs without severe structural/functional defects at the restrictive temperature.** The γ-tubulin–depleted wild-type or *MTT1::gtu1*-A101G-HA cells were shifted to 15°C in MEPPS containing 0.5 μg/ml CdCl$_2$ for the indicated times, fixed, and stained with anticentrin antibody to visualize and count BBs (A and C). (A) The percentage of recovering cells with BB rows. (B) Immunofluorescence staining with anti-KF (red) and anticentrin (green) shows one fully recovered wild-type cell (inset) and five *MTT1::gtu1*-A101G-HA mutant cells showing a high density of BBs in some areas and BB rows in different orientations. Bar, 10 μm. (C) The number of BBs/cell in wild-type and *MTT1::gtu1*-A101G-HA cells was counted 29 d after recovery. Cells with different number of BBs were grouped into seven categories, and the percentage of cells in each category was determined. (D) Immunofluorescence staining with antipolyglutamic acid antibody (green) and anticentrin (red) shows that many BBs synthesized at 15°C have nucleated cilia.
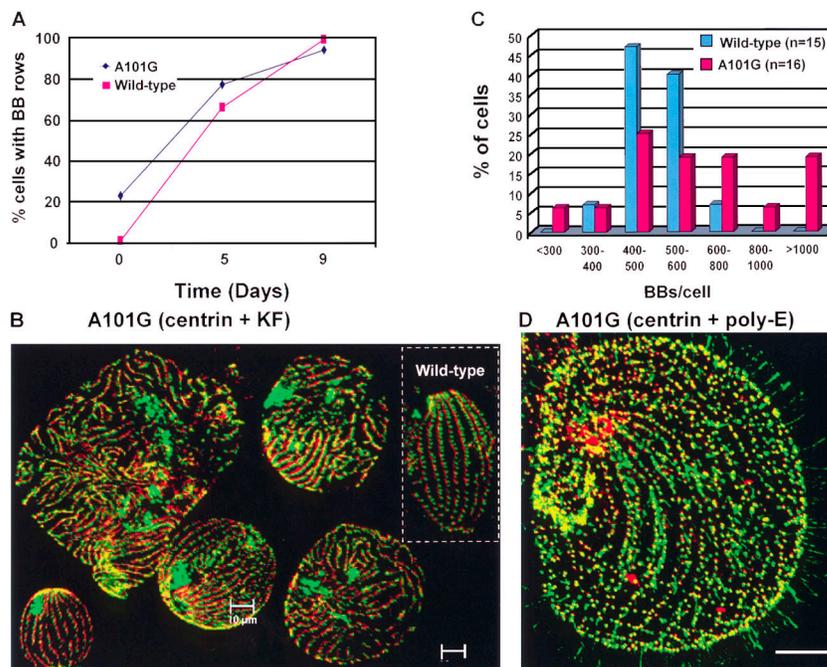
nuclear division and a normal fission furrow. In addition, when mutant strains were shifted from 15 to 30°C, they resumed growth within 5–7 h without detectable synchrony in micronuclear division. Moreover, very few mutant cells contained extra OAs or nuclei in any of the conditions tested. Finally, a strain with a control mutation (ΔRFT2) that blocks cytokinesis and produces enlarged cells did not show increased BB density or disorganization of BB rows. Thus, we conclude that overproduction of individual BBs in the NBD mutants is unlikely to result from a prolonged cell cycle stage.

Finally, the depletion–reinduction experiments showed that most BBs formed at restrictive temperature (15 or 20°C) were able to be assembled into BB rows and form cilia, and we did not detect any obvious defects in immunofluorescent studies with antibodies that detect either BBs or BB-associated structures. These results indicate that both BB maturation and stability in the mutant cells at the restrictive condition are not severely affected, arguing that the mutations do not affect the function of BBs.

Therefore, we conclude that mutations in the NBD of the *T. thermophila* γ-tubulin gene lead to deregulation of BB formation, and, as a result, mutant cells are able to make BBs with random orientations at ectopic locations where BB assembly normally is not allowed. This process of unregulated BB duplication likely causes the organization of cortical rows to be disrupted.

