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# Suppression of tubulin tyrosine ligase during tumor growth

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## SUMMARY

**The C terminus of the tubulin α-subunit of most eukaryotic cells undergoes a cycle of tyrosination and detyrosination using two specific enzymes, a tubulin tyrosine ligase (TTL) and a tubulin carboxypeptidase. Although this enzyme cycle is conserved in evolution and exhibits rapid turnover, the meaning of this modification has remained elusive. We have isolated several NIH-3T3 derived clonal cell lines that lack TTL (TTL$^-$). TTL$^-$ cells contain a unique tubulin isotype (Δ2-tubulin) that can be detected with specific antibodies. When injected into nude mice, both TTL$^-$ cells and TTL$^-$ cells stably transfected with TTL cDNA form sarcomas. But in tumors formed from TTL rescued cells, TTL is systematically lost during tumor growth. A strong selection process has thus acted during tumor growth to suppress TTL activity. In accord with this result, we find suppression of TTL activity in the majority of human tumors assayed with Δ2-tubulin antibody. We conclude there is a widespread loss of TTL activity during tumor growth in situ, suggesting that TTL activity may play a role in tumor cell regulation.**

Key words: Microtubule, Cytoskeleton, Post-translational modification, Cell cycle, Tumorigenesis, Tumor suppressor

## INTRODUCTION

Tubulin is subject to several post-translational modifications, including acetylation at a specific lysine (LeDizet and Piperno, 1987), polyglutamylation near the C terminus of the α-subunit (Eddé et al., 1990), truncation of the C terminus of α-tubulin by two amino acids (Δ2 tubulin) (Paturle-Lafanechère et al., 1991), and a cycle of tyrosine addition and removal at the C terminus of the α-subunit (Arce et al., 1991; Argaraña et al., 1980; Barra et al., 1988; MacRae, 1997; Raybin and Flavin, 1977; Wehland et al., 1986).

The cycle of tyrosination/detyrosination is of particular interest, as the reaction is completely specific to the tubulin α-subunit (Arce et al., 1991; Argaraña et al., 1977; Wehland and Weber, 1987a), and it is evolutionarily conserved (Gabius et al., 1983; Preston et al., 1979; Steiger et al., 1984; Thompson, 1982). Two enzymes unique to tubulin metabolism, a tubulin tyrosine ligase (TTL) and a tubulin carboxypeptidase (TCP), maintain the modification cycle (Argaraña et al., 1980; Ersfeld et al., 1993; Hallak et al., 1977; Martensen, 1982; Wehland et al., 1986). TTL shows preference for the free tubulin subunit and does not appear to tyrosinate tubulin in microtubules (Arce et al., 1978; Bré et al., 1987; Gundersen et al., 1987). In contrast, TCP appears to act with preference on the assembled polymer (Arce and Barra, 1985; Kumar and Flavin, 1981). As a result, newly assembled microtubules of the interphase array are composed largely of tyr-tubulin (α-tubulin containing C-terminal tyrosine), while stable microtubules are composed largely of glu-tubulin (α-tubulin with a C-terminal glutamate exposed by tyr excision) (Gundersen and Bulinski, 1986; Gundersen et al., 1984; Kreis, 1987; Schulze et al., 1987; Wehland and Weber, 1987b; Wehland et al., 1983).

The importance of this tubulin modification cycle to the physiology of the cell has remained elusive. Extensive analysis of the in vitro properties of microtubules has failed to reveal any difference in assembly or dynamics between polymers composed of tyr-tubulin or glu-tubulin (Paturle et al., 1989). While stable microtubules in interphase arrays contain glu-tubulin (Gundersen et al., 1984; Kreis, 1987; Wehland and Weber, 1987b), it is evident that detyrosination of tubulin does not itself alter microtubule stability in the cell (Khawaja et al., 1988; Webster et al., 1990). Further, antibodies to TTL, microinjected into mammalian cells, prevent tyrosination but do not appear to have significant impact on the cytoskeleton, nor on cell function over a short time course (Webster et al., 1990; Wehland and Weber, 1987b).

We have isolated several subclonal lines of mouse NIH-3T3 cells which, through spontaneous mutation, are devoid of the tubulin tyrosine ligase. These cells have allowed us to study the long-term effects of variance in TTL expression. Although TTL loss has no obvious effects on microtubule dynamics or the cell cycle, the cells show evidence of transformed behavior. When introduced into nude mice, both $TTL^-$ cells and $TTL^-$ cells rescued by TTL cDNA produce sarcomas. Interestingly, the tumors resulting from rescued cells contain predominantly glu- and Δ2-tubulin microtubule networks, indicating that TTL activity is suppressed during the growth of sarcomas from rescued cells.

The phenomenon of loss of TTL activity appears to be general during tumor growth. Human tumors of several different tissue origins lose TTL activity during sarcoma growth in nude mice. Further, a survey of human breast tumor biopsies shows that most have suppressed TTL activity. These results demonstrate that TTL is commonly suppressed during tumor growth. Although the reason for this suppression is presently unknown, it is possible that TTL may play a role as a tumor suppressor in situ.

## MATERIALS AND METHODS

### Buffers and media

Pipes, EGTA, phosphate-buffered saline (PBS; reconstituted from tablets), bovine serum albumin (BSA), BES and Hoechst 33258 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Culture medium (DME), calf serum, fetal calf serum, geneticin, collagenase II, penicillin and streptomycin were from Life Technologies, Gibco BRL (Cergy-Pontoise, France). Paraformaldehyde, $CaCl_2$, $MgCl_2$, $Na_2HPO_4$, and sucrose were from Merck (Darmstadt, Germany). NaCl was from Carlo Erba Reactifs (Nanterre, France). Tween-20 was from Bio-Rad Laboratories (Hercules, CA, USA). Glycerol was from Fluka (Buchs, Switzerland).

### Antibodies

Monoclonal anti-tyr-tubulin antibody (YL1/2) was a generous gift from Dr J. V. Kilmartin (MRC, Cambridge, UK). Monoclonal anti-tubulin tyrosine ligase antibody (ID3) was as described (Wehland and Weber, 1987a). Monoclonal anti-α-tubulin antibody (DM1A) was from Amersham (Les Ulis, France). Polyclonal anti-glu-tubulin and anti-Δ2-tubulin antibodies (named, respectively, L3 and L7) were produced by Dr L. Lafanechère (Paturle-Lafanechère et al., 1994). Rhodamine-conjugated goat anti-rat and fluorescein-conjugated anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Peroxidase-conjugated anti-mouse antibody was from Cappel (Durham, NC, USA). Peroxidase-conjugated anti-rabbit antibody was from Tago (Camarillo, CA, USA).

