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# Regulation of Tubulin Synthesis and Cell Cycle Progression in Mammalian Cells by γ-Tubulin-Mediated Microtubule Nucleation

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**Abstract** We have previously shown that γ-tubulin, the third member of the tubulin family that functions in microtubule nucleation, when overexpressed, accumulates throughout the cytoplasm and forms numerous ectopic microtubule nucleation sites in mammalian cells (Shu and Joshi [1995] J. Cell. Biol. 130:1137–1147). We now show that overexpression of γ-tubulin differentially upregulates the synthesis of α- and β-tubulins in mammalian cells. Surprisingly, despite a dramatic increase in the level of γ-tubulin protein in transfected cells, there is no obvious alteration in the level of endogenous γ-tubulin mRNA, suggesting that synthesis of γ-tubulin might employ a regulatory mechanism other than the autoregulatory pathway shared by α- and β-tubulins. Interestingly, a significant number of mammalian cells transfected with γ-tubulin fail to form normal bipolar mitotic spindle during mitosis; instead, numerous microtubules occur in the cytoplasm intermingled with the condensed chromosomes. In addition, they reduplicate their DNA after an abnormal mitotic exit. These results thus suggest that the number of microtubule nucleation sites, or even γ-tubulin itself, might play an important role in the regulation of tubulin synthesis as well as cell cycle progression.
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**Key words:** microtubule assembly; microtubule nucleation; tubulin synthesis; γ-tubulin; mitosis

Microtubules are highly dynamic cytoskeletal components essential for many cellular events in eukaryotes, such as organelle distribution, cell division, and cell motility. The building blocks of microtubules are heterodimers of α- and β-tubulin subunits. They are arranged in a head-to-tail fashion to form a 13-protofilament sheet first, which then closes up to form the cylindrical microtubule filament. Essential to microtubule functions is how microtubules are assembled. Microtubules can self-assemble from purified α- and β-tubulins at a high concentration in a test tube, but these in vitro assembled microtubules are randomly organized. However, in eukaryotic cells, microtubules are precisely organized due to their anchorage at the microtubule organizing center (MTOC, called the centrosome in mammalian cells) [Pickett-Heaps, 1969; Berns et al., 1977; Gould and Borisy, 1977; Kirschner, 1978; Brinkley, 1985].

The synthesis of α- and β-tubulins has been well known to respond reciprocally to the concentration of unpolymerized tubulin, a process referred to as autoregulation of tubulin synthesis [Ben-Ze'ev et al., 1979; Cleveland et al., 1981, 1983; Caron et al., 1985]. Since the number of cellular microtubule nucleation sites might contribute to the partitioning of tubulin between polymer and monomer by controlling the rate of the initiation of microtubule assembly, the natural hypothesis that the number of microtubule nucleation sites might play an indirect role in the regulation of tubulin expression has gained widespread support [McIntosh, 1983; Mitchison and Kirschner, 1987]. Although very reasonable and of obvious biological importance, unfortunately this hypothesis has not been experimentally addressed thus far due to the lack of a suitable in vivo system.

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γ-Tubulin, the third member of the tubulin family, has been well known to localize predominantly at the centrosome, where it is essential for the nucleation of microtubule assembly [Oakley et al., 1990; Horio et al., 1991; Stearns et al., 1991; Joshi et al., 1992; Stearns and Kirschner, 1994; Sobel and Snyder, 1995; Sunkel et al., 1995; Marschall et al., 1996]. We have previously shown that γ-tubulin, when overexpressed, accumulates throughout the cytoplasm and forms numerous ectopic microtubule nucleation sites in mammalian cells [Shu and Joshi, 1995]. This offers an exciting opportunity to test whether the number of microtubule nucleation sites plays a role in the regulation of α- and β-tubulin expression. In addition, since purification of functional monomeric γ-tubulin has not been successful so far [Shu and Joshi, 1995; Vassilev et al., 1995; Zheng et al., 1995; Stearns, personal communication], which makes a direct microinjection of γ-tubulin to study its synthetic regulation impossible, overexpression of γ-tubulin provides a useful tool to examine whether an autoregulatory mechanism exists to control γ-tubulin expression similar to that of α- and β-tubulins. Such efforts have had limited success in the overexpression of α- and β-tubulins [Burke et al., 1989; Sisodia et al., 1990].

Another intriguing issue in the field of microtubule biology is the involvement of microtubules in cell cycle regulation as pointed out earlier by Teng et al. [1977]. It has been hypothesized that an exquisite control of the number of microtubule nucleation sites might be required for normal cell cycle progression [McIntosh, 1983; Gliksman et al., 1993]. For example, a significant increase in the number of microtubule nucleation sites might be crucial for the rearrangement of the microtubule network at the onset of mitosis. Since overexpression of γ-tubulin can produce abundant microtubule nucleation sites throughout the cytoplasm [Shu and Joshi, 1995], it allows a straightforward investigation of cell cycle progression in the presence of remarkably increased microtubule nucleation sites.

Here, we show that in mammalian cells, the expression levels of α- and β-tubulin mRNAs are differentially upregulated by γ-tubulin overexpression. Interestingly, the expression of endogenous γ-tubulin mRNA is not changed by the overexpression of exogenous γ-tubulin. In addition, a significant amount of mammalian cells overexpressing γ-tubulin fail in the formation of bipolar mitotic spindle and reduplicate their DNA after an abnormal mitotic exit. These results thus provide novel insights into the mechanisms by which the tubulin family genes are regulated and suggest a role for the number of microtubule nucleation sites, or even γ-tubulin itself, in the control of tubulin expression and cell cycle progression.

## MATERIALS AND METHODS

### Vector Construction

Construction of the γ-tubulin expression vectors pRSV-hγT and pRSV-hγT/myc has been previously described in detail [Shu and Joshi, 1995]. Briefly, Rous Sarcoma Virus long terminal repeat sequence was used to drive the transcription of human γ-tubulin cDNA. In pRSV-hγT/myc, a short DNA sequence encoding the 14-amino acid myc epitope was fused in frame with the 3′ end of γ-tubulin cDNA.

### Cell Culture and Transfection

HeLa (human cervical carcinoma), 293 (human embryonic kidney), and COS-7 (monkey kidney) cells were maintained in DMEM medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL). Cells cultured either in tissue culture plates or on glass coverslips were transfected using the FUGENE 6 reagent according to the protocol of the manufacturer (Roche Molecular Biochemicals).

### Microinjection

Microinjection was carried out by a Narashigi micromanipulator following the method of Capecchi [1980]. Cells were cultured on glass coverslips with photoetched locator grids (Belco Glass, Inc.). Approximately 50 copies of the plasmid in ~50 femtoliters of the injection solution (48 mM $K_2HPO_4$, 14 mM $NaH_2PO_4$, 45 mM $KH_2PO_4$, pH 7.2) were injected directly into the nucleus. Morphological changes of cells were captured at different time points with phase contrast optics.

