

# $\gamma$ -Tubulin Can Both Nucleate Microtubule Assembly and Self-assemble into Novel Tubular Structures in Mammalian Cells

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**Abstract.**  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tubulins are evolutionarily highly conserved members of the tubulin gene superfamily. While the abundant members,  $\alpha$ - and  $\beta$ -tubulins, constitute the building blocks of cellular microtubule polymers,  $\gamma$ -tubulin is a low abundance protein which localized to the pericentriolar material and may play a role in microtubule assembly. To test whether  $\gamma$ -tubulin mediates the nucleation of microtubule assembly *in vivo*, and co-assembles with  $\alpha$ - and  $\beta$ -tubulins into microtubules or self-assembles into macro-molecular structures, we experimentally elevated the expression of  $\gamma$ -tubulin in the cell cytoplasm. In most cells,

overexpression of  $\gamma$ -tubulin causes a dramatic reorganization of the cellular microtubule network. Furthermore, we show that when overexpressed,  $\gamma$ -tubulin causes ectopic nucleation of microtubules which are not associated with the centrosome. In a fraction of cells,  $\gamma$ -tubulin self-assembles into novel tubular structures with a diameter of  $\sim 50$  nm (named  $\gamma$ -tubules). Furthermore, unlike microtubules,  $\gamma$ -tubules are resistant to cold or drug induced depolymerization. These data provide evidence that  $\gamma$ -tubulin can cause nucleation of microtubule assembly and can self-assemble into novel tubular structures.

**I**N most eukaryotic cells the ordered distribution of many cellular organelles, including the Golgi apparatus and the endoplasmic reticulum, is mediated by a highly organized radial array of tubular fibers, microtubules (Dabora and Sheetz, 1988; Kreis, 1990; Terasaki, 1990). Although these polymers can self assemble from purified subunits at high concentration in the test tube, in animal cells their *in vivo* assembly is initiated by the centrosome, a specialized structure at the center of a cell (Gould and Borisy, 1977; Berns et al., 1977; Kirschner, 1978; Brinkley, 1981). Nucleation of microtubule assembly at the centrosome occurs with a distinct polarity: in most cells, the slow-growing minus end of the microtubule remains attached to the centrosome and thus the rapidly growing plus end is distally disposed toward the cell periphery (Heidemann and McIntosh, 1980). Therefore, the microtubule nucleation activity of the centrosome results in the formation of a radial array of microtubules in interphase mammalian cells. In addition, during mitosis, the duplicated centrosomes form a bipolar microtubule array, the mitotic spindle, that is responsible for the correct segregation of chromosomes into nascent cells (for reviews see Bajer, 1972; Brinkley, 1985; McIntosh, 1991).

Microtubules are comprised of  $\alpha$ - and  $\beta$ -tubulin subunits in the form of heterodimers (Bryan and Wilson, 1971; Feit et al., 1971). Both  $\alpha$ - and  $\beta$ -tubulins are globular

molecules with a molecular weight of  $\sim 50$  kD. Sequence comparison suggests that  $\alpha$ - and  $\beta$ -tubulins are highly conserved proteins during evolution (Burns, 1991). Several isoforms of either  $\alpha$ - or  $\beta$ -tubulin can be expressed in a single species and even in a single cell. The amino acid sequences of the isoforms in a single cell are also highly conserved (Sullivan, 1988). It has been shown that microtubules are heterogeneous polymers assembled from different isoforms under physiological conditions (Lewis et al., 1987; Lopata and Cleveland, 1987; Joshi et al., 1987; Joshi, 1993b; Joshi and Cleveland, 1989; for a review see Raff, 1994). Furthermore, tubulins from divergent species can co-assemble into microtubules *in vitro* and *in vivo* (for a review see Raff, 1994).

During the biogenesis of microtubules, the tubulin subunits assemble into protofilaments which form sheets. The protofilament sheets finally close to form microtubules with a diameter of  $\sim 25$  nm (Kirschner, 1978). Under certain experimental conditions,  $\alpha$ - and  $\beta$ -tubulins can also assemble into macro-tubules with a diameter of  $\sim 50$  nm or even bigger (Tilney and Porter, 1967; Burton and Fernandez, 1973; Tyson and Bulger, 1973; Hinkley, 1976; Larson et al., 1976; Suprenant and Rebhun, 1984). It has been proposed that macro-tubules may be formed by sliding of the protofilaments of microtubules (Tilney and Porter, 1967; Hinkley, 1976), or alternatively, macro-tubules may simply consist of more protofilaments than the normal 13 protofilaments in microtubules (Tilney et al., 1973).

$\gamma$ -Tubulin is a new member of the tubulin gene family with  $\sim 35\%$  sequence identity to the classical  $\alpha$ - and  $\beta$ -tubulins,

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while  $\alpha$ - and  $\beta$ -tubulins share 35–40% sequence identity with each other (Oakley and Oakley, 1989). Unlike  $\alpha$ - and  $\beta$ -tubulins which assemble to form microtubule polymers,  $\gamma$ -tubulin is excluded from the bulk of the microtubule lattice. Instead,  $\gamma$ -tubulin is enriched in mammalian centrosomes and in other microtubule organizing centers of distinct morphologies found in many divergent species and cell types (Stearns et al., 1991; Horio et al., 1991; Zheng et al., 1991; Joshi et al., 1992; Baas and Joshi, 1992; Liu et al., 1993; Palacios et al., 1993; Muresan et al., 1993; for reviews see Oakley, 1992; Joshi, 1993a, 1994). Using quantitative immunoblot analysis (Stearns et al., 1991) found that  $\gamma$ -tubulin is present in mammalian cells at a low copy number of only  $10^4$  molecules per cell, <1% the level of either  $\alpha$ - or  $\beta$ -tubulin. Three lines of experimental evidence have implicated  $\gamma$ -tubulin as a key functional component of the centrosome and other microtubule organizing centers. First, disruption of the  $\gamma$ -tubulin gene in a filamentous fungus, *Aspergillus nidulans*, causes depletion of microtubules and mitotic arrest (Oakley et al., 1990); second, microinjection of a  $\gamma$ -tubulin antibody into mammalian cells disrupts microtubule nucleation by the centrosome in interphase cells and disrupts the morphogenesis of the spindle in mitotic cells (Joshi et al., 1992); third, depletion of a  $\gamma$ -tubulin containing heteromeric protein complex from *Xenopus* egg extract diminishes the ability of the extract to support the formation of sperm-mediated microtubule asters (Felix et al., 1994; Stearns and Kirschner, 1994). These experiments provide compelling evidence for an important role of  $\gamma$ -tubulin in centrosome-dependent microtubule assembly and organization. However, all the available evidence is indirect, and a definite and direct role of  $\gamma$ -tubulin in the centrosome as the nucleator of microtubule assembly or in the organization of the resulting microtubule arrays has yet to be demonstrated.