### Animals

Swiss (nu/nu) and Balb C female mice, 6-8 weeks old, were purchased from IFFA-CREDO (St Germain sur l'Arbresle, France). The animals were maintained under pathogen-free conditions.

### Cells

NIH-3T3 (ATCC CRL 1658) used as control cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA), and were grown in DME complemented with 10% calf serum. The NIH-3T3 cells containing a subpopulation of $TTL^-$ cells were originally obtained from ATCC, but have been maintained independently for a substantial period of time. They were grown in DME complemented with 10% calf serum and 1% fetal calf serum. Human breast tumor cell line Cal 51 (Gioanni et al., 1990) was a generous gift from Dr Claude Cochet. All cell types were incubated at 37°C in a humidified incubator with 6% $CO_2$.

### Cell cloning

$TTL^-$ cell subclonal lines were cloned from the NIH-3T3 parental line by three cycles of growth from limiting dilution. They were screened for uniformity of phenotype by double immunofluorescence staining, using either anti-glu-tubulin or anti-Δ2-tubulin polyclonal antibodies in combination with monoclonal anti-tyr-tubulin antibody (YL1/2). Several subclones, named LL4C2, LL5C1, LLA607, LLAA6, LLA222, 1A6C4, all expressing the $TTL^-$ phenotype, were obtained. The experiments described in this publication were done using the clones 1A6C4 and LLA607.

### Cell transfection

Complete TTL cDNA from domestic pig (Ersfeld et al., 1993), was inserted into the pcDNA3 eukaryotic expression vector (Invitrogen, San Diego, CA), which contains a neomycin resistance gene. Plasmids containing the insert were then transfected into $TTL^-$ cells following the procedure described in Chen and Okoyama (1987). Selection for stable transformants was initiated by addition of geneticin (750 μg/ml).

After 2 weeks of selection, isolated clones were picked using cloning rings and incubated separately in growth medium with 750 μg/ml geneticin for amplification and for immunofluorescence screening.

### Immunofluorescence procedures

Cells grown for 1-4 days on glass coverslips were processed for immunofluorescence as described in Paturle-Lafanechère et al. (1994). Coverslips were observed using a Zeiss Axioskop microscope (Carl Zeiss, Germany). Photographs were taken with Kodak (TMY 400) black and white film or with Fujicolor (400 Super G plus).

### Confocal microscopy

Cells were double-stained for immunofluorescence using FITC- and TRITC-conjugated secondary antibodies as described in Paturle-Lafanechère et al., (1994). Optical section series were obtained with a confocal scanning laser apparatus MRC 600 (Bio-Rad Microsciences Division, Herts, England) attached to a Nikon Optiphot microscope. The machine was set for a Z-axis shift of 0.5 μm for each optical section, and the starting optical section (0.0 μm) was defined as the level of contact between the cells and the coverslip.

### In vivo experiments in nude mice

Cells transfected with TTL cDNA (or with the plasmid alone) were subcloned in growth medium with 750 μg/ml geneticin and checked for tyr-, glu- and Δ2-tubulin content by immunofluorescence. Apparently pure clones (containing 100% Tyr cells or 100% Glu cells) were selected and amplified.

Exponentially growing cells were trypsinized and centrifuged (1,250 rpm, 5 minutes, room temperature). Cells were resuspended in growth medium and 400 μl of each cell suspension (i.e. 1 to $2.5\times10^6$ cells) were injected subcutaneously into the scapular area of nude mice, under mild ether anesthesia. For each different clone, two mice were simultaneously injected.

Animals were observed every day and the day of appearance of the tumor was noted. Animals were killed by cervical dislocation after a period of tumor growth, then tumors were aseptically dissected out, weighed and cut in small pieces. These pieces were divided into three samples. One sample was immediately frozen in liquid nitrogen for storage before extraction of tubulin for western blots.

The second tissue sample was transferred into growth medium and treated with collagenase II (200 i.u./ml) for 15 hours at 37°C in a cell

culture incubator. The dissociated cells were then washed three times in growth medium without serum, and a final wash was made with growth medium containing serum. The cells were then transferred into 75 $cm^2$ flasks containing 20 ml of complete culture medium and restored to in vitro culture.

The third tissue sample was transferred into PBS containing 4% paraformaldehyde, pH 7.4, for 12 hours at 4°C. The fixed tissue was then incubated for an additional 12 hours in a 20% sucrose buffer, and frozen in isopentane on dry ice. Sections (10 μm thick) were then cut in a cryotome at −18°C. Immunofluorescence of these tissue sections was then performed as described in Paturle-Lafanechère et al. (1994).

The Cal 51 cell line was grown in vitro in DMEM supplemented with 10% calf serum, then cells were injected into nude mice as described above. The tumors elicited by these cells were dissected out aseptically, and frozen in liquid nitrogen prior to homogenization and extraction for western blots.

Human tumor samples that were passaged in nude mice had been directly excised from patients during routine surgery (CHU Hospital, Grenoble). Directly after excision, the tissue samples were washed extensively with cell culture medium containing antibiotics, then were cut into 3 mm blocks and xenografted subcutaneously into the scapular area of nude mice. After a period of tumor growth, the tumors were aseptically dissected out and processed as above.

### Generation of extracts for western blots

To produce cytosolic extracts from cells in culture, cells from one 75 $cm^2$ flask were trypsinized and washed twice with PBS. 50 μl of lysis buffer (Lieuvin et al., 1994) containing 5 mM $CaCl_2$ was added to the pellet of cells and the mixture was incubated 10 minutes at 4°C, in order to depolymerize the microtubules. EGTA (10 mM, final concentration) was then added from a stock and the sample was centrifuged (200,000 ***g***, 15 minutes, 4°C). Electrophoresis sample buffer was added to the supernatant and the mixture was boiled for 3 minutes at 100°C. Samples were stored at −80°C before use.