### Western Blot Analysis

Proteins extracted from mammalian cells were analyzed by polyacrylamide gel electrophoresis as described [Laemmli, 1970], and the protein bands were electrophoretically transferred onto nitrocellulose membranes. The membranes were first incubated with primary

antibodies against α-, β-, or γ-tubulin and then incubated with horseradish peroxidase-labeled secondary antibodies. Specific proteins were visualized using enhanced chemiluminescence following manufacturer's instructions (Amersham). The relative protein levels were determined by densitometric analysis using a Lynx video densitometer.

### Northern Blot Analysis

Total RNA was isolated from mammalian cells as described [Sambrook et al., 1989], and RNA concentrations were determined by absorbance at 260 nm. Samples (a 1.25-fold serial dilution starting from 20 μg) were electrophoresed on 1% agarose gels and then transferred to nylon membranes (Amersham) following the standard procedures [Sambrook et al., 1989]. To probe the total γ-tubulin mRNA, the KS-hγT vector [Zheng et al., 1991] was digested with EcoR I, and the 1.6 kb fragment of the γ-tubulin coding sequence was isolated and radiolabeled. To detect the endogenous γ-tubulin mRNA, we amplified the γ-tubulin cDNA 3′ untranslated region (UTR) from KS-hγT plasmid by PCR with the following two primers: 5′ GAGTCCCCCAGGACAGG 3′ and 5′ CGCTCTAGAACTAGTGGATC 3′. The PCR product of the γ-tubulin 3′-UTR was isolated and radiolabeled. To detect α-tubulin mRNA, a plasmid harboring Chinese hamster α-tubulin cDNA [Elliott et al., 1986] was digested with BamH I and Pst I to release the 1.6 kb fragment corresponding to α-tubulin coding region. To make β-tubulin probe, a β-tubulin plasmid, pβG5 [Sullivan and Cleveland, 1986], was digested with EcoR I to release the 1.6 kb β-tubulin coding region. All probes were labeled with $^{32}$P-dCTP using the Megaprime DNA labeling system following procedures recommended by the manufacturer (Amersham). Hybridizations were performed in Rapid-hyb buffer (Amersham) at 65°C for 2 h.

### BrdU Incorporation Assay

Sixty hours after transfection, 50 μM BrdU (Sigma) was applied to cell culture medium for 1.5 h. Cells were then fixed with 3% paraformaldehyde for 15 min, prestained with 5 mg/ml BSA, 10 mM $MgCl_2$, and 0.5% NP-40 in PBS for 5 min, and incubated with a monoclonal rat antibody against BrdU (Accurate Chemical and Scientific Corp.) for 3 h. Incorporated BrdU was visualized under the Zeiss Axiovert microscope after incubation with rhodamine-conjugated anti-rat IgG (Jackson ImmunoResearch Laboratories).

### Immunofluorescence Microscopy

Cells cultured on glass coverslips were washed for 15 s at 37°C with the buffer that prevents microtubule depolymerization (0.1 M PIPES, pH 6.9, 1 mM EGTA, and 4 M glycerol) and incubated for 1 min at 37°C with 0.2% Triton X-100. Coverslips were then rinsed with the microtubule depolymerization preventing buffer and plunged into methanol at −20°C for 5 min. Coverslips were rehydrated in PBS and then incubated with mouse anti-myc or anti-γ-tubulin monoclonal antibody and rabbit anti-β-tubulin polyclonal antibody (Amersham) at 37°C for 2 h. They were then rinsed with 2% BSA in PBS and incubated with fluorescein-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Finally, they were stained with DAPI (Sigma) and mounted in Aqua Mount (Lerner Laboratories). Images were taken using a Zeiss Axiovert microscope with epifluorescence optics.

### Electron Microscopy

Cells were fixed and permeabilized simultaneously with 2% glutaraldehyde and 0.5% Triton X-100. These cells were then processed for γ-tubulin immunogold staining and flat embedding as previously described [Joshi et al., 1986; Baas and Joshi, 1992; Joshi, 1993a]. The gold-labeled γ-tubulin antibodies were purchased from Janssen Biochimica, Inc. Embedded cells were sectioned parallel to the substratum using a Reichert Jung Ultracut S microtome. Serial thin sections (80–100 Å thick) were photographed with a JEOL electron microscope.

## RESULTS

### Differential Upregulation of α- and β-Tubulin mRNAs by γ-Tubulin Overexpression

We have previously shown that overexpression of γ-tubulin in mammalian cells, by transient transfection of γ-tubulin expression vector pRSV-hγT or pRSV-hγT/myc, leads to the accumulation of exogenous γ-tubulin throughout the cytoplasm, which forms abundant sites functional for microtubule nucleation, resulting in an increase in microtubule polymer mass