This study was designed to address several basic questions regarding  $\gamma$ -tubulin function. First, can  $\gamma$ -tubulin mediate the nucleation of microtubule assembly independently of the centrosome in mammalian cells? Second, based on the fact that  $\gamma/\alpha$  and  $\gamma/\beta$  tubulins share a similar degree of sequence identity as do  $\alpha/\beta$  tubulins, we wondered why  $\gamma$ -tubulin is partitioned into the pericentriolar material and not being incorporated into the microtubule lattice in mammalian cells. We also wondered whether  $\gamma$ -tubulin, when overexpressed, can co-assemble with  $\alpha$ - and  $\beta$ -tubulins or self-assemble to form similar microtubules. We show here that overexpression of  $\gamma$ -tubulin causes a dramatic reorganization of the normal cellular microtubule network in mammalian cells. Using an *in vivo* microtubule nucleation assay, we demonstrate that overexpressed  $\gamma$ -tubulin causes ectopic microtubule nucleation outside the centrosome in mammalian cell cytoplasm. We also found, surprisingly, that in a fraction of cells, overexpressed  $\gamma$ -tubulin can polymerize into novel tubular structures with distinct morphological, thermodynamic, and pharmacological properties.

## Material and Methods

### Vector Construction

To construct the plasmid RSV-h $\gamma$ T, a 1,568 bp full-length human  $\gamma$ -tubulin cDNA fragment (from 24-bases upstream of the translation initiating site to 308-bases downstream of the stop codon) was excised from KSII-

hyT vector (Zheng et al., 1991) with the restriction enzyme EcoRI and the plasmid vector was excised from RSV- $\beta$ -globin (Gorman et al., 1982) with the restriction enzymes HindIII and BglII. The staggered ends were repaired by filling in the nucleotides with the Klenow fragment of DNA polymerase I. The  $\gamma$ -tubulin cDNA fragment was ligated with the vector and plasmids with correct orientation were screened.

For the construction of RSV-h $\gamma$ T/tag, human  $\gamma$ -tubulin cDNA was amplified by PCR with following two primers, 5' primer: 5'CGAGGTCCGACGGTATCG3', 3' primer: 5'CCTGGGTCGACCACTGCTCTGGGGTGCC3'. The resultant product carrying the stop codon mutation was digested with Sall, and then inserted into a Sall site of pMyc-O (Monteiro and Cleveland, 1990) vector to produce phyT/myc-tag which carries the h $\gamma$ T/myc fusion gene. phyT/myc was then digested with EcoRI, blunted with Klenow, and digested with HindIII to isolate the 1.7-kb h $\gamma$ T/myc fusion fragment. This fusion fragment was further cloned into the HindIII/BglII (BglII site was blunted) sites of the RSV- $\beta$ -globin vector to produce RSV-h $\gamma$ T/tag.

### Antibodies

A rabbit polyclonal antibody specific to mammalian  $\gamma$ -tubulin was prepared by covalently cross-linking keyhole limpet haemocyanin with a 17-amino acid polypeptide, EEFATEGTRDKDVFFYC (the amino terminal 16 residues of which are conserved among all known  $\gamma$ -tubulin sequences), followed by injection into rabbits. The characterization of this antibody is given in Joshi et al. (1992). Antibodies specific to  $\alpha$ -tubulin (Cat No. N356) and  $\beta$ -tubulin (Cat No. N357) were purchased from Amersham Corp. (Arlington Heights, IL). Antibodies against the myc-tag were kindly provided by Dr. D. Cleveland (UCSF, La Jolla, CA). Human autoantiserum (H1) was provided by Dr. T. Medsger (University of Pittsburgh, Pittsburgh, PA). The fluorescent labeled secondary antibodies were purchased from Cooper Biomedical, Inc. (Malvern, PA).

### Cell Culture and DNA Transfection

Monkey kidney COS cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Sigma Chemical Co.). Cells on glass coverslips were transfected with 20  $\mu$ g of plasmid DNA in 100 mm culture dishes using the calcium phosphate precipitation procedure (Graham and van der Eb, 1973). The transfection efficiency we routinely achieved was ~5%. Cells were analyzed at 36–60-h posttransfection. For immunoblot analysis cell extracts were prepared in 0.1 M Tris (pH 6.8) containing 1% SDS as previously described (Joshi et al., 1992). Protein concentrations were determined by a bicinchoninic acid assay (Smith et al., 1985).

### Gel Electrophoresis and Immunoblotting

Polyacrylamide gel electrophoresis for analyses of protein samples were performed as previously described (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) in one-half strength Laemmli gel running buffer containing 20% methanol. Typical transfer was for 4 h at 50 V. Filters containing transferred samples were stained with Ponceau S (0.2% Ponceau S in 3% trichloroacetic acid) to identify the positions of molecular weight standards. Nonspecific protein binding was blocked by incubation in TBS-T blocking buffer (5% dry milk, 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 10 min. For immunological detection,  $\gamma$ -tubulin antibody (4  $\mu$ g/ml) in TBS-T was allowed to react for 1 h at room temperature. The filters were then washed five times (3-min each wash) in TBS-T. Horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody in TBS-T was added and incubated for 1 h at room temperature followed by five washes in TBS-T to remove unbound secondary antibody. Binding was detected by chemiluminescence (Western Light; Amersham Corp.) followed by autoradiography using Eastman Kodak Co. (Rochester, NY) XAR film. The relative protein levels were determined by densitometric analysis with a Dage-Mti densitometer and Image 1 Software (Universal Imaging Incorporation, West Chester, PA).

### Indirect Immunofluorescence Microscopy

Cells on glass coverslips were washed for 15 s at 37°C with microtubule stabilization buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA, 4 M glycerol, and 1 mM GTP) (Soloman et al., 1989) and then incubated for 1 min at 37°C in stabilizing buffer containing 0.5% Triton X-100. Coverslips were rinsed with stabilizing buffer and then plunged into methanol at –20°C for 5 min. Coverslips were rehydrated in PBS and stained with primary antibodies

diluted 1:100 in PBS for 1 h at room temperature. They were then rinsed with PBS and secondary antibodies were applied for 45 min at room temperature. The coverslips were rinsed with PBS containing the DNA dye, DAPI, and mounted in Aqua mount (Lerner Laboratories, New Haven, CT). The cells were examined on Zeiss Axiovert microscope with epifluorescence optics.

### Microtubule Nucleation Assays

These assays were adapted from Osborn and Weber (1976). Cells grown on glass coverslips were transfected transiently with RSV-h $\gamma$ T plasmid using calcium phosphate precipitation (Graham and van der Eb, 1973). 36 h later, cells were incubated on ice for 1 h to depolymerize microtubules, and either processed immediately for immunofluorescence (0 time point of recovery), or the cold medium was replaced with warm (30°C) medium for the initiation of microtubule recovery. Coverslips were harvested for immunofluorescence analysis 30 s, 1 min, 2 min, 5 min, or 15 min after warming. Immunofluorescence analysis was performed as described earlier.

### Drug Treatment

For drug induced depolymerization, cells were treated with 1  $\mu$ g/ml Nocodazole for 1 h. Then the treated cells were lysed in MTSB buffer containing 0.5% Triton X-100 (Solomon et al., 1979) for 1–2 min, fixed in cold methanol, and processed for immunocytochemistry as described earlier.

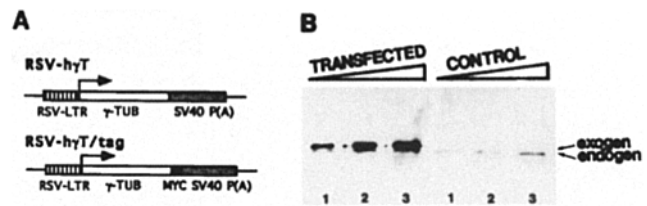
### Immunogold Electron Microscopy

COS cells transiently transfected with RSV-h $\gamma$ T were fixed and permeabilized simultaneously with 2% glutaraldehyde and 0.5% Triton X-100 in TBS (Joshi, 1993). Cells were processed for  $\gamma$ -tubulin immunogold staining and flat embedded as previously described (Joshi et al., 1993), except here we depolymerized microtubules by incubation with nocodazole (1  $\mu$ g/ml) or cold (0°C) for 1 h prior to processing. Embedded cells were sectioned parallel to the substratum using a Reichert Jung Ultracut S microtome. Serial thin sections (80–100-nm thick) were photographed with a JOEL electron microscope.