### Tissue extracts

To prepare extracts of normal mouse tissues, they were dissected out and stored frozen in liquid nitrogen. Samples were weighed and homogenized in 1% SDS (1 ml/g of tissue), boiled for 5 minutes, then centrifuged (200,000 ***g***, 15 minutes, 20°C). After addition of sample buffer, the supernatant was again boiled for 3 minutes, then stored at −80°C before use. To prepare extracts of nude mouse tumors, they were frozen in liquid nitrogen, then reduced to powder using a Freezer mill (Spex, Bioblock, Illkirch, France). Lysis buffer (1 ml/g of frozen cells) was added and the resulting homogenate was centrifuged (200,000 ***g***, 15 minutes, 4°C), then processed as above for sample preparation for western blots. Human breast tumor samples were obtained during surgery at the CHU hospital of Grenoble, frozen immediately in liquid nitrogen, then later reduced to powder as described above for mouse tissue, with the exception that the extraction buffer was 100 mM Tris-HCl, 1 mM EDTA, 2.5 mM $MgCl_2$, 10% glycerol, 25 mg/ml leupeptin, 0.5 mM PMSF, 1 mM vanadate and 0.5 mM DTT at pH 7.4. Protein content of the samples varied from 1 to 3 mg/ml.

### Western blots

Gel electrophoresis of protein samples was performed according to Laemmli (1970). The separated proteins were then transferred onto nitrocellulose sheets (Schleicher & Schuell, CeraLabo, Aubervilliers, France) according to Towbin et al. (1979). The dilution for most antibodies was as described previously (Paturle-Lafanechère et al. (1994). Additionally, the monoclonal anti-tubulin tyrosine ligase antibody (ID3) was diluted 1/1,000. To reveal the blots, the membranes were incubated with peroxidase-labeled secondary antibodies (1/5,000) for 30 minutes, washed three times as above and developed using an Amersham ECL kit (Les Ulis, France).

## RESULTS

### Clonal selection of glu-tubulin ($TTL^-$) cells

On examining a laboratory derived line of NIH-3T3 cells for its cytoskeletal characteristics, we found that the microtubule networks of a substantial number of interphase cells reacted with glu-tubulin antibody (Fig. 1A). This was a surprising result, as this tubulin isoform had previously been detected only in the centrosomes of mammalian cells in culture, or in a distinct subset of stable microtubules (Gundersen et al., 1984, 1987; Schulze et al., 1987; Wehland and Weber, 1987b). Double-label experiments, comparing the distribution of glu-tubulin microtubules with those containing tyr-tubulin, showed clearly that these cells constituted two discrete populations. Cells containing glu-tubulin microtubules (glu-tubulin cells) did not react prominently with tyr-tubulin antibody (Fig. 1A, arrows), and cells reacting with tyr-tubulin antibody exhibited few glu-tubulin microtubules. Despite the clear distinction between the different subpopulations with respect to the state of tubulin modifications, we found no consistent differences in morphology between the different cells.

Another post-translational modification of tubulin, designated Δ2-tubulin, is characterized by the absence of the two C-terminal α-subunit residues (Paturle-Lafanechère et al., 1991). This modification is prominent among microtubules in neuronal cells, but absent in cycling cells (Paturle-Lafanechère et al., 1994). It was therefore of interest to us that a substantial subpopulation of these NIH-3T3 cells contained Δ2-tubulin microtubules (Fig. 1B). The subpopulation containing Δ2-tubulin microtubules was also distinct from those cells displaying tyr-tubulin microtubules (Fig. 1B, arrows).

We purified several subclonal lines from the parent population, selecting clones that displayed a uniform and strong glu-tubulin signal in microtubules. One such subclone is shown in Fig. 2A (left). The glu-tubulin signal is strong, whereas the same cells contain only a weak and scattered tyr-tubulin signal. When the same glu-tubulin subclone was analysed for the presence of Δ2-tubulin microtubules, they were found in abundance in interphase cells (Fig. 2A, right).

The results obtained by immunocytochemistry correlate with western blot analysis of levels of the different tubulins in the different cell lines. When gels are loaded on an equal protein basis, α-tubulin is approximately equivalent in NIH-3T3 cells and in an isolated glu-tubulin subclone (Fig. 3A). However, the cell lines are dramatically different with respect to the nature of the α-tubulin C terminus. The glu-tubulin subclone contains little apparent tyr-tubulin, all of which is probably the unmodified primary translation product. By contrast, this subclone contains abundant glu- and Δ2-tubulin (Fig. 3A).

### Glu-tubulin ($TTL^-$) cells lack TTL, and can be rescued by transfection with TTL

Cell extracts were blotted with TTL antibody to determine whether the abundance of glu-tubulin microtubules in the subclonal lines correlated with the lack of TTL activity. Using an antibody specific for TTL, we found that the enzyme is not detectable in glu-tubulin subclones. The result for one subclone is shown in Fig. 3B, and the result is comparable with that found for all subclonal isolates. As a result of finding no TTL