In *T. thermophila*, γ-tubulin is required to nucleate and maintain centriolar MTs and MTs that are required for micro- and macronuclear division (Shang et al., 2002a). Strikingly, the only severe defects we observed in the two NBD mutant cells described in this study are overproduction and ectopic formation of new BBs. Thus, the γ-tubulin NBD is specifically required for both temporal and spatial restriction of BB formation, and the activity of γ-tubulin in the initiation of BB/centriole formation can be uncoupled from its MT-nucleating activity.

In the templated pathway of BB formation, daughter BBs/centrioles are always formed perpendicular to old ones (for review see Beisson and Wright, 2003). In *T. thermophila*, the spatial regulation is further restricted so new BBs are only formed just anterior to old ones (Nanney, 1975; Kaczanowski, 1978). In the NBD mutants, this spatial restriction is severely compromised. One possibility is that without the normal γ-tubulin NBD, new BBs can form de novo and in a more spatially unrestricted manner relative to global cell coordinates. Alternatively, but not necessarily mutually exclusive, new BBs may still be formed near old ones but at unconstrained positions. We favor the first hypothesis because we observed many new BBs both outside of BB rows and underneath the cell surface, not adjacent to preexisting BBs.

Mechanistically, we propose that to prevent overproduction and ectopic nontemplated formation of BBs, the initiation of BB assembly must normally be suppressed by an inhibitory process that acts through the NBD (Fig. 7, step 0). In mammalian tissue culture cells, both templated and de novo centriole biogenesis begin with the formation of multiple precentriolar particles containing centrin and γ-tubulin, which can form multiple centrioles (de novo) or can coalesce to form a single centriole (templated) depending on whether the cell contains a preexisting centriole (La Terra et al., 2005). A single centriole can prevent the de novo formation of centrioles, indicating that there is a trans-acting signal that inhibits de novo formation. Our studies suggest that the NBD of γ-tubulin is the cis-acting target of this signal. Because normal templated assembly and experimentally induced de novo assembly of centrioles both occur in the same cell cycle stage (S phase; Ruiz et al., 1999; Marshall et al., 2001; Khodjakov et al., 2002; La Terra et al., 2005) and γ-tubulin is involved in both pathways (Khodjakov et al., 2002; Shang et al., 2002a; Suh et al., 2002), this inhibitory machinery is likely to suppress both pathways, and
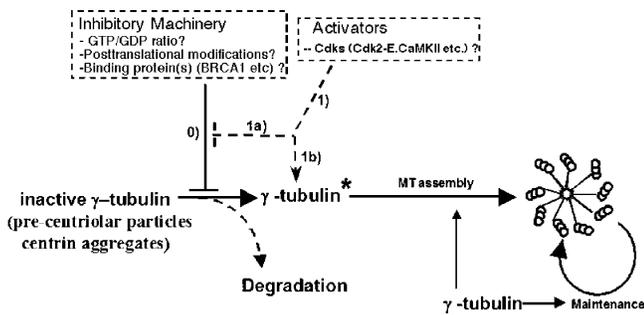
Figure 7. A model for the role of γ-tubulin in centriole/BB biogenesis. We propose that the initiation activity of γ-tubulin is inhibited by a mechanism acting through the NBD (step 0). Nonexclusive possibilities include mechanisms that monitor or are sensitive to the GTP/GDP ratio in γ-tubulin or posttranslational modifications (that might be mimicked by NBD mutations) that regulate the association–dissociation of inhibitory complexes with the NBD. During initiation, an activating signal either inactivates the inhibitors (step 1a) or acts on γ-tubulin (step 1b) to convert it from an inactive to a licensed form that is competent to initiate duplication. The precise site of the active γ-tubulin complex depends on whether cells contain a preexisting BB/centriole. The precentriole particles containing inactive γ-tubulin will be degraded. Activation and recruitment of other BB/centriole components are then initiated. γ-Tubulin is also known to be involved in MT nucleation and long-term maintenance of BBs. γ-Tubulin indicates an inactive γ-tubulin complex; γ-tubulin* indicates an active form. MT, microtubule.

the initiation of both pathways probably requires the same trans-activators (Hinchcliffe and Sluder, 2001; Matsumoto and Maller, 2002) to release the inhibition and enable (license) the γ-tubulin complex to function (Fig. 7, steps 1a and 1b). Additional cell cycle–regulated steps also are required for newly formed centrioles to mature into MTOCs (La Terra et al., 2005).