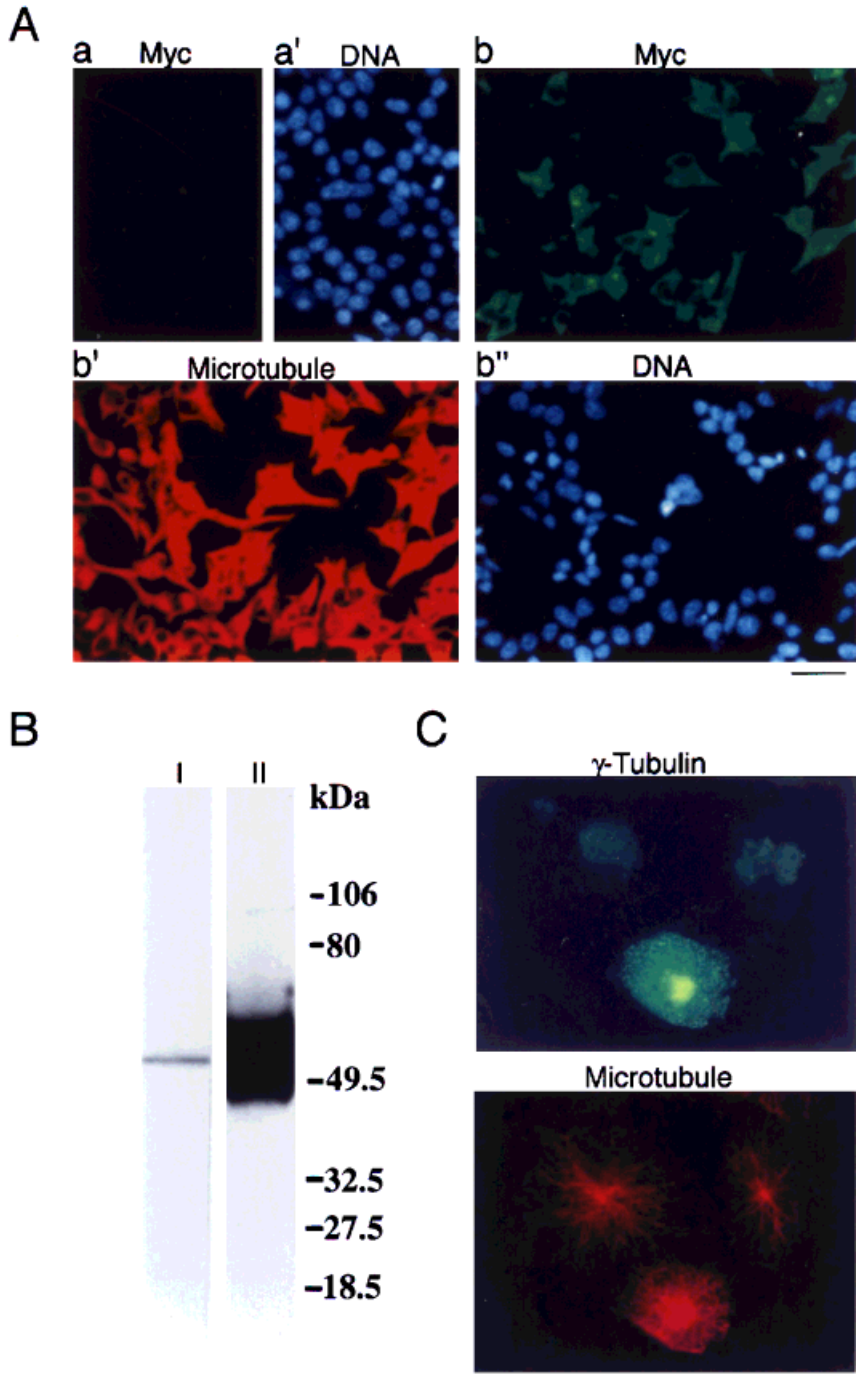


**Fig. 1.** Transient transfection and overexpression of γ-tubulin in mammalian cells. **A**: Immunofluorescence staining of γ-tubulin-myc (green), microtubule (red), and DNA (blue) in untransfected (a & a′) and transfected 293 cells (b, b′, & b″). Bar, 50 μm. **B**: Western blot analysis of γ-tubulin expression in untransfected 293 cells (**lane I**) and those transfected with the γ-tubulin expression vector (**lane II**). Fifty micrograms of cellular proteins were loaded for both lanes. Protein levels were measured by densitometry and corrected for transfection efficiency. **C**: Microtubule reorganization following overexpression of γ-tubulin in COS-7 cells. Microtubules (red) are grossly reorganized in the transfected cell (lower cell), while microtubules in the neighbouring untransfected cells are radially organized at the center of the cells (upper cells). Bar, 15 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

[Shu and Joshi, 1995; also see Fig. 1C]. This would transiently deplete the pool of free α- and β-tubulin subunits. It is reasonable to assume that these cells will continuously compensate for depleting the free tubulin subunit pool by stabilizing α- and β-tubulin mRNAs and will

increase the synthetic rates of α- and β-tubulins to continuously replenish the depleting pool of tubulin subunits. To test this hypothesis, we first documented γ-tubulin overexpression by transient transfection of γ-tubulin expression vector into HeLa (human cervical carcinoma), COS-7 (monkey kidney), and 293 (human embryonic kidney) cells. The transfection efficiency routinely achieved in our laboratory was 35, 31, and 75% on average for these three cell lines respectively (Fig. 1A). Western blot analysis showed that the level of γ-tubulin in cells transfected with γ-tubulin expression vector was 140-fold as high as that in untransfected cells (Fig. 1B).

Strikingly, α- and β-tubulin protein levels increased 53 and 45%, respectively in cells transfected with pRSV-hγT or pRSV-hγT/myc in comparison to the control (Fig. 2A). On the surface, this extent of increase might appear modest, but given that 1–2% of total cellular proteins are α- and β-tubulins, this is in fact a big change. The modest up-regulation of α- and β-tubulin levels in cells overexpressing γ-tubulin might result either from direct stabilization and therefore accumulation of α- and β-tubulin proteins or from accumulation of α- and β-tubulin mRNAs. To test this, we performed Northern blot analysis to examine the levels of α- and β-tubulin mRNAs. Surprisingly, α-tubulin mRNA level was increased 87-fold on average in cells overexpressing γ-tubulin, yet β-tubulin mRNA level was increased only 1.2-fold (Fig. 2B). Thus, the increase in the number of microtubule nucleation sites, or even γ-tubulin itself, could upregulate cellular α- and β-tubulin mRNA levels to very different extents.

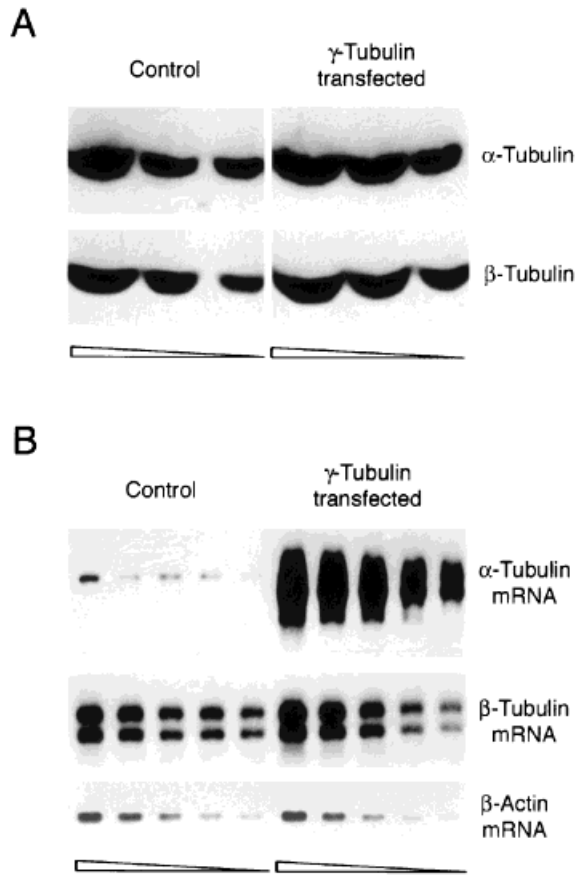


**Fig. 2.** Differential upregulation of α- and β-tubulin synthesis by γ-tubulin overexpression. **A**: Western blot analysis of α- and β-tubulin protein levels in 293 cells. To ensure measurement in the linear range of detection, total extracts of cells were loaded at a 1.25-fold serial dilution starting from 30 μg of protein. Blots were detected with monoclonal antibodies against α-tubulin or β-tubulin. **B**: Northern blot analysis of α- and β-tubulin mRNA levels in 293 cells following transfection. mRNAs were loaded at a 1.25-fold serial dilution starting from 20 μg. Blots were probed with α- and β-tubulin, or β-actin cDNA fragments. Protein and mRNA levels were both determined by densitometry and corrected for transfection efficiency.