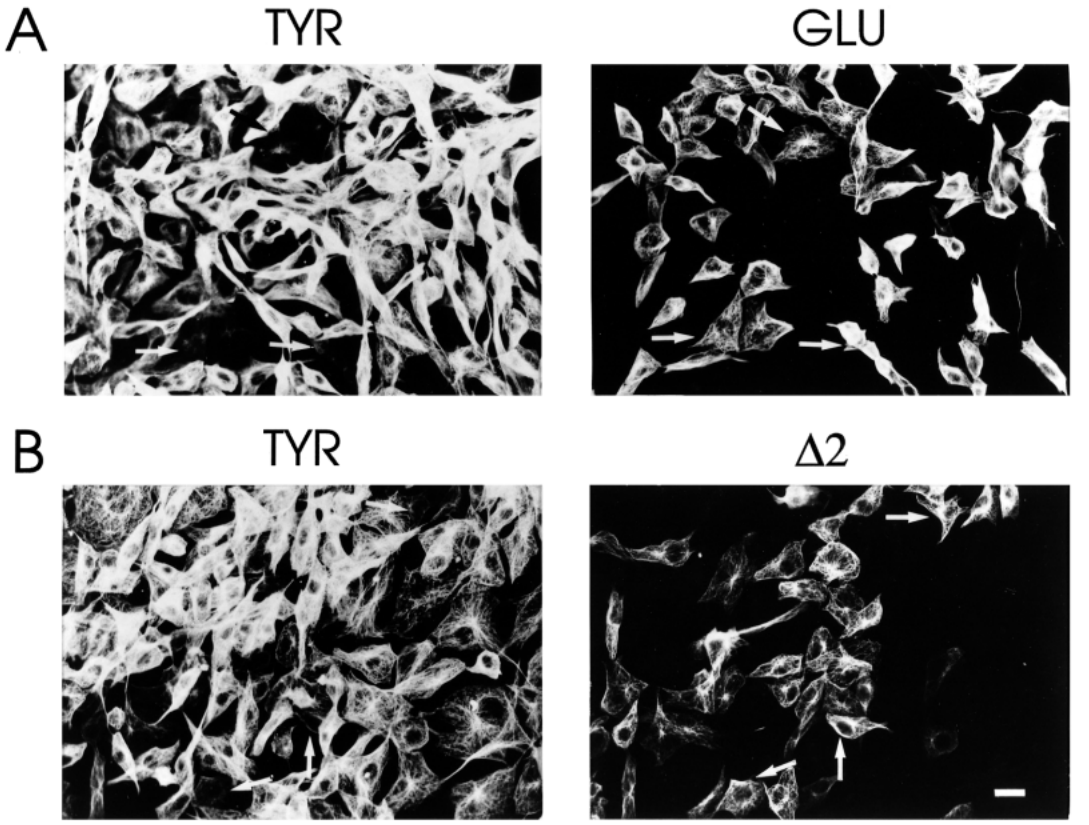


**Fig. 1.** Immunofluorescence staining of NIH-3T3 cells before subcloning of TTL⁻ cells. (A) Double staining of tyr- and glu-microtubules using monoclonal anti-tyr-tubulin antibody ('Tyr', left) and polyclonal anti-glu-tubulin antibody ('Glu', right). (B) Double staining of tyr- and Δ2-microtubules using monoclonal anti-tyr-tubulin antibody ('Tyr', left) and polyclonal anti-Δ2-tubulin antibody ('Δ2', right). Some of the cells faintly stained by the anti-tyr-tubulin antibody and brightly stained by either the anti-glu-tubulin antibody or the anti-Δ2-tubulin antibody are indicated by arrows. Bar, 20 μm.

enzyme in these cell lines, we will henceforth refer to glu-tubulin cells as TTL⁻ cells.

Transfection of TTL⁻ cells with the plasmid carrying TTL cDNA completely restored the native level of TTL expression (Fig. 3B). Transfection also restored TTL activity to apparently normal levels. Thus, after transfection with a plasmid restoring expression of TTL, the TTL⁻ subclonal line exhibits native levels of tyr-tubulin, very little glu-tubulin, and no apparent Δ2-tubulin (Fig. 3A). These results demonstrate that the abundance of glu-tubulin and of Δ2-tubulin in the subclonal lines is due to the absence of TTL, and that restoration of TTL can fully restore tyr-tubulin levels.

The effect of transfection with the cDNA expressing TTL is also evident by immunofluorescence. A stable subclonal line derived from a TTL⁻ subclone now exhibits abundant tyr-tubulin, but normal low levels of glu-tubulin microtubules (Fig. 2B, left). It shows no apparent reaction with the antibody specific for Δ2-tubulin (Fig. 2B, right).

## Expression of TTL is lost during cell overgrowth in vitro and during tumor growth in nude mice

In long-term culture, we have found no detectable difference between normal and TTL⁻ cells with respect to microtubule dynamics or cell morphology. TTL-rescued cells cultured in standard conditions and passaged before confluency maintain a stable tyr-tubulin phenotype. However, when they become confluent, TTL-rescued cells show a striking relationship between ligase expression and cell behavior. In this condition, TTL⁻ cells reappear, and the newly emerging TTL⁻ cells routinely grow above the TTL rescued cells (Fig. 4), suggesting that loss of ligase favors cell overgrowth.

These observations raised the possibility that TTL suppression may be important to substrate-independent growth, and thus to tumorigenesis. To test the possibility that TTL loss is linked to tumorigenesis, we injected TTL⁻ cells rescued with TTL cDNA into nude mice. The rationale of these experiments was based on the previous demonstration that proteins expressed as transgenes are lost during tumorigenesis in nude mice if they have a tumor-suppressor function (Harris et al., 1996). Such loss is in accord with the recently published definition of tumor suppressors as genes that sustain loss-of-function mutations during the development of a tumor (Haber and Harlow, 1997).

NIH-3T3 cells are not normally tumorigenic, but can spontaneously transform in vitro (Rubin, 1994). Both TTL⁻ cells and TTL⁻ cells rescued with TTL cDNA, injected into nude mice, always produced tumors. These tumors always arose at the site of injection and were sarcomas. However, when we examined the tumors derived from TTL⁻ cells rescued with TTL, we found they had largely reverted to a TTL⁻ state. Western blot analysis showed that such reverted cells contained both glu-tubulin and Δ2-tubulin in abundance (Fig. 5A). This