In conclusion, we have demonstrated that mutations in the NBD of γ-tubulin lead to nontemplated formation of BBs and uncoupling of BB biogenesis from the cell cycle. We have shown that this is not likely to be a secondary effect caused by cell cycle arrest, supporting the hypothesis that γ-tubulin is directly involved in intiating centiole/BB assembly and that its nucleation activity is normally suppressed by an unidentified negative regulating complex through interaction with its NBD. Future studies will hopefully identify the components of this complex. Unfortunately, methods are not yet available in *T. thermophila* for performing screens for allele-specific suppressors, and the unique genetic code (TAA/G = gln) makes traditional two-hybrid approaches difficult.

Materials and methods

Strains, culture growth, and conjugation

All mutant and *MTT1::GTU1*-HA strains are under control of the Cd^{2+}-inducible *MTT1* promoter and are described in Table I. The knockout heterokaryon strains GTUKO5 (*GTU1::neo2-5*) and GTUKO6 (*GTU1::neo2-6*; Shang et al., 2002a) contain disrupted *GTU1* genes encoding γ-tubulin in their germinal micronuclei and wild-type *GTU1* genes in their macronuclei. When mated (conjugated), these strains produce nonviable progeny that contain only disrupted *GTU1* genes in their somatic nucleus unless they are rescued by transforming them with a functional *GTU1* gene during conjugation. The *GTU1*-HA strain (tG2NHA) encodes γ-tubulin tagged by HA at its COOH terminus at the *GTU1* locus (Shang et al., 2002a). *T. thermophila* cells were grown in 1× SPP (1% protease peptone, 0.2% glucose, 0.1% yeast extract, and 0.003% EDTA–ferric sodium salt) or MEPPS medium (2% proteose peptone, 2 mM Na₃ citrate–2H₂O, 1 mM ferric chloride, 12.5 mM cupric sulfate, 1.7 mM folinic acid, and Ca salt; Orias and Rasmussen,

1976) at 30, 15, or 20°C as indicated. For conjugation, midlogarithmic phase cultures of two different mating types were pelleted by centrifugation, washed twice, 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.

Creation of mutant strains by rescue of knockout heterokaryons

PCR mutagenesis was used to construct rescuing plasmids containing mutated gtu1 genes fused at the COOH terminus of the coding region to sequences encoding an HA tag (YPYDVPDYA) under the control of the MTT1 promoter (Shang et al., 2002b). For the preparation of rescuing DNA, plasmid DNA was digested with KpnI and SacI. The knockout heterokaryon strains GTUKO5 and GTUKO6 (Shang et al., 2002a), each containing disrupted GTU1 genes in their germinal micronuclei and wild-type GTU1 genes in their macronuclei, were starved, mixed to initiate mating, and transformed by biolistic bombardment at 24 h after mixing (Cassidy-Hanley et al., 1997; Hai and Gorovsky, 1997). Rescued conjugation progeny were selected with 60 μg/ml paromomycin in the presence of 1.0 μg/ml CdCl₂. The macronuclear genotype of the mutants was confirmed by PCR analysis, restriction enzyme digestion, and sequencing of the mutated region. The MTT1::GTU1-HA strain, which contains a wild-type GTU1 gene with a COOH-terminal HA tag under the control of the MTT1 promoter (Shang et al., 2002a), grows to similar density at a similar growth rate at 15°C in 0.5 μg/ml CdCl₂ compared with the GTU1-HA strain and, therefore, will be referred to as wild type.