## Results

### Overexpression of $\gamma$ -Tubulin Results in a Reorganized Microtubule Network in Mammalian Cells

To overexpress  $\gamma$ -tubulin in mammalian cells, we constructed two plasmids RSV-h $\gamma$ T and RSV-h $\gamma$ T/tag, in which a full length human  $\gamma$ -tubulin cDNA with or without a COOH-terminal myc tag (Evans et al., 1985) were ligated downstream of RSV-LTR promoter (Fig. 1 A). Following transfection of either RSV-h $\gamma$ T (not shown) or RSV-h $\gamma$ T/tag plasmid (Fig. 1 B) into COS cells by calcium phosphate precipitation, immunoblot analysis confirmed a robust expression of  $\gamma$ -tubulin in transfected cells. The molecular weight of overexpressed  $\gamma$ -tubulin is slightly higher than the endogenous  $\gamma$ -tubulin due to in-frame fusion of a 14-amino acid tag sequence. As shown in Fig. 1 B, the  $\gamma$ -tubulin level in cells transfected transiently with RSV-h $\gamma$ T/tag plasmid is approximately sixfold higher than control cells transfected with RSV- $\beta$ -gal vector as measured by densitometry. Since the transfection efficiency in these experiments was about 5% (determined by direct counting of transfected cells), these levels represent an approximately 120-fold increase in expression relative to control cells. Immunofluorescence microscopy revealed that overexpressed  $\gamma$ -tubulin was distributed throughout the cytoplasm in transfected cells (Fig. 2 B). In contrast, in 20–30% of transfected cells overexpressed  $\gamma$ -tubulin polymerized into filamentous structures (see later part of this paper). To clearly visualize microtubules in cells overexpressing  $\gamma$ -tubulin, we extracted transfected cells with mi-

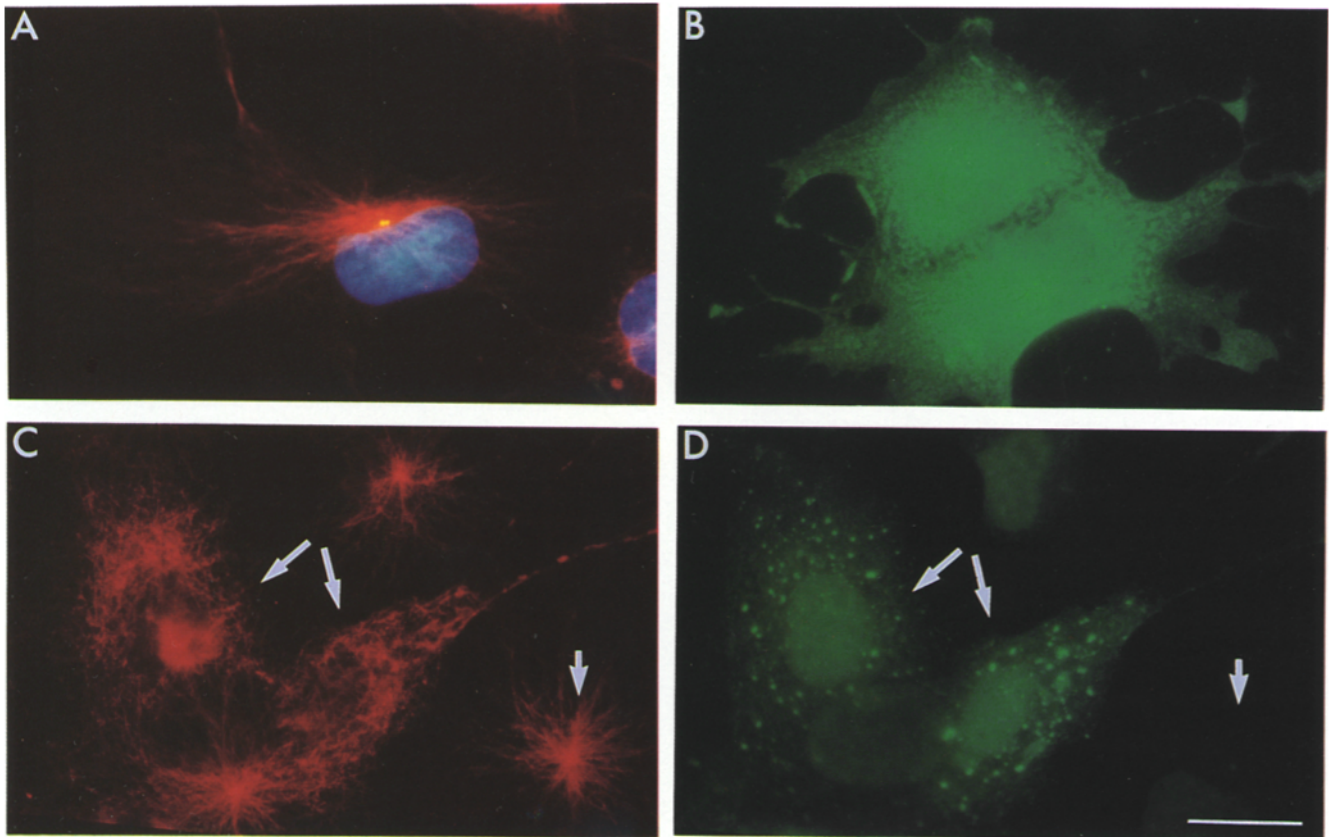


**Figure 1.** Quantitative immuno-blot analysis of  $\gamma$ -tubulin overexpression in COS cells. (A) Schematic presentation of  $\gamma$ -tubulin overexpression vectors. (B) Immuno-blot analysis of  $\gamma$ -tubulin overexpression in COS cells (monkey kidney epithelial cell line). For quantitative measurements, serial dilutions of total protein extracts from RSV- $\beta$ -gal (Shu et al., 1993) (Control) and RSV-h $\gamma$ T/tag transfected cells were loaded. The two thin arrows point to endogenous and overexpressed  $\gamma$ -tubulin. The overexpressed  $\gamma$ -tubulin is larger than the endogenous  $\gamma$ -tubulin due to the in frame fusion of a 14-amino acid myc tag. Because the transient transfection efficiency was 5% as determined by immunofluorescence staining with  $\gamma$ -tubulin antibody followed by direct counting of positive cells, the overexpressed  $\gamma$ -tubulin is about 120-fold higher than the endogenous  $\gamma$ -tubulin in transfected cells (measured by laser scanning densitometry).

crotochule stabilizing buffer containing 0.5% Triton X-100 prior to fixation and immunocytochemistry. This procedure removes most free tubulins and lowers the background staining caused by these free tubulins (Fig. 2, C and D). However, some large  $\gamma$ -tubulin aggregates were insoluble which could be used to identify transfected cells (Fig. 2 D). The major phenotypic consequence of  $\gamma$ -tubulin overexpression was a pronounced reorganization of cellular microtubule distribution as visualized by immunofluorescence using  $\alpha$ - or  $\beta$ -tubulin antibodies (Fig. 2 C, long arrows). In contrast to the radial microtubule array emanating from the centrosome found in untransfected cells (Fig. 2 A) or neighboring untransfected control cells (Fig. 2, C and D, short arrows), 53% (34 out of 64) of transfected cells contained a disorganized microtubule network (Fig. 2 C, long arrows). Further, transfected cells with disorganized microtubules did not organize functional mitotic spindles and became polynucleate due to abortive mitosis and failure of cytokinesis (Shu, H.-B., and H. C. Joshi, manuscript submitted for publication). In addition, cells transfected with a bacterial reporter enzyme  $\beta$ -galactosidase expression vector, RSV- $\beta$ -gal (Shu et al., 1993), displayed normal radial microtubule organization (not shown).