### Synthesis of Endogenous γ-Tubulin Is Not Changed by the Overexpression of Exogenous γ-Tubulin

γ-Tubulin overexpression also allows us to examine the expression of endogenous γ-tubulin in the presence of abundant exogenous γ-tubulin. Such efforts have had limited success in the overexpression of α- and β-tubulins [Burke et al., 1989; Sisodia et al., 1990]. Since γ-tubulin shares about 35% sequence identity to α- and β-tubulins [Burns, 1995], it is possible that the synthesis of γ-tubulin is also regulated by its concentration in the cell, similar to the autoregulatory mechanism shared by α- and β-tubulins. To test this idea, we performed Northern blot analysis to examine γ-tubulin mRNA levels in cells transfected with pRSV-hγT or pRSV-hγT/myc. Surprisingly, despite a remarkable increase in γ-tubulin protein level in mammalian cells transfected with γ-tubulin expression vector (Fig. 1B), there was no obvious change in the level of endogenous γ-tubulin mRNA in these cells (Fig. 3). In contrast, the level of total γ-tubulin mRNA in cells transfected with γ-tubulin vector was $>$ 500-fold higher than that of the control (Fig. 3), accounting for the 140-fold increase in the level of total γ-tubulin protein (Fig. 1B). Thus, the synthesis of γ-tubulin must employ a

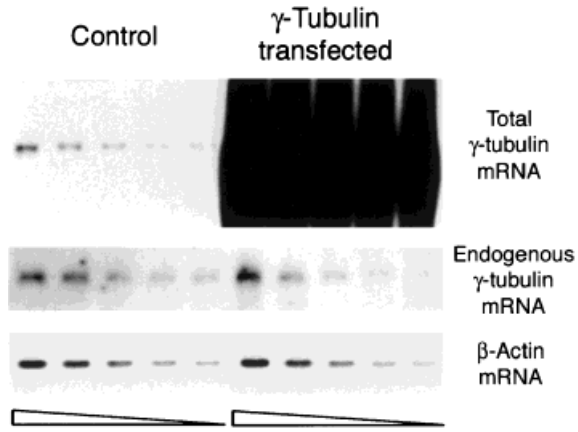


**Fig. 3.** Northern blot analysis of γ-tubulin mRNA levels following transfection of the γ-tubulin expression vector. Human γ-tubulin cDNA coding region and a PCR amplified fragment of γ-tubulin cDNA 3′ (untranslated region (~450 base pair) were used as probes for the examination of total mRNA levels and endogenous γ-tubulin mRNA levels respectively. The expression of β-actin was also examined as a loading control. mRNAs were loaded at a 1.25-fold serial dilution starting from 20 μg. mRNA levels were determined by densitometry and corrected for transfection efficiency. Data shown here were representative blots obtained using 293 cells.

regulatory mechanism other than the autoregulatory pathway shared by α- and β-tubulins.

### Formation of Multilobed Nuclei in Cells Overexpressing γ-Tubulin

The number of microtubule nucleation sites has been suggested to play a regulatory role in the initiation and progression of mitosis [McIntosh, 1983; Gliksman et al., 1993]. However, a lack of suitable mechanistic strategy has prevented a direct test of this idea. Since γ-tubulin overexpression can lead to the formation of abundant cytoplasmic sites for microtubule nucleation [Shu and Joshi, 1995], it allows an examination of the cell cycle progression in the presence of extracentrosomal microtubules. Interestingly, 60 h after transfection of the γ-tubulin expression vector pRSV-hγT or pRSV-hγT/myc, as many as 59% (48 out of 81) of the cells overexpressing γ-tubulin developed large multilobed nuclei, which had intact nuclear lamina (Fig. 4). These cells failed to undergo cytokinesis.

To determine whether the multilobed nuclear phenotype in cells overexpressing γ-tubulin was a consequence of abnormal cell division, we microinjected the γ-tubulin expression vector into the nuclei of mammalian cells and followed them by phase contrast microscopy. Microinjection of cells plated on photoetched glass coverslips resulted in a high efficiency of gene transfer (> 95%), and thus enabled to follow the fate of the injected cells prior to their death caused by γ-tubulin overexpression. Strikingly, cells injected with γ-tubulin expression vector cyclically progressed into a mitotic-like state characterized by a round contracted morphology, followed by interphase with a multilobed nuclear morphology (Fig. 5B). Usually, these cells died after 2–3 cell cycles. In contrast, in the mean time, cells injected with the control vector underwent completion of the normal cell cycle resulting in the proper doubling of cells (Fig. 5A).

We then asked whether these multinuclear cells resulting from γ-tubulin overexpression could progress into the subsequent S-phase. To do this, we let the majority of the transfected cells undergo at least one round of cell cycle producing the characteristic multilobed nuclear morphology in the absence of cell cleavage. The transfected cells were then exposed to a 90-min pulse of thymidine analog BrdU, incorporation of which into nascent DNA usually shows the occurrence of S-phase. As shown in Figure 6, transfected cells with the multilobed nuclear phenotype incorporated BrdU, indicating that these cells passed through mitosis and then entered S-phase.

### Kinetochore-Microtubule Interactions in γ-Tubulin Transfected Cells

To understand the mitotic progression in mammalian cells transfected with γ-tubulin, we examined the morphology of mitotic microtubules and chromosomes in these cells. Strikingly, a small fraction of transfected cells was in a mitotic-like state with the characteristic round morphology of mitotic cells; however, the bipolar mitotic spindle and the metaphase plate were absent in these cells (Fig. 7). Instead, microtubules in these cells were distributed throughout the cytoplasm intermingled with the condensed chromosomes.

We then performed a high-resolution immunogold electron microscopic study to examine the interactions between kinetochores and microtubules in cells transfected with γ-tubulin. Serial thin section analyses of these mitotic-like cells revealed, as expected, ectopic microtubules throughout the cytoplasm with γ-tubulin aggregates at their free ends (Fig. 8B). The kinetochores of the condensed chromosomes were indeed attached to the extracentrosomal