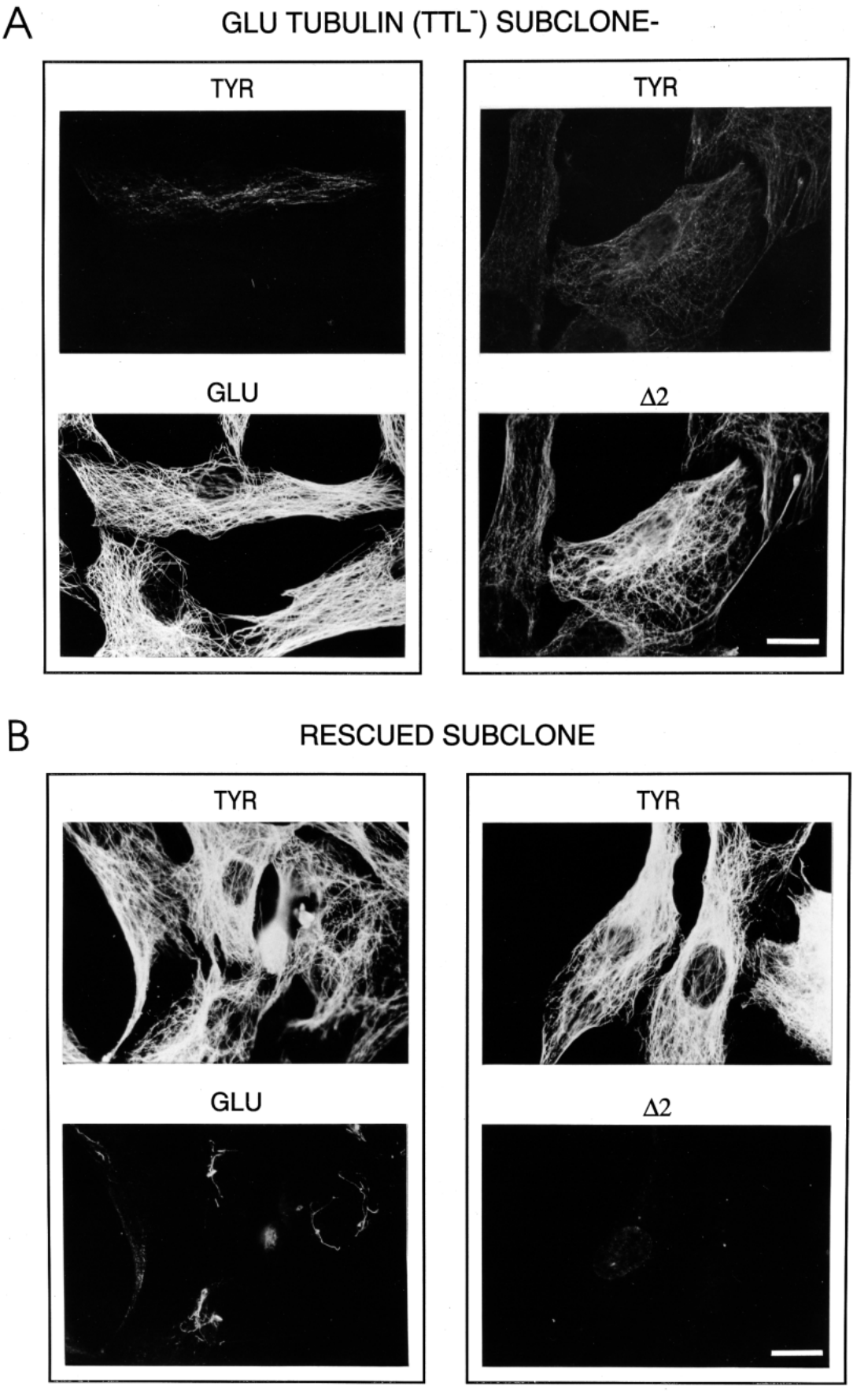


**Fig. 2.** Comparison by immunofluorescence analysis of subcloned glu tubulin (TTL⁻) cells, and of TTL⁻ cells stably transfected with TTL cDNA, for their content of tyr-, glu- and Δ2-tubulin. Glu tubulin (TTL⁻) subclonal cells (A) and stable TTL expressing transfectants of the same cell line (B) were double stained either with monoclonal anti-tyr-tubulin antibody ('Tyr') and polyclonal anti-glu-tubulin antibody ('Glu'), or with monoclonal anti-tyr-tubulin antibody ('Tyr') and polyclonal anti-Δ2-tubulin antibody ('Δ2'), as indicated. Bars, 20 μm.

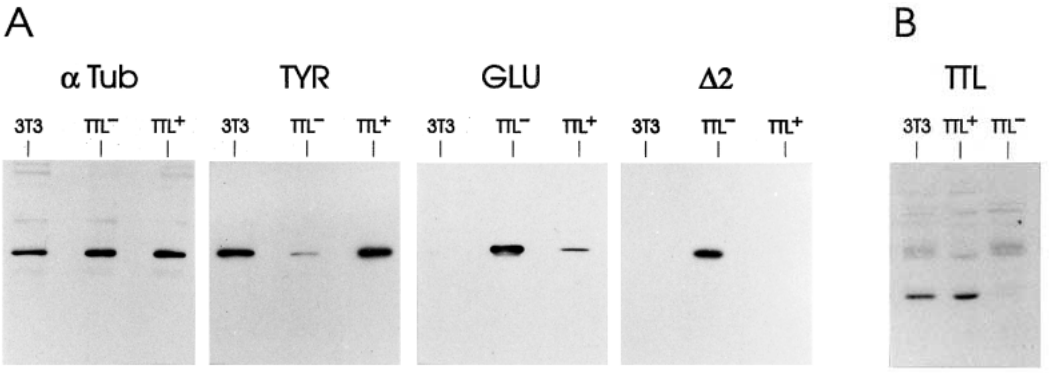


**Fig. 3.** (A) Immunoblot analysis of tyr-, glu- and Δ2-content of normal NIH-3T3 cells ('3T3'), TTL⁻ cells ('TTL⁻') and TTL⁻ cells transfected with TTL cDNA ('TTL⁺'). Cellular extracts from the different cell lines were prepared as described in Materials and Methods. Samples (5 µl) were analyzed by immunoblot procedure for total α-tubulin content using monoclonal anti-α-tubulin antibody (DM1A: 'α-tub'); for tyr-tubulin content using monoclonal anti-tyr-tubulin as primary antibody (YL1/2: 'Tyr'); for glu-tubulin content using polyclonal anti-glu-tubulin as primary antibody (L3: 'Glu') and for Δ2-tubulin content using polyclonal anti-Δ2-tubulin as primary antibody (L7: 'Δ2'), as indicated. The prominent 50 kDa band is tubulin. (B) Immunoblot analysis of TTL content of normal NIH-3T3 cells ('3T3'), TTL⁻ cells transfected with TTL cDNA ('TTL⁺') and of TTL⁻ ('TTL⁻') cells. Samples (5 µl) of cellular extracts were analyzed using monoclonal anti-tubulin tyrosine ligase antibody (ID3: 'TTL') as primary antibody. The prominent 43 kDa band is TTL (Wehland and Weber, 1987a).