Analysis of mutant phenotypes

To study the phenotypes of cold-sensitive mutants at restrictive temperatures (15 or 20°C), log phase wild-type cells and mutant cells were shifted from 30 to 15 or 20°C either in SPP or MEPPS medium (as indicated) with 0.5 μg/ml CdCl₂. To study the regeneration of BBs at 15°C, log phase cells of wild-type or mutant strains were first grown in CdCl₂-free SPP medium for ∼40 h and were shifted to 15°C in MEPPS with 0.5 μg/ml CdCl₂. Growth rates were measured by counting cells using a ZB1 cell counter (Coulter Electronics, Inc.) and plotted using Cricket Graph III (Computer Associates). In some studies, the mean number of BBs of each cell was determined by counting the number of BBs from 10 squares (25 μm² each). The mean number from 8–10 cells for each strain was then calculated. To study the spatial pattern of new BB formation, each cell was divided into four sectors as shown in Fig. 4 C, and the percentage of new BBs in each sector in each cell was calculated. Cells were then grouped into four categories based on which sector was the most active region for new BB assembly, and the number of cells in each category was determined. For the MTT1::gtu1-A101G-HA mutant, the new BBs inside or outside BB rows were calculated separately.

Immunocytochemistry

To study the phenotypes of mutant cells under various conditions (as indicated below), both wild-type and mutant cells were fixed in either 2% PFA or 35% EtOH as described previously (Nelsen et al., 1994; Gaertig et al., 1995; Shang et al., 2002a). PFA-fixed samples were double stained with anticentrin mAbs (provided by J.L. Salisbury, Mayo Clinic School of Medicine, Rochester, MN) and antipolyglutamic acid polyclonal antibody (R-polyE; Shang et al., 2002a) for visualizing BBs. Nuclei were stained with the DNA-specific dye DAPI at 100 ng/ml. Secondary antibodies were goat anti–mouse FITC (Sigma-Aldrich) at a 1:200 dilution or AlexaFluor568 goat anti–mouse IgG (Invitrogen) at a 1:500 dilution. The EtOH-fixed samples were double stained with anticentrin polyclonal antibodies and either mAb FI-5D8 (provided by J. Frankel, University of Iowa, Iowa City, IO; Jerka-Dziadosz et al., 1995) for KF (also known as striated rootlets) or mAb anti-K (10D12; provided by N. Williams, University of Iowa; Williams et al., 1990) for mature BB marker K antigens. All primary antibodies were used at a 1:1,000 dilution except FI-5D8 (1:250) and anti-K (1:50). Secondary antibodies were goat anti–rabbit FITC (Sigma-Aldrich) at a 1:200 dilution and AlexaFluor568 goat anti–mouse IgG (Invitrogen) at a 1:500 dilution. 11–12 stacked images of stained cells were obtained using a confocal microscope (model TCS SP; Leica) with a PL Apo 100× NA 1.4 lens and 1.8× or 1.6× zoom.

Western blot analysis

To demonstrate the expression level of HA-tagged γ-tubulin, MTT1::GTU1-HA or the two gtu1 mutant strains were grown to 2×10^5 cells/ml in 0.5 μg/ml SPP/CdCl₂ at 15°C, harvested, and washed in 10 mM Tris-Cl, pH 7.5, containing protease inhibitors (Complete Protease Inhibitor Cocktail; Roche Diagnostics). The cells were pelleted and dissolved in 1× SDS sample buffer containing 5% β-mercaptoethanol, 10% glycerol, 2% SDS, and

60 mM Tris-Cl, pH 6.8. Proteins from 0.5–1 × 10⁵ cells per lane were separated on 10% SDS-PAGE and transferred onto an Immobilon-P membrane (Millipore) by a semidry electrophoretic transfer unit (Gelman Biotrans). The membrane was blocked in 5% dry milk in 1× TBST (100 mM Tris-HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20) for 1 h at room temperature and incubated overnight at 4°C with anti-HA mAb (16B12; Covance). The membrane was washed in 1× TBST at room temperature, incubated in rabbit anti–mouse IgG HRP-conjugated secondary antibody (Zymed Laboratories) at a 1:20,000 dilution for 1 h at room temperature, and washed five times for 5 min each in TBST and then once in 1× TBS. Proteins were detected using Western Blot Chemiluminescence Reagent (NEN Life Science Products).

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