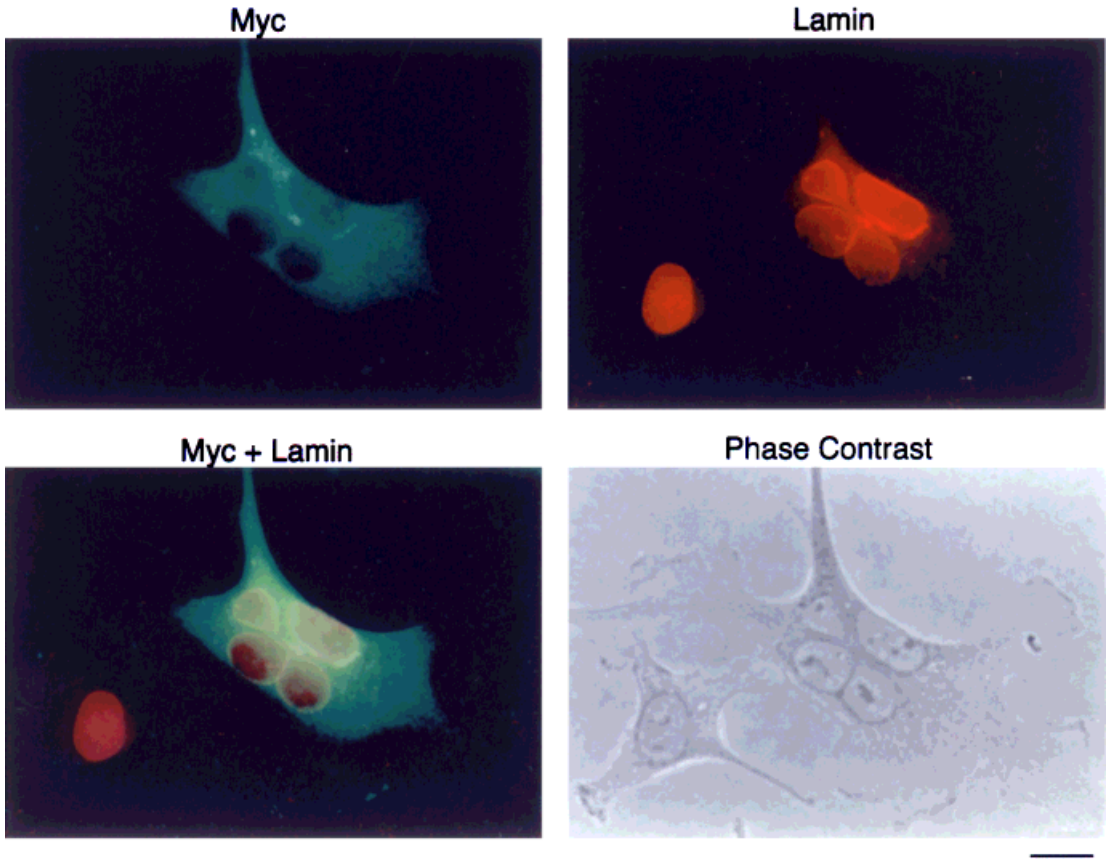


**Fig. 4.** Formation of multilobed nuclei (polynuclei) with intact nuclear lamina in COS-7 cells overexpressing γ-tubulin. Sixty hours after transfection with the γ-tubulin expression vector, immunofluorescence microscopy was carried out using monoclonal antibodies against human γ-tubulin or myc (green) and polyclonal antibodies against nuclear lamin A (red). Phase contrast microscopy of the same field was also performed. The cell overexpressing γ-tubulin (right cell) formed polynuclei, while the neighboring untransfected cell had a normal nucleus. Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

microtubules, and the occupancy of kinetochores by microtubules was as high as 98% (114 out of 116, with 2 ambiguous). However, compared with normal cells (Fig. 8A), cells overexpressing γ-tubulin exhibited randomly oriented kinetochore-microtubule interactions (Fig. 8C).

## DISCUSSION

The synthesis of α- and β-tubulins has been suggested to respond to the levels of free tubulin subunits [Ben-Ze'ev et al., 1979; Cleveland et al., 1983], which can be described by the following equation derived from Mitchison and Kirschner [1987]. This equation:

$$C_p = \frac{Nk}{n\Omega_\gamma \mathrm{V}} C_m^{\gamma+1} \quad \text{or} \quad C_m = \left(\frac{n\Omega_\gamma \mathrm{V} C_p}{Nk}\right)^{\frac{1}{\gamma+1}}$$

where γ is an arbitrary exponent, $\Omega_\gamma$ is the rate constant, and $k$ is the on-rate constant for microtubule polymerization, allows an ideal explanation for how the tubulin polymer concentration ($C_p$) and monomer concentration ($C_m$) change with each other as well as with the number of microtubule nucleation sites ($N$) within a cell that has a fixed volume (V). For example, at the onset of mitosis, the rapid increase in $C_p$ and decrease in $C_m$ might mainly result from a significant increase in the number of microtubule nucleation sites [McIntosh, 1983; Mitchison and Kirschner, 1987], which drives the tubulin monomer into polymer. In this study, the numerous microtubule nucleation sites, resulting from γ-tubulin overexpression, efficiently promote a shift of tubulin from monomer to polymer, thus depleting the monomer pool. As an autoregulatory feedback, the cell must enhance the rate of tubulin synthesis to continuously replenish the monomer pool. Thus, our finding that the overexpression of γ-tubulin upregulates the expression of α- and

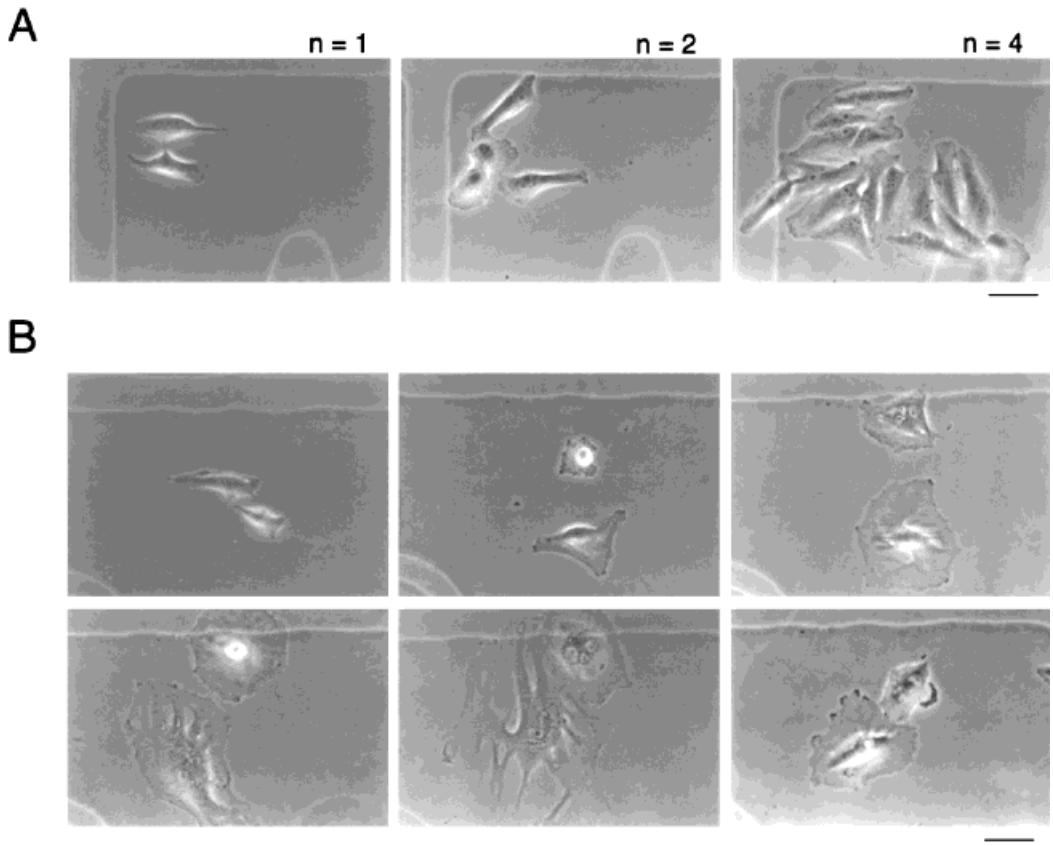


**Fig. 5.** Time-lapse observation of the cell cycle progression following γ-tubulin overexpression in HeLa cells. Phase contrast microscopy of the cells was carried out at variable time points after they were injected with the control vector pRSV (**A**) or the γ-tubulin expression vector (**B**). In (A), n refers to the number of cell cycles completed after injection. In (B), the two injected cells were asynchronous, and the typical cell cycle events are shown by one of them (upper cell). Bars, 20 μm.