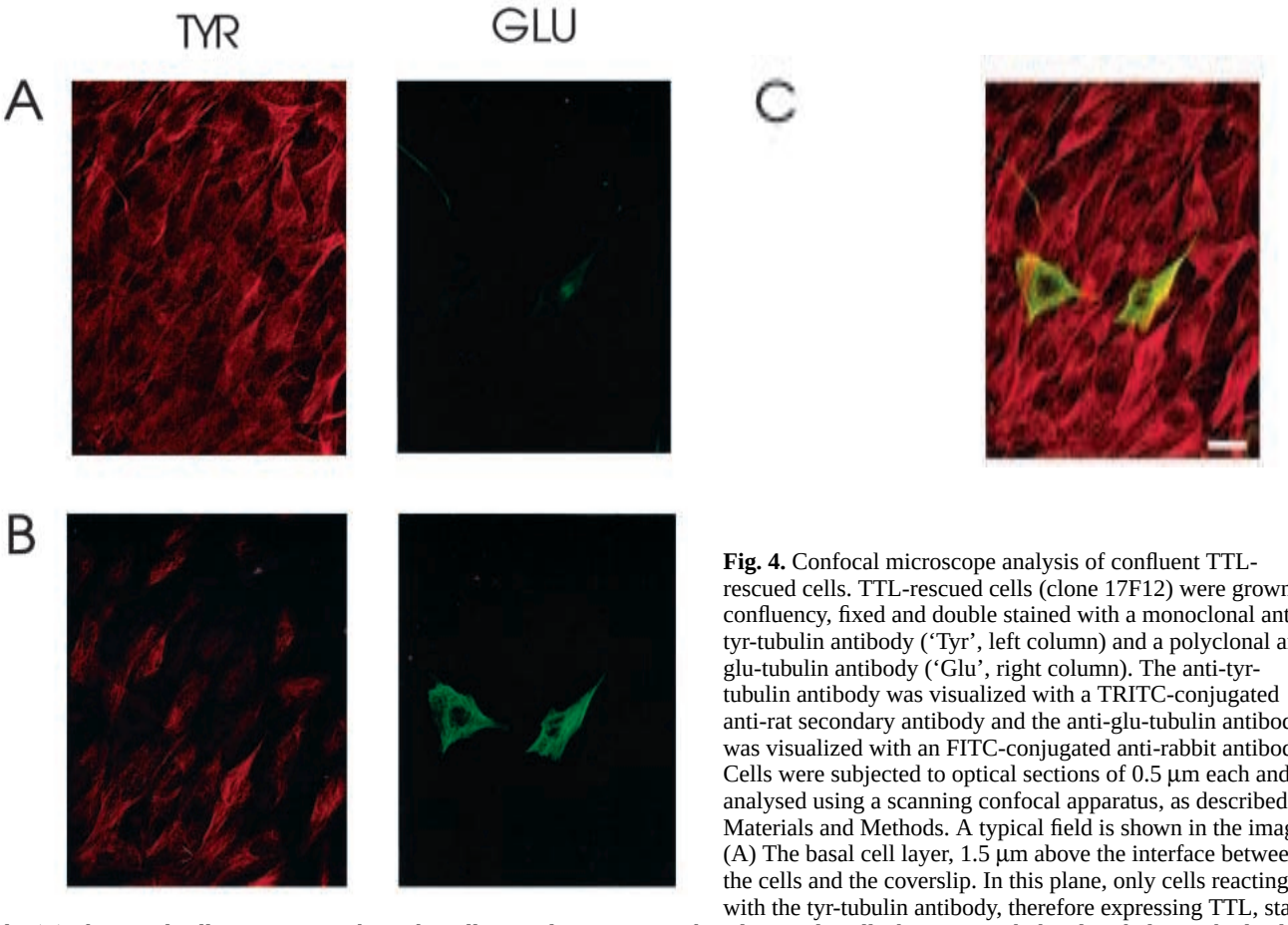


**Fig. 4.** Confocal microscope analysis of confluent TTL-rescued cells. TTL-rescued cells (clone 17F12) were grown to confluency, fixed and double stained with a monoclonal anti-tyr-tubulin antibody ('Tyr', left column) and a polyclonal anti-glu-tubulin antibody ('Glu', right column). The anti-tyr-tubulin antibody was visualized with a TRITC-conjugated anti-rat secondary antibody and the anti-glu-tubulin antibody was visualized with an FITC-conjugated anti-rabbit antibody. Cells were subjected to optical sections of 0.5 µm each and analysed using a scanning confocal apparatus, as described in Materials and Methods. A typical field is shown in the images. (A) The basal cell layer, 1.5 µm above the interface between the cells and the coverslip. In this plane, only cells reacting with the tyr-tubulin antibody, therefore expressing TTL, stain brightly. (B) The apical cell region, 5 µm above the cell-coverslip contact. In this plane, only cells that stain with the glu-tubulin antibody alone, therefore lacking TTL, are present. (C) Superimposition of all optical sections taken during this analysis. Bar, 20 µm.