### Overexpressed $\gamma$ -Tubulin Causes Ectopic Nucleation of Microtubules In Vivo

We sought to determine the nucleation sites of microtubule polymerization in transfected cells by monitoring recovery of microtubules following complete disassembly induced by lowering temperature to 0°C for 1 h. Cells were then incubated in fresh medium at 30°C for varying time periods ranging from 0 s to 30 s to 5 min, and lysed to remove free tubulin and fixed to visualize the initiation of microtubule assembly sites in the red channel and  $\gamma$ -tubulin in the green channel by double immunofluorescence microscopy (Fig. 3). As early as 30 s after return to 30°C, short microtubules emerged throughout the cytoplasm in 91% (93 out



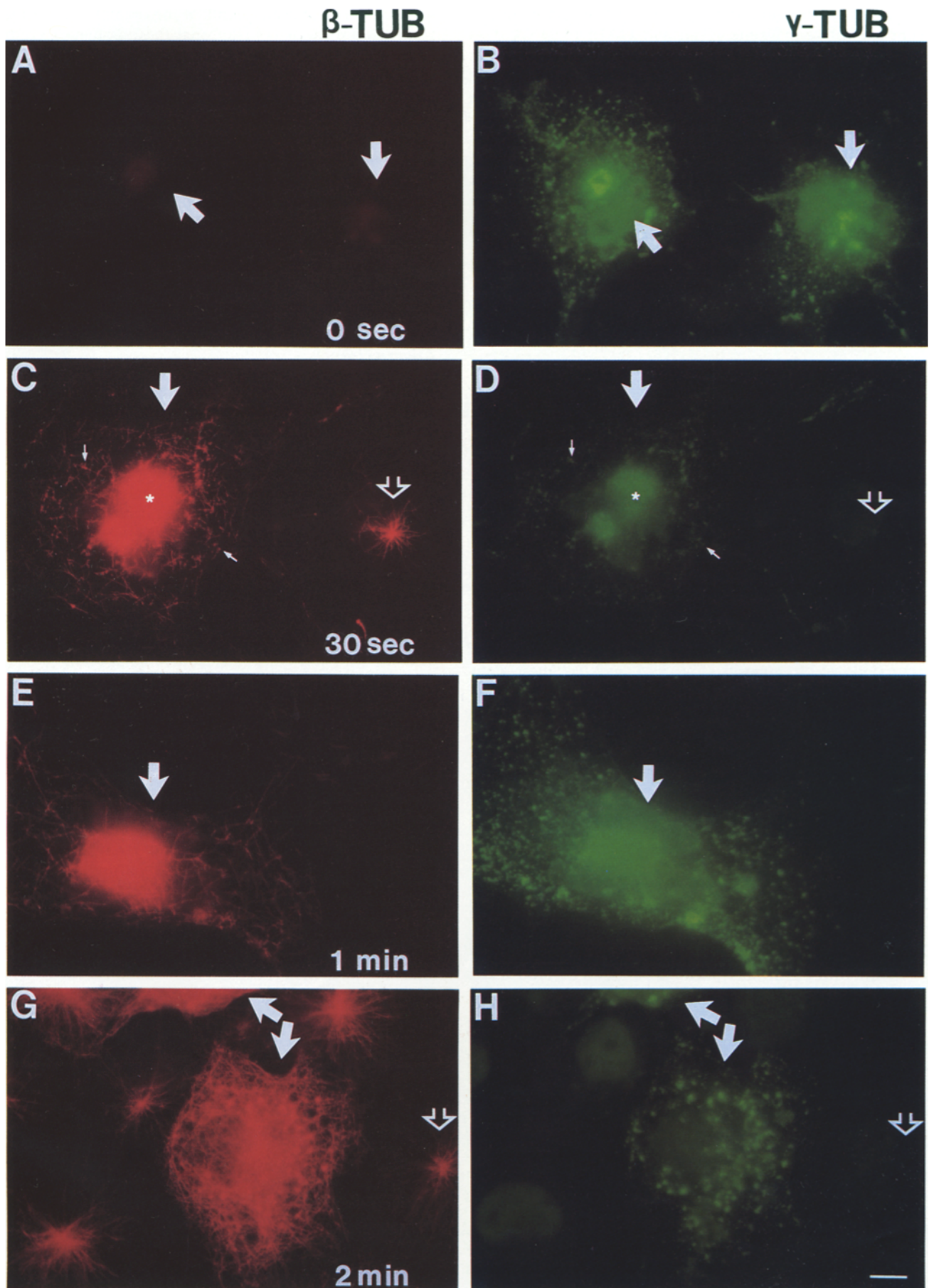
**Figure 2.** Microtubule reorganization following overexpression of  $\gamma$ -tubulin in mammalian cells. (A) Triple immunofluorescence staining of  $\gamma$ -tubulin (green),  $\beta$ -tubulin (red), and DNA (blue) in untransfected COS cells. (B) Transfected cells were stained with  $\gamma$ -tubulin antibody, without detergent treatment prior to immunocytochemistry (C and D). Transfected cells were lysed in microtubule stabilizing buffer containing 0.5% Triton X-100 prior to fixation and immunocytochemistry to visualize microtubules clearly. Microtubules (C, long arrows) are grossly reorganized in cells overexpressing  $\gamma$ -tubulin (D, long arrows). In contrast, microtubules in the neighboring untransfected cells are radially organized at the centers of the cells (C and D, short arrows). In D the  $\gamma$ -tubulin aggregates rather than the centrosomes are in focus. Bar, 20  $\mu$ M.

of 102) of transfected cells (Fig. 3 C, *small arrows*). At early stage of microtubule regrowth, distinct  $\gamma$ -tubulin staining spots were often associated with microtubules (Fig. 3, C and D, *small arrows*). In these transient transfection experiments, the neighboring untransfected cells served as internal controls which showed longer microtubules emerging mostly from the center of the cell where centrosome is localized (Fig. 3, *open arrows*). The number of microtubules nucleated appeared greatly increased in transfected cells than untransfected cells (Fig. 3, C, E, and G). As expected, after this depolymerization and repolymerization procedures, 90% (65 out of 76) of transfected cells had grossly disorganized microtubule network while microtubules in all the neighboring untransfected cells were organized radially by the centrosome (Fig. 3, G and H). We conclude that overexpressed  $\gamma$ -tubulin in transfected cells can cause ectopic nucleation of microtubule assembly in vivo.