β-tubulins supports very well that the number of microtubule nucleation sites (*N*) plays a regulatory role in the maintenance of tubulin subunit levels presumably by increasing their synthesis.

Cells have acquired both transcriptional and post-transcriptional mechanisms during evolution to maintain the appropriate expression levels of proteins, which are crucial for these proteins to carry out their functions. As for tubulin, its synthesis can be regulated in the transcriptional level where different tissues have different patterns of tubulin gene expression [Sullivan, 1988]. However, autoregulation through the modulation of mRNA instability pathway is the best known mechanism for the regulation of tubulin synthesis in metazoan cells [Cleveland, 1988, 1989]. In fact, many other proteins, such as histones [Capasso et al., 1987; Graves et al., 1987] and proteins encoded by proto-oncogenes [Shaw and Kamen, 1986], also utilize this mechanism for the regulation of their synthesis. It has been suggested that cells might employ the autoregulatory mechanism to maintain constant cellular tubulin levels for microtubule assembly [Theodorakis and Cleveland, 1992]. Our results show that despite a comparable increase in α- and β-tubulin protein levels upon γ-tubulin transfection, the increases in mRNA levels for α- and β-tubulin are greatly different, in that α-tubulin mRNA was increased up to 87-fold on average, while β-tubulin mRNA was increased only 1.2-fold. This result thus reinforces the previous observation that autoregulations of α- and β-tubulin synthesis, although both involve mRNA degradation mechanisms, are not mediated through a common pathway [Bachurski et al., 1994]. The comparable increase in α- and β-tubulin protein levels, on the other hand, might be due to the translational repression of α-tubulin synthesis, which coordinates production of α- and β-tubulins as suggested by Gonzalez-Garay and Cabral [1996]. Alternatively, it might be due

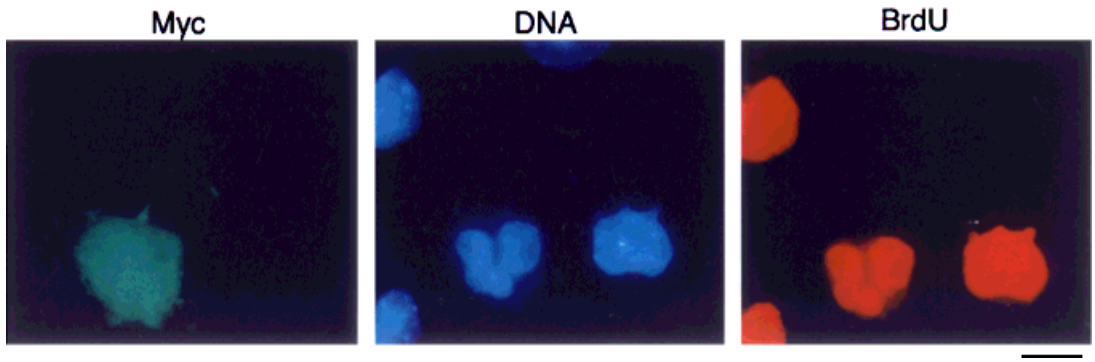


**Fig. 6.** Progression into S-phase following abnormal mitotic exit in COS-7 cells overexpressing γ-tubulin. Sixty hours after transfection, γ-tubulin overexpressing cells developed polynuclei. Subsequent to this, cells were pulse-treated with BrdU for 90 min and then fixed for immunocytochemistry to visualize γ-tubulin or myc (green), DNA (blue), and BrdU (red). Bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to an increased rate of degradation of newly formed α-tubulin [Sisodia et al., 1990]. Our result is very consistent with the generally accepted idea that α- and β-tubulins must be stoichiometrically maintained to ensure equivalent amounts for microtubule assembly; overexpression of either α- or β-tubulin alone is toxic to the cell [Burke et al., 1989].

In this study, we also found that the endogenous γ-tubulin mRNA level did not have an observable change upon overexpression of exogenous γ-tubulin. This indicates that γ-tubulin synthesis does not follow the traditional autoregulatory pathway of α- and β-tubulins, although it shares ~35% sequence homology in amino acid level with α- and β-tubulins [Burns, 1995]. This result is understandable considering the different subcellular localizations and functions of γ-tubulin from α- and β-tubulins. Furthermore, the first three amino acids, Met-Arg-Glu (MRE), of α- and β-tubulins are thought to be responsible for the autoregulatory mechanisms of their synthesis, which involve cotranslational recognition of the nascent amino-terminus of tubulin as it emerges from the ribosome [Yen et al., 1988; Bachurski et al., 1994]. However, this might not be the full story, although α- and β-tubulins share the first three amino acids, they themselves are differentially regulated. γ-Tubulin, on the other hand, has an inserted proline at position 2 of this sequence, i.e., making it MPRE [Joshi, 1993b]. Plus, the fact that there is a high degree of sequence divergence among α-, β-, and

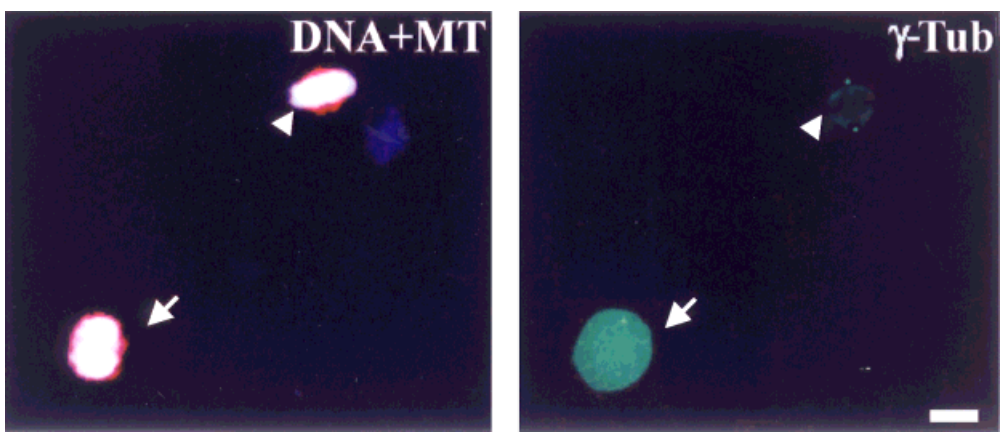


**Fig. 7.** Interference of morphogenesis of the mitotic spindle by γ-tubulin overexpression. While the control HeLa cell formed a tightly arranged bipolar mitotic spindle during mitosis, as seen as red cones on both sides of the aligned DNA, the transfected cell failed to form a bipolar spindle. Instead, microtubules in the transfected cell could be seen intermingled with a ball of condensed DNA, as shown by red microtubule signal mixed with the un-aligned DNA signal and red rim all around the un-aligned DNA. Bar, 15 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