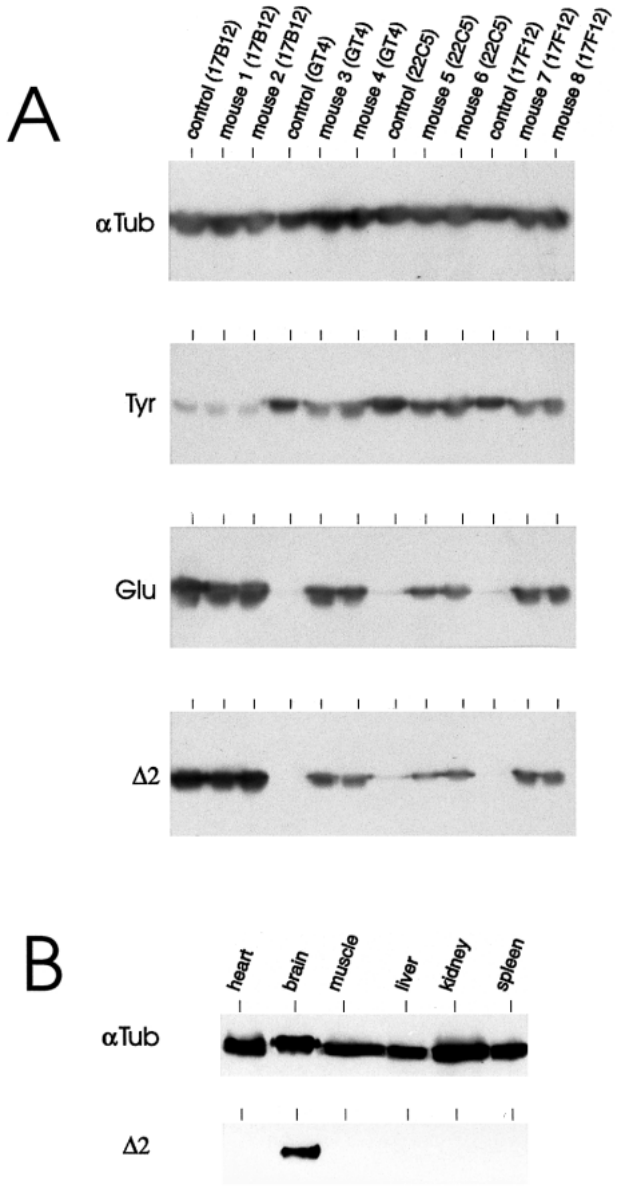


**Fig. 5.** Immunoblot analysis of total α-tubulin, and of tyr-, glu- and Δ2-tubulin content of nude mouse tumors and of different normal mouse tissues. (A) Control cellular extracts and tumor cell extracts were prepared as described in Materials and Methods. Samples (5 μl) were analyzed on western blots for total α-tubulin content using monoclonal anti-α-tubulin antibody (DM1A), for tyr-tubulin content using monoclonal anti-tyr-tubulin antibody (YL1/2), for glu-tubulin content using polyclonal anti-glu-tubulin antibody (L3) and for Δ2-tubulin content using polyclonal anti-Δ2-tubulin antibody (L7). Four experimental series, each shown in three lanes, were performed. In each series, a control lane is shown, representing extracts from the cells used for injection, and the two following lanes correspond to extracts of tumors derived from duplicate injections into two different mice. In the first experimental series, which used TTL⁻ cells to elicit tumors, the first lane (control 17B12) corresponds to an extract of the TTL⁻ cells obtained just before injection into two different mice. The second (mouse 1, 17B12) and the third lane (mouse 2, 17B12) of this series correspond to extracts of the tumors elicited in these two mice by the cells injected. All subsequent series used TTL-rescued cells. In each of the other three experimental series, two mice each received duplicate samples of a TTL⁻ subclonal cell line that had been transfected with TTL cDNA. Three different subclonal lines of TTL-transfected cells (designated GT4, 22C5 and 17F12) were tested in duplicate for each series. (B) Several mouse tissue extracts, as indicated, were loaded on an 8% SDS gel at levels that yielded equivalent total α-tubulin content. The separated proteins were subjected to western blot analysis using either anti-α-tubulin (DM1A) or Δ2-tubulin (L7) antibody. Even in heavily overloaded gels (not shown), Δ2-tubulin is present only in brain.

result was highly reproducible. Two mice injected with the same clonal cell line gave the same result with respect to the content of tyr-, glu- and Δ2-tubulin in the tumors. Further, three independently derived subclonal TTL⁻ cell lines rescued with TTL cDNA yielded comparable results, indicating a loss of TTL activity during tumor growth in situ. The presence of Δ2-tubulin, indicative of loss of TTL activity, is unique to tumor cells, in striking contrast to results on assay of samples from a variety of mouse tissues. Normal tissue biopsies show tyr- and glu-tubulin on western blots, but are completely devoid of Δ2-tubulin, with the exception of brain (Fig. 5B), and Δ2-tubulin is present only in neurons in the brain (Paturle-Lafanechère et al., 1994).

Careful analysis of one representative TTL⁻ cell line rescued with TTL cDNA showed that it contained glu- and Δ2-tubulin in abundance when excised as a tumor, as detected by western blot, although it had contained only tyr-tubulin prior to injection into a nude mouse (Fig. 6A). This outcome was not the result of plasmid instability in the absence of geneticin. Repeated experiments with the different TTL⁻ cell lines rescued with TTL cDNA showed that expression of tyr-tubulin remained stable indefinitely when cells were maintained in culture either in the presence or in the absence of geneticin (Fig. 6A). The increased presence of glu- and Δ2-tubulin was apparently due to loss of TTL from the rescued cells during tumor growth, as shown by western blots with TTL antibody (Fig. 6B).

The change in the status of tubulin within TTL⁻ cells reverted with TTL cDNA following injection into a mouse is dramatically evident in immunofluorescence images (Fig. 6C). A TTL⁻ cell line reverted with TTL cDNA contains abundant tyr-tubulin microtubules and no evident glu-tubulin microtubules in culture, whether grown with or without geneticin (Fig. 6C, top). However, a tumor slice shows the same cell line contains abundant glu-tubulin and little tyr-tubulin after growth in situ (Fig. 6C, bottom left).

Delay in onset of tumor growth from NIH-3T3 cells was highly variable, whether the cells initially were TTL⁻ or were rescued (data not shown). We do not know why the initial presence or absence of TTL did not influence the rate of tumor growth. However, the NIH-3T3 cells were almost certainly transformed (Rubin, 1994) regardless of TTL background, and as plasmid-expressed TTL can apparently be rapidly suppressed on overgrowth (Fig. 4), the initial presence or absence of TTL may not have had a decisive influence on growth in situ.