#### ***Polymerization of $\gamma$ -Tubulin into Novel Filamentous Structures In Vivo***

As mentioned earlier in this paper, one of the surprising discoveries of  $\gamma$ -tubulin overexpression in COS cells is that overexpressed  $\gamma$ -tubulin can polymerize into filamentous

structures in 20–30% of transfected cells. We observed that this novel array of regular filaments was radially organized at the center of the transfected cells as revealed by immunofluorescence microscopy with affinity purified  $\gamma$ -tubulin antibody (Fig. 4 B). In other transfected cells which did not form the filaments, overexpressed  $\gamma$ -tubulin was present as granular aggregates throughout the cytoplasm with a pronounced accumulation around the centrosome (see earlier sections). Cells untransfected or transfected with RSV- $\beta$ -gal vector did not display any filaments upon staining with the affinity purified  $\gamma$ -tubulin antibody. To confirm that the filaments detected by  $\gamma$ -tubulin antibody staining in transfected cells were formed from overexpressed  $\gamma$ -tubulin, we constructed another  $\gamma$ -tubulin overexpression vector, RSV-h $\gamma$ T/tag, in which a DNA sequence encoding an epitope tag (Evans et al., 1985) of 14 amino acids was appended, in frame, at the most variable 3' extreme codon of the  $\gamma$ -tubulin cDNA. Transfection with this vector followed by immunofluorescence microscopy with a mouse monoclonal antibody against the epitope tag revealed that the epitope tagged  $\gamma$ -tubulin also polymerizes into filamentous structures in COS cells, while the neighboring untransfected cells did not show any such filaments (Fig. 4 C). Triple label immunofluorescence analy-



sis using a  $\gamma$ -tubulin antibody, a human autoimmune serum against centrosome (H1), and DAPI (a DNA dye) suggested that these  $\gamma$ -tubulin filaments emanated from the centrosomal region (Fig. 4, *E* and *F*). To reveal the spatial arrangement of these  $\gamma$ -tubulin filaments in relation to microtubules, we performed immunofluorescence staining with the rabbit  $\gamma$ -tubulin antibody, a mouse  $\beta$ -tubulin antibody, and a DNA dye (DAPI). We found that  $\gamma$ -tubulin staining filaments and microtubules did not overlap spatially (Fig. 4 *D*). The number of the filaments that can be resolved changes slightly in different transfected cells with an average number of 65 ( $n = 10$ , STD = 14), which is much fewer than the number of microtubules in a normal COS cells (see Fig. 4 *A*). These results suggest for the first time that  $\gamma$ -tubulin, like  $\alpha/\beta$  tubulins, has the ability to assemble into filamentous polymers.

### ***Thermodynamic and Pharmacological Properties of $\gamma$ -Tubulin Filaments***

One of the important properties of cellular microtubules is the dynamic instability (Mitchison and Kirschner, 1984; for a recent review see Wordeman and Mitchison, 1994). It has also been long recognized that cold temperature (0°C) or drug (such as nocodazole) treatments can depolymerize microtubule polymers into free  $\alpha/\beta$  tubulin dimers. To compare these properties of microtubules with the  $\gamma$ -tubulin filaments, we treated transfected cells with nocodazole (1  $\mu\text{g}/\text{ml}$ ) or incubated them on ice for 1 h, and then followed by immunofluorescence analysis. While microtubules depolymerized under these conditions (Fig. 5, *C* and *D*), we found that  $\gamma$ -tubulin filaments were resistant to either cold or nocodazole treatments (Fig. 5, *A* and *B*). These data suggest that the  $\gamma$ -tubulin filaments and microtubules have different thermodynamic and pharmacological properties. In these cold or nocodazole treated and lysed transfected cells,  $\beta$ -tubulin antibodies (Fig. 5, *C* and *D*), and  $\alpha$ -tubulin antibodies (not shown) only recognized the centriole tubulins, but no staining of the  $\gamma$ -tubulin filaments was observed. These data strongly indicate that  $\gamma$ -tubulin filaments contain no  $\alpha$ - and  $\beta$ -tubulin.

### ***Immunogold Electron Microscopic Observation of $\gamma$ -Tubulin Filaments***

To determine the structure of the  $\gamma$ -tubulin filaments at higher resolution, we performed immunogold electron microscopy with the affinity purified  $\gamma$ -tubulin antibody. We found that the  $\gamma$ -tubulin filaments are hollow tubular structures (Fig. 6). Therefore, we named these filaments

$\gamma$ -tubules. The measurements of the diameter of  $\gamma$ -tubules from cross sections revealed that these polymers are two-fold wider ( $\sim 50$  nm) than the classical microtubules (Fig. 6, *A* and *F*). The inner diameter of  $\gamma$ -tubules is  $\sim 25$  nm, much larger than that of microtubules, suggesting  $\gamma$ -tubules are not formed by adhesion of overexpressed  $\gamma$ -tubulin to the surface of microtubules. We also performed immunogold electron microscopy on cells transfected with the epitope-tagged  $\gamma$ -tubulin using anti-tag antibody. These experiments showed similar results (not shown).

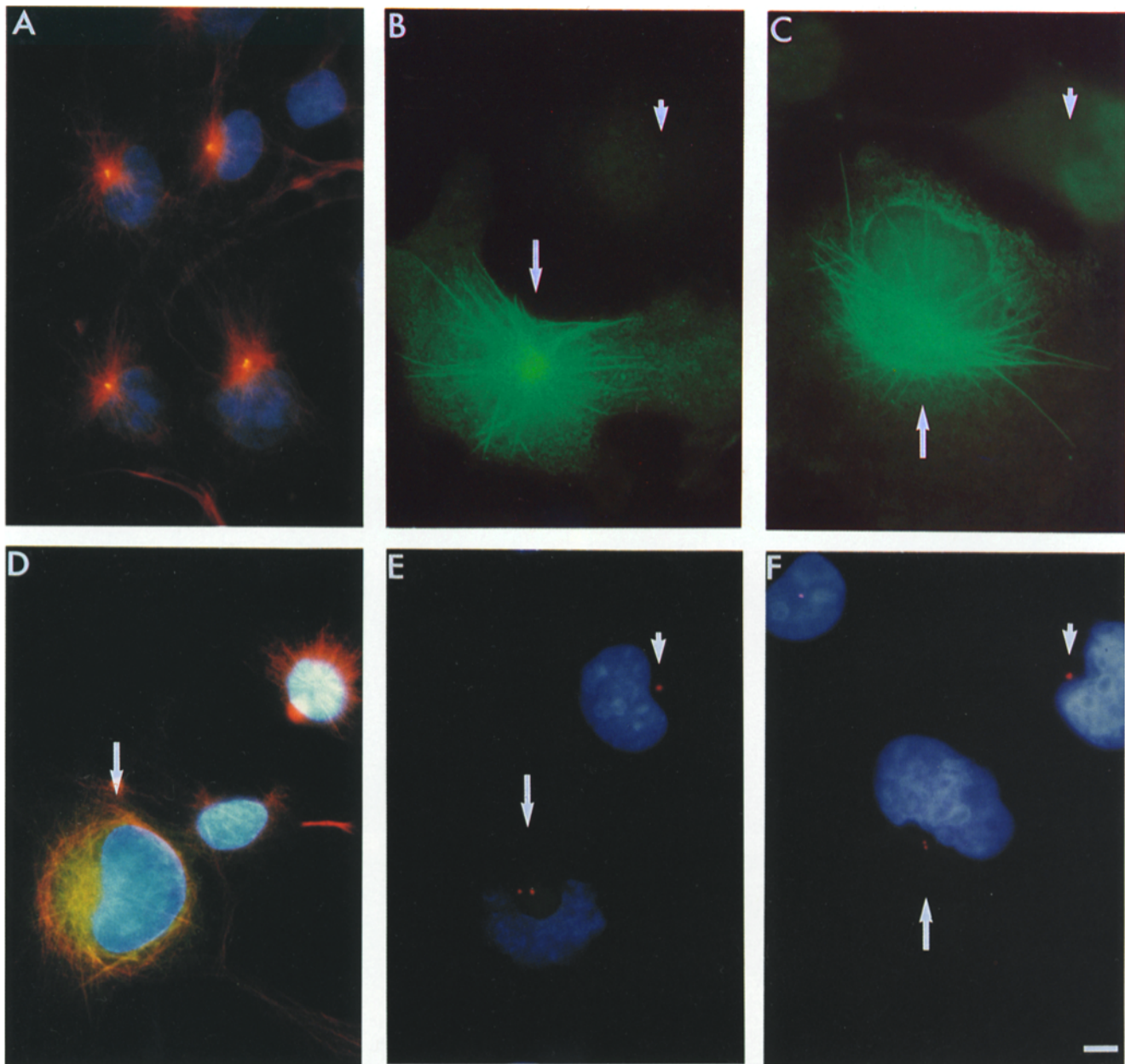
Taken together, our data show that, when overexpressed,  $\gamma$ -tubulin can form novel tubular structures which are distinct from microtubules in subunit composition, morphology, and thermodynamic (cold-stability) and pharmacological properties (nocodazole-stability).