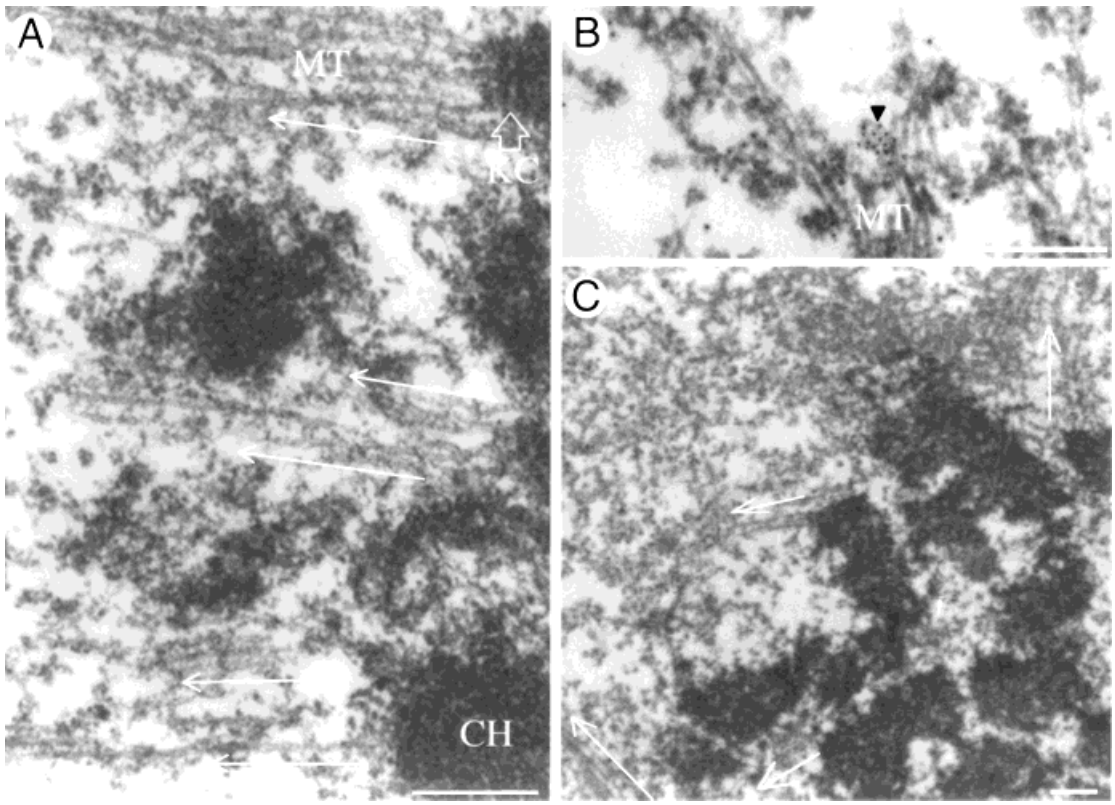


**Fig. 8.** Kinetochore-microtubule interactions in HeLa cells. **A**: Alignment of chromosomes at the metaphase plate in a control metaphase cell. **B**: γ-Tubulin overexpression causes ectopic microtubule nucleation from γ-tubulin aggregates in the cytoplasm identified by immunogold staining (arrowhead). **C**: Attachment of kinetochores to extracentrosomal microtubules in mitotic-like cells with γ-tubulin overexpression. Note that in cells overexpressing γ-tubulin, kinetochores interact with microtubules with random orientations (C, long arrows), whereas in control cells, they interact with a single orientation (A, long arrows). Bars, 250 nm.

γ-tubulins at their carboxyl termini indicate that the differences in tubulin regulation might be directly due to the differences in the amino acid sequences. Considering the critical role of γ-tubulin in microtubule nucleation, it will be of great importance to further investigate how γ-tubulin is regulated in mammalian cells.

In normal cells, the onset of anaphase and subsequent exit from mitosis are triggered only after all kinetochores are attached by spindle microtubules, a process controlled by a surveillance mechanism called the spindle assembly checkpoint [Amon, 1999]. It thus seems to be a paradox why in this study γ-tubulin overexpressing cells underwent mitotic exit in the absence of a normal bipolar spindle. However, this result is understandable given that in these cells kinetochores were attached to the ectopically nucleated microtubules, which might "satisfy" the requirement of the spindle checkpoint.

Another surprising finding here is that the cells overexpressing γ-tubulin abrogate cytokinesis and midbody formation. It has been previously shown that in addition to the centrosome, γ-tubulin also localizes transiently at the midbody (at the minus ends of midbody-microtubules) [Julian et al., 1993; Shu et al., 1995]. Recently, our laboratory has isolated several mutations in γ-tubulin that cause defects in cytokinesis in the fission yeast, *Schizosaccharomyces pombe* [Hendrickson et al., 2001]. Although the precise role of γ-tubulin in cytokinesis is obscure at this point, it seems increasingly clear that it plays a role in the regulation of cytokinesis probably through microtubule nucleation, although other alternative mechanisms may also be involved [Julian et al., 1993; Shu et al., 1995; Hendrickson et al., 2001].

Collectively, in this study we show that overexpression of γ-tubulin up-regulates the levels of α- and β-tubulin mRNAs to dramatically different extents, yet their protein levels are up-regulated more or less stoichiometrically. However, the synthesis of endogenous

γ-tubulin is not significantly altered. In addition, cells overexpressing γ-tubulin undergo abnormal mitotic exit, abrogate cytokinesis, and then enter the next round of DNA duplication. These results thus suggest that γ-tubulin-mediated microtubule nucleation might play an important role in tubulin synthesis and cell cycle progression.

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## REFERENCES

Amon A. 1999. The spindle checkpoint. Curr Opin Cell Biol 9:69–75.

Baas PW, Joshi HC. 1992. Gamma-tubulin distribution in the neuron: Implications for the origins of neuritic microtubules. J Cell Biol 119:171–178.

Bachurski CJ, Theodorakis NG, Coulson RMR, Cleveland DW. 1994. An amino-terminal tetrapeptide specifies cotranslational degradation of beta-tubulin, but not alpha-tubulin mRNAs. Mol Cell Biol 14:4076–4086.

Ben-Ze'ev A, Farmer SR, Penman S. 1979. Mechanisms of regulating tubulin synthesis in cultured mammalian cells. Cell 17:319–325.

Berns MW, Rattner JB, Brenner S, Meredith S. 1977. The role of the centriolar region in animal cell mitosis. A laser microbeam study. J Cell Biol 72:351–367.

Brinkley BR. 1985. Microtubule organizing centers. Annu Rev Cell Dev Biol 1:145–172.

Burke D, Gasdaska P, Hartwell L. 1989. Dominant effects of tubulin overexpression in *Saccharomyces cerevisiae*. Mol Cell Biol 9:1049–1059.

Burns RG. 1995. Analysis of the gamma-tubulin sequences: Implications for the functional properties of gamma-tubulin. J Cell Sci 108:2123–2130.

Capasso O, Bleecker GC, Heintz N. 1987. Sequences controlling histone H4 mRNA abundance. EMBO J 6: 1825–1831.