The mechanism of TTL suppression during in situ growth is not known, although it appears that cell selection occurs in the specific environment of in situ growth. If selection has occurred during tumor growth, TTL suppression should remain relatively stable upon regrowth of these tumor cells in culture. This was observed. When tumor cells are regrown in culture after explanting from the tumors, about half retain the TTL⁻

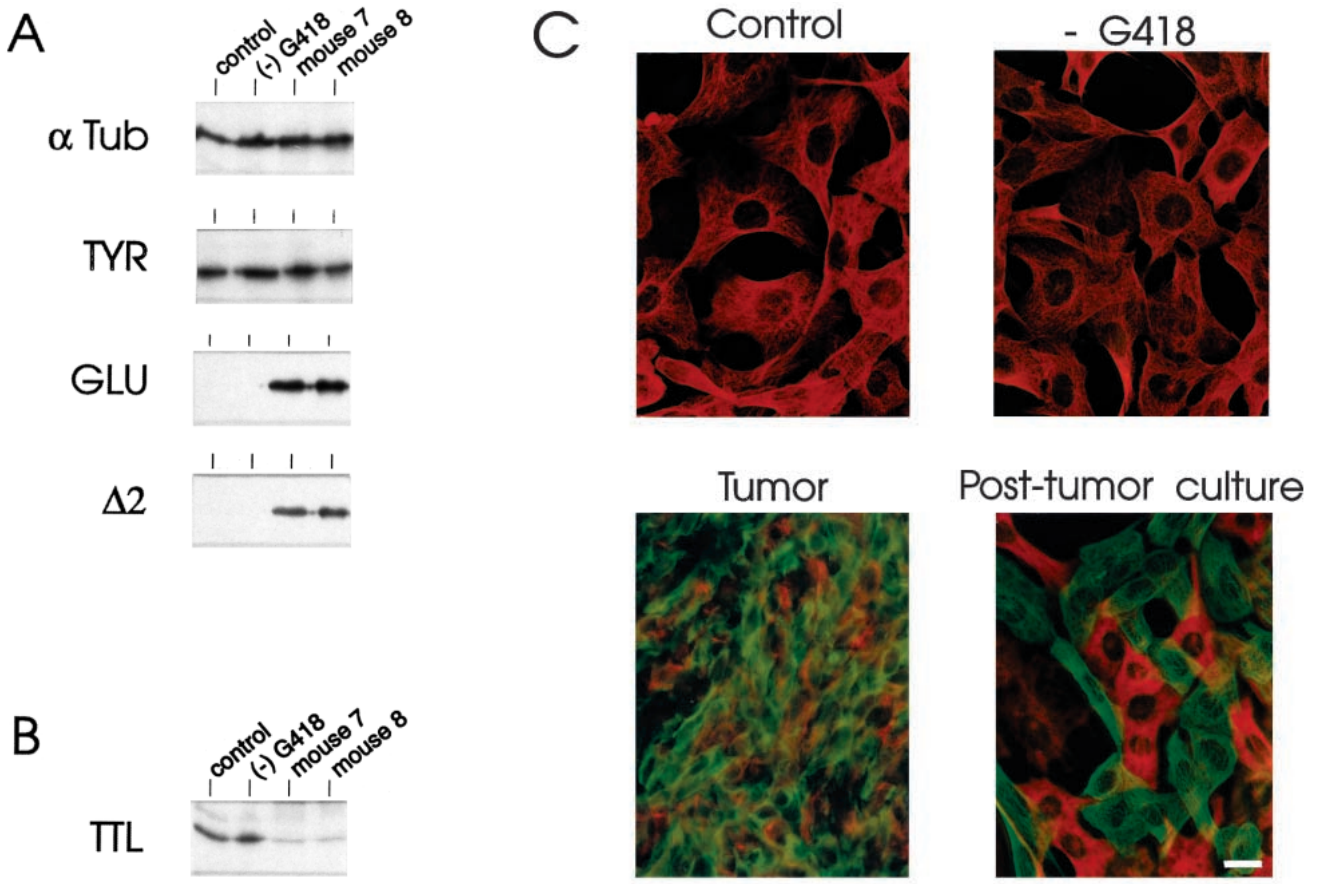


**Fig. 6.** Comparison of tubulin isoform and TTL levels in TTL cDNA-rescued TTL⁻ cells either grown in vitro or grown as sarcomas in situ. (A) TTL cDNA-rescued cells (clone 17F12) were grown either with (control) or without (–G418) geneticin for 6 weeks, and tumors were generated by injection of cells from the same clone (mouse 7 and mouse 8). Samples were then extracted as described in Materials and Methods. Samples (5 μl) were analysed on western blots for total α-tubulin (anti-α-tubulin antibody DM1A), for tyr-tubulin content (monoclonal anti-tyr-tubulin antibody YL1/2), for glu-tubulin content (polyclonal anti-glu-tubulin antibody L3) and for Δ2-tubulin content (polyclonal anti-Δ2-tubulin antibody L7). (B) The same samples were analysed by western blot for TTL content using monoclonal anti-TTL antibody (ID3). (C) Cells of the same clone (17F12) were adhered to coverslips after growth for 6 weeks either with ('Control') or without ('–G418') geneticin. They were then fixed and processed for immunofluorescence as described in Materials and Methods. The tumor sample was obtained from a sarcoma produced by injection of 17F12 cells into a nude mouse. After excision, the tumor samples were either processed immediately for immunofluorescence ('tumor') or dissociated and restored to in vitro culture. The bottom right image ('post tumor culture') shows such cells excised from a tumor then restored to culture for 5 weeks, then processed for immunofluorescence. For analysis, all cell samples were double-labeled with a monoclonal anti-tyr-tubulin antibody and a polyclonal anti-glu-tubulin antibody. Secondary antibodies were TRITC-conjugated anti-rat and FITC-conjugated anti-rabbit, respectively. All pictures were taken at the same magnification and camera settings, using a double filter which allowed simultaneous visualization of both the FITC and TRITC signals. Bar, 20 μm.

phenotype after 5 weeks of continuous culture in the absence of G418 (Fig. 6C, bottom right). We conclude that TTL is specifically lost during tumor growth, even when expressed by plasmid transfection. Its loss thus appears to result from a true selection process during tumorigenesis.

### Loss of TTL and appearance of Δ2-tubulin are common among different human tumor cells

The reversion of cells containing the TTL plasmid to TTL⁻ status was a striking result, and this led us to ask if TTL is suppressed in other tumor cells. To determine the generality of the suppression of TTL during tumor growth, we assayed an established human transformed cell line (Cal 51) both during in vitro culture and following passage through a nude mouse. No mammalian tumor cell that we have assayed has shown suppression of TTL during growth in cell culture. In keeping with this observation, Cal 51 contained normal levels of tyr- and glu-tubulin and no Δ2-tubulin, as determined by western blot (Fig. 7A) or by immunofluorescence analysis (data not shown). However, after introduction into a nude mouse, it generated Δ2-tubulin during in situ growth as a tumor (Fig. 7A), indicative of loss of TTL activity.

Following this result, we assayed a series of human tumor biopsies of various