### ***Discussion***

In this study, we observed that overexpressed  $\gamma$ -tubulin causes ectopic microtubule nucleation and self-assembles into novel tubular structures in mammalian cells. These results were not observed when human  $\gamma$ -tubulin was overexpressed in *Schizosaccharomyces pombe* (Horio and Oakley, 1994). Several reasons may account for these differences, for example, the species difference and the difference in the levels of expression. The study in *S. pombe* showed a fivefold increase of  $\gamma$ -tubulin, in contrast to a 120-fold increase of  $\gamma$ -tubulin level in mammalian cells reported in this study (Fig. 1). Alternatively, the differences in these two studies may simply result from the resolution with which the microtubules and  $\gamma$ -tubules can be visualized in a *S. pombe* cell versus a mammalian cell.

To test whether  $\gamma$ -tubulin can mediate microtubule nucleation, one approach is to purify  $\gamma$ -tubulin and reconstitute with pure  $\alpha/\beta$  tubulin and assay copolymerization in vitro. However, our effort at purifying soluble  $\gamma$ -tubulin from heterogeneous expression systems or natural sources have so far been unsuccessful. Thus, we took an in vivo approach by taking advantage of the information that  $\gamma$ -tubulin is a low abundance centrosomal protein. Our data presented here show that the overexpressed  $\gamma$ -tubulin can cause ectopic nucleation of cellular microtubules independently of centrosome. In the microtubule regrowth experiments directed to map the intracellular locations of the initiation of microtubule assembly, we observed that some  $\gamma$ -tubulin staining spots are often associated with the ectopically nucleated microtubules at early stage of regrowth. We also noticed that not all  $\gamma$ -tubulin staining spots were associated with microtubules (Fig. 3). Since the

**Figure 3.** Double label immunofluorescence visualization of the sites of microtubule nucleation. After depolymerization of microtubules by cold treatment, cells were rewarmed to 30°C for variable periods: 0 s (*A* and *B*); 30 s (*C* and *D*); 1 min (*E* and *F*); and 2 min (*G* and *H*). (*A* and *B*) microtubules in transfected cells are completely depolymerized after cold treatment for 1 h. (*C*) Microtubules (red) emerge throughout the cytoplasm (small arrows) and at the cell center (asterisk) of the transfected cell (large solid arrows). As expected, the neighboring untransfected cells show microtubule nucleation majorly from the cell center (open arrows). (*D*) Distribution of  $\gamma$ -tubulin in the same cells as shown in *C*. Small arrows in *D* indicate  $\gamma$ -tubulin staining spots at the ends of short microtubules shown in *C*. (*E* and *F*) Increasing the time of repolymerization resulted in elongated microtubules in the peripheral cytoplasm. (*G* and *H*) Transfected cells (large solid arrows) have many more microtubules than the neighboring untransfected cells (open arrows). Also, transfected cells show disorganization of microtubule architecture after depolymerization/repolymerization procedure (large solid arrows), while microtubules in the neighboring untransfected cell are organized radially exclusively at the centrosomes. In this figure, the  $\gamma$ -tubulin aggregates rather than the centrosomes are in focus. Bar, 6  $\mu\text{m}$ .