Capecchi MR. 1980. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell 22:479–488.

Caron JM, Jones AL, Kirschner MW. 1985. Autoregulation of tubulin synthesis in hepatocytes and fibroblasts. J Cell Biol 101:1763–1772.

Cleveland DW. 1988. Autoregulated instability of tubulin mRNAs: A novel eukaryotic regulatory mechanism. Trends Biochem Sci 13:339–343.

Cleveland DW. 1989. Autoregulated control of tubulin synthesis in animal cells. Curr Opin Cell Biol 1:10–14.

Cleveland DW, Lopata MA, Sherline PA, Kirschner MW. 1981. Unpolymerized tubulin modulates the level of tubulin mRNAs. Cell 25:537–546.

Cleveland DW, Pittenger MF, Feramisco JR. 1983. Elevation of tubulin levels by microinjection suppresses new tubulin synthesis. Nature 305:738–740.

Elliott EM, Henderson G, Sarangi F, Ling V. 1986. Complete sequence of three alpha-tubulin cDNAs in Chinese hamster ovary cells: Each encodes a distinct alpha-tubulin isoprotein. Mol Cell Biol 6:906–913.

Gliksman NR, Skibbens RV, Salmon ED. 1993. How the transition frequencies of microtubule dynamic instability (nucleation, catastrophe, and rescue) regulate microtubule dynamics in interphase and mitosis: Analysis using a Monte Carlo computer simulation. Mol Biol Cell 4: 1035–1050.

Gonzalez-Garay ML, Cabral F. 1996. Alpha-tubulin limits its own synthesis: Evidence for a mechanism involving translational repression. J Cell Biol 135:1525–1534.

Gould RR, Borisy GG. 1977. The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. J Cell Biol 73:601–615.

Graves RA, Pandey NB, Chodchoy N, Marzluff WF. 1987. Translation is required for regulation of histone mRNA degradation. Cell 48:795–804.

Hendrickson TW, Yao J, Bhadury S, Corbett AH, Joshi HC. 2001. Conditional mutations in gamma-tubulin reveal its involvement in chromosome segregation and cytokinesis. Mol Biol Cell 12:2469–2481.

Horio T, Uzawa S, Jung MK, Oakley BR, Tanaka K, Yanagida M. 1991. The fission yeast gamma-tubulin is essential for mitosis and is localized at microtubule organizing centers. J Cell Sci 99:693–700.

Joshi HC. 1993a. Immunogold electron microscopy: Mapping tubulin isotypes on neurite microtubules. Methods Cell Biol 37:259–281.

Joshi HC. 1993b. Gamma-tubulin: The hub of cellular microtubule assemblies. Bioessays 15:637–643.

Joshi HC, Baas P, Chu DT, Heidemann SR. 1986. The cytoskeleton of neurites after microtubule depolymerization. Exp Cell Res 163:233–245.

Joshi HC, Palacios MJ, McNamara L, Cleveland DW. 1992. Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. Nature 356:80–83.

Julian M, Tollon Y, Lajoie-Mazenc I, Moisand A, Mazarguil H, Puget A, Wright M. 1993. Gamma-tubulin participates in the formation of the midbody during cytokinesis in mammalian cells. J Cell Sci 105:145–156.

Kirschner MW. 1978. Microtubule assembly and nucleation. Int Rev Cytol 54:1–71.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Marschall LG, Jeng RL, Mulholland J, Stearns T. 1996. Analysis of Tub4p, a yeast gamma-tubulin-like protein: implications for microtubule-organizing center function. J Cell Biol 134:443–454.

McIntosh JR. 1983. The centrosome as an organizer of the cytoskeleton. Mod Cell Biol 2:115–142.

Mitchison TJ, Kirschner MW. 1987. Some thoughts on the partitioning of tubulin between monomer and polymer

under conditions of dynamic instability. Cell Biophys 11:35–55.

Oakley BR, Oakley CE, Yoon Y, Jung MK. 1990. Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. Cell 61:1289–1301.

Pickett-Heaps JD. 1969. The evolution of the mitotic apparatus: An attempt at comparative ultrastructural cytology in dividing plant cells. Cytobios 3:257–280.

Sambrook J, Fritch EF, Maniatis T. 1989. Molecular Cloning, a laboratory manual, 2nd Edn. New York: Cold Spring Harbor Laboratory.

Shaw G, Kamen R. 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:659–667.

Shu HB, Joshi HC. 1995. Gamma-tubulin can both nucleate microtubule assembly and self-assemble into novel tubular structures in mammalian cells. J Cell Biol 130:1137–1147.

Shu HB, Li Z, Palacios MJ, Li Q, Joshi HC. 1995. A transient association of gamma-tubulin at the midbody is required for the completion of cytokinesis during the mammalian cell division. J Cell Sci 108:2955–2962.

Sisodia SS, Gay DA, Cleveland DW. 1990. In vivo discrimination among beta-tubulin isotypes: Selective degradation of a type IV beta-tubulin isotype following overexpression in cultured animal cells. New Biol 2:66–76.

Sobel SG, Snyder M. 1995. A highly divergent gamma-tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. J Cell Biol 131:1775–1788.

Stearns T, Kirschner MW. 1994. In vitro reconstitution of centrosome assembly and function: The central role of gamma-tubulin. Cell 76:623–638.

Stearns T, Evans L, Kirschner M. 1991. Gamma-tubulin is a highly conserved component of the centrosome. Cell 65:825–836.

Sullivan KF. 1988. Structure and utilization of tubulin isotypes. Annu Rev Cell Biol 4:687–716.

Sullivan KF, Cleveland DW. 1986. Identification of conserved isotype-defining variable region sequences for four vertebrate beta-tubulin polypeptide classes. Proc Natl Acad Sci USA 83:4327–4331.

Sunkel CE, Gomes R, Sampaio P, Perdigao J, Gonzalez C. 1995. Gamma-tubulin is required for the structure and function of the microtubule organizing center in drosophila neuroblasts. EMBO J 14:28–36.

Teng MH, Bartholomew JC, Bissell MJ. 1977. Synergism between anti-microtubule agents and growth stimulants in enhancement of cell cycle traverse. Nature 268:739–741.

Theodorakis NG, Cleveland DW. 1992. Physical evidence for cotranslational regulation of beta-tubulin mRNA degradation. Mol Cell Biol 12:587–600.

Vassilev A, Kimble M, Silflow CD, LaVoie M, Kuriyama R. 1995. Identification of intrinsic dimer and overexpressed monomeric forms of gamma-tubulin in Sf9 cells infected with baculovirus containing the *Chlamydomonas* gamma-tubulin sequence. J Cell Sci 108: 1083–1092.

Yen TJ, Machlin PS, Cleveland DW. 1988. Autoregulated instability of beta-tubulin mRNAs by recognition of the nascent amino terminus of beta-tubulin. Nature 334:580–585.

Zheng Y, Jung MK, Oakley BR. 1991. Gamma-tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with the centrosome. Cell 65:817–823.

Zheng Y, Wong ML, Alberts B, Mitchison T. 1995. Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. Nature 378:578–583.