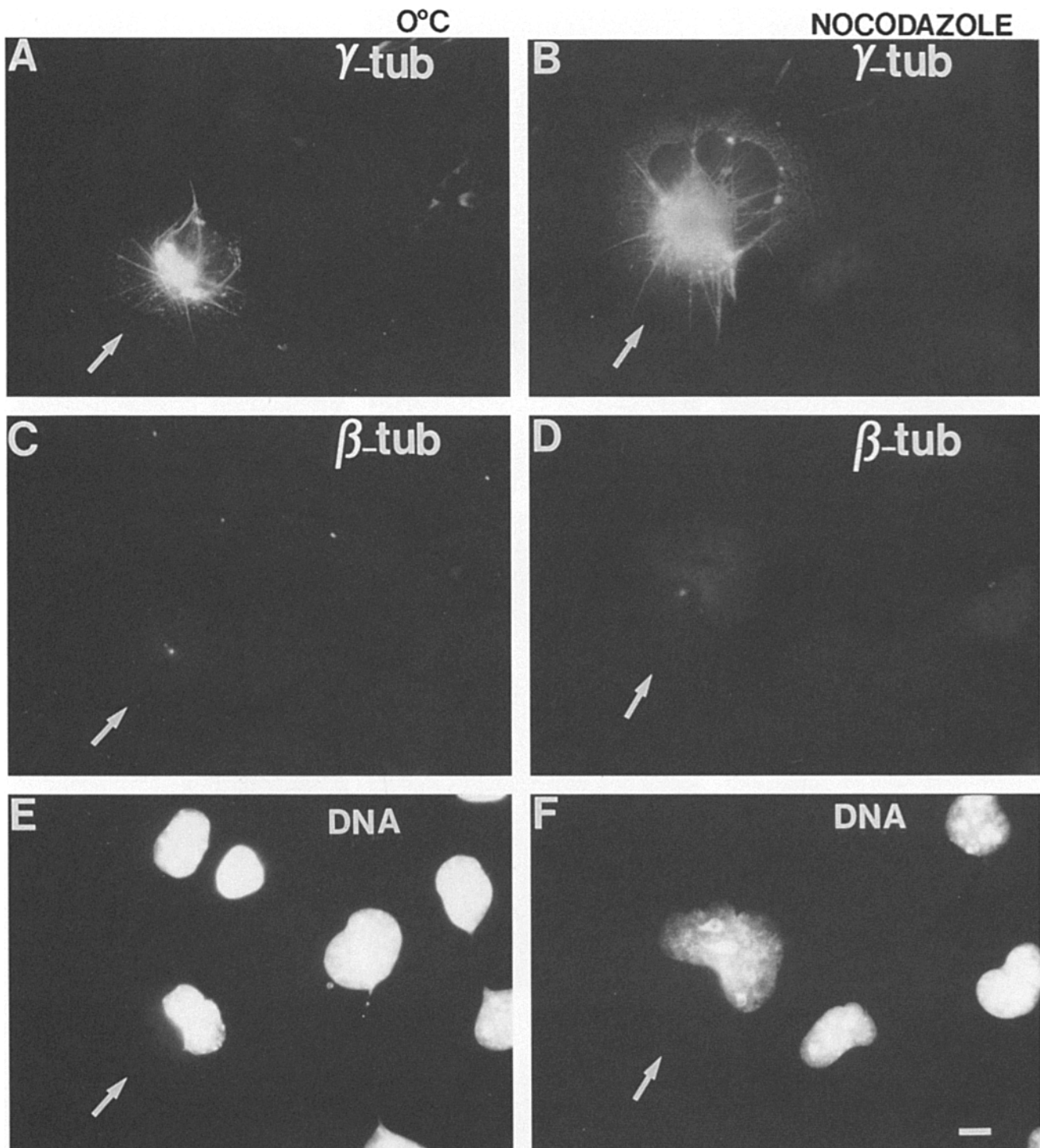


**Figure 4.** Polymerization of overexpressed  $\gamma$ -tubulin into filamentous structures in vivo. (A) Triple staining of  $\gamma$ -tubulin (yellow),  $\beta$ -tubulin (red), and DNA (blue) in untransfected cells in comparison to a RSV-h $\gamma$ T transfected cell (D, long arrow). (B and E) Cells were transfected with RSV-h $\gamma$ T, and then stained with  $\gamma$ -tubulin antibody (B), an autoimmune serum (H1) against an 85-kD centrosomal antigen and DAPI (E). (C and F) Cells were transfected with RSV-h $\gamma$ T/tag, then stained with a monoclonal antibody against the myc tag (C), H1 serum and DAPI (F). Long arrows point to transfected cells, while short arrows point to untransfected cells in the same field of view in these transient transfection experiments. Bar, 4  $\mu$ m.

nucleation step of microtubule assembly reaction is energetically less favored than the elongation of the existing polymers, only a fraction of the nucleation sites may out compete others by recruiting the available free tubulins. It is also possible that a certain structural configuration of the  $\gamma$ -tubulin aggregates may be more efficient in microtubule nucleation activity than others (Melki et al., 1993). A third possibility is that microtubules can be released from the sites of their nucleation. Finally, it is also possible that there are biochemical distinctions among the  $\gamma$ -tubulin sites competent for microtubule nucleation (see below). In

the microtubule regrowth experiments, we also found that some microtubules were not seen converging on  $\gamma$ -tubulin spots. Several possible mechanisms may account for this observation. For example, after nucleation, microtubules may be released from the  $\gamma$ -tubulin spots. It has been observed that microtubules can be released from centrosome after nucleation (Mastronarde et al., 1993). In addition, the detergent extraction procedure required to visualize short nucleated microtubules may dislodge some of these  $\gamma$ -tubulin aggregates from the microtubule ends.

What is the precise mechanism by which overexpressed

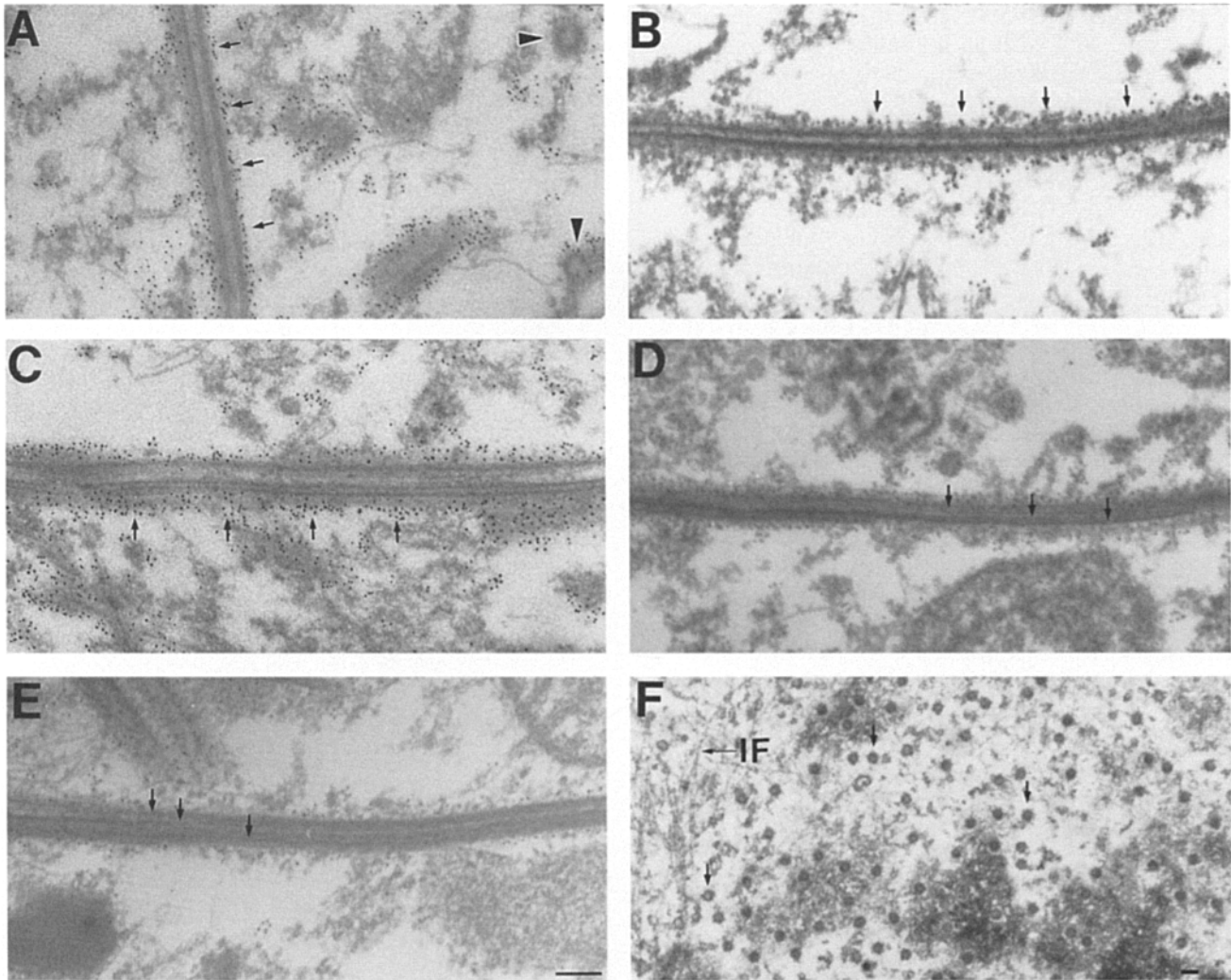


**Figure 5.**  $\gamma$ -Tubules are resistant to cold and nocodazole treatment. Cold (A, C, and E) or nocodazole (B, D, and F) treated transfected cells were stained with  $\gamma$ -tubulin antibody (A and B),  $\beta$ -tubulin antibody (C and D), and DAPI (E and F). Bar, 4  $\mu$ m.

$\gamma$ -tubulin cause ectopic nucleation of microtubules? One possibility is that  $\gamma$ -tubulin may directly act as a primer for the nucleation of microtubule assembly. This would demand the assembly of a precise oligomeric template primer which can extend by the addition of the  $\alpha\beta$ -tubulin dimers within the cytoplasm in the absence of other centrosomal components. Or, other necessary unknown centrosomal components may coassemble with the overex-

pressed  $\gamma$ -tubulin to align the  $\gamma$ -tubulin molecules in these template primers. However, this scenario would necessitate a coordinated upregulation of these other obligatory components following  $\gamma$ -tubulin overexpression. It is also possible that instead of forming a precise template primer,  $\gamma$ -tubulin self association might form a site for the aggregation of  $\alpha\beta$  tubulin dimers increasing the local concentration sufficiently high enough to initiate microtubule as-





**Figure 6.** Immunogold electron microscopy of the  $\gamma$ -tubules. Transfected cells were labeled with anti- $\gamma$ -tubulin antibody and a gold conjugated anti-rabbit secondary antibody. Gold particles were heavily concentrated along hollow regular tubular structures (A–C, *small arrows*). Triangles in A point to cross sections of  $\gamma$ -tubules. (D and F) Protofilaments (*small arrows*) rather than the gold particles are focused on in the grazing thin sections shown in these panels. (F) A low magnification of  $\gamma$ -tubule cross-sections. Bar, 80 nm.

sembly (Stearns et al., 1991). Although our data do not distinguish among these possible mechanisms, and we can not rule out the possibility that the ectopic microtubule nucleation in transfected cells is a result of upregulation of  $\alpha\beta$  tubulins by the overexpressed  $\gamma$ -tubulin, we can nevertheless conclude that overexpressed  $\gamma$ -tubulin cause ectopic nucleation of microtubules in vivo.

These results are consistent with recent in vitro studies of microtubule aster formation by sperm centrioles in *Xenopus* egg extracts. Two studies demonstrate a tight correlation between the aster forming activity and the accumulation of  $\gamma$ -tubulin in sperm centrioles incubated in *Xenopus* egg extract which contains soluble  $\gamma$ -tubulin complexed with other proteins in a heteromeric 25S protein particle. Removal of these protein complexes from the egg extract, either by antibodies or by sequestration with taxol-mediated microtubule asters, removes the active components from the egg extract; and these extracts fail to support microtubule aster formation by sperm centrioles (Felix et al., 1994; Stearns and Kirschner, 1994). In addition, there have

been recent reports of the association of  $\gamma$ -tubulin with other proteins in *Drosophila* embryos (Raff et al., 1993) and mammalian cells (Marchesi and Ngo, 1993). In light of these observations, questions remain about the biochemical complexity of the reaction leading to microtubule nucleation in vivo. In fact, there may be no decisive way so far to determine the minimally required biochemical component(s) for the nucleation of microtubule assembly in vivo.

In this study, we also tested the hypothesis that  $\gamma$ -tubulin may form structures analogous to microtubules. We found surprisingly that, when overexpressed,  $\gamma$ -tubulin can polymerize into novel tubular structures with a diameter of  $\sim 50$  nm in a fraction of transfected cells. This is an intriguing result because previous studies have suggested that, under certain conditions,  $\alpha$ - and  $\beta$ -tubulins can assemble into macrotubules with a similar diameter of  $\sim 50$  nm in vitro and in vivo (Tilney and Porter, 1967; Burton and Fernandez, 1973; Tyson and Bulger, 1973; Hinkley, 1976; Larson et al., 1976; Suprenant and Rebhun, 1984).

Taken together, these data suggest that  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tubulins may have evolved from the same ancestor molecule and they have retained the potential to assemble into similar macromolecular assemblies. One may wonder whether  $\gamma$ -tubules are the same structures as microtubules? We do not believe that this is likely for the following reasons: First,  $\gamma$ -tubules can only be stained by  $\gamma$ -tubulin antibody, but not by  $\alpha$ - and  $\beta$ -tubulin antibodies, suggesting that  $\alpha$ - and  $\beta$ -tubulins are not components of  $\gamma$ -tubules (Fig. 5). On the other hand, microtubules are primarily comprised of  $\alpha$ - and  $\beta$ -tubulins, and they can be immunostained by  $\alpha$ - and  $\beta$ -tubulin antibodies (Suprenant and Rebhun, 1984). Secondly,  $\gamma$ -tubules are resistant to cold treatment (Fig. 5), but microtubules are cold labile (Suprenant and Rebhun, 1984). Thirdly, we only observed  $\gamma$ -tubules in transfected cells overexpressing  $\gamma$ -tubulin. Because the inner diameter of  $\gamma$ -tubules is much wider than that of microtubules, it is unlikely that  $\gamma$ -tubules are formed by adhesion of  $\gamma$ -tubulin to the surface of microtubules.

In our preliminary experiments, we tried to establish a stable cell line which can overexpress  $\gamma$ -tubulin or can be induced to overexpress  $\gamma$ -tubulin. However, these efforts were unsuccessful even when using several available tightly regulated gene expression systems (Shu, H.-B., and H. C. Joshi, unpublished data). We concluded that constitutive expression of even low levels of foreign  $\gamma$ -tubulin is lethal to mammalian cells. In the absence of such a stable cell line, we are not able to produce enough  $\gamma$ -tubules to purify for biochemical analysis due to the low transient transfection efficiency (<5%). Although we do not exclude the rare possibility that  $\alpha\beta$  tubulins may be present in the  $\gamma$ -tubules and the failure to stain  $\gamma$ -tubules with  $\alpha$ - and  $\beta$ -tubulin antibodies may be due to masking of these tubulins (Fig. 5), however, the simplest explanation of our data is that  $\alpha$ - and  $\beta$ -tubulins are not incorporated into  $\gamma$ -tubules.

That overexpressed  $\gamma$ -tubulin can polymerize into tubule-like structures poses a further question. Why do only a fraction of transfected cells develop  $\gamma$ -tubules? In addition to the monkey kidney COS cells, we have also transfected human HeLa cells, CHO cells, and rodent PtK2 cells. Overexpression of  $\gamma$ -tubulin in these cells rarely produced  $\gamma$ -tubules. Previous studies suggested that  $\gamma$ -tubulin exists in two distinct pools (Felix et al., 1994; Stearns and Kirschner, 1994). A centrosome-associated pool that may be active in nucleating microtubule assembly, and a cytoplasmic pool, that may be rendered inactive for the nucleation of microtubule assembly by association with other inactivating components in a heteromeric 25S protein complex (Stearns and Kirschner, 1994). It is possible that after overwhelming these putative inactivating components, the overexpressed  $\gamma$ -tubulin is free to polymerize. In some transfected cells, the level of overexpressed  $\gamma$ -tubulin might not be high enough to overwhelm the available inactivating components. On the other hand, lower expression levels could result in a higher percentage of correctly folded  $\gamma$ -tubulin, perhaps promoting the formation of  $\gamma$ -tubules. In this regard, it should be noted that the expression levels of genes varies in these transient transfections. It has been suggested that  $\gamma$ -tubulin associates with chaperonin complexes and MAPs both in vivo and in vitro (Marchesi and Ngo, 1993; Melki et al., 1993; Raff et al.,

1993). Thus, it is possible that overexpressed  $\gamma$ -tubulin can saturate the limited amounts of chaperonins allowing unbound  $\gamma$ -tubulin to polymerize into  $\gamma$ -tubules. Alternatively, it is possible that the assembly of  $\gamma$ -tubules is assisted by specialized chaperonin-like complexes that happen to be abundantly expressed in COS cells. Whatever the precise mechanism, the data presented provide the first evidence that  $\gamma$ -tubulin can assemble into novel tubular structures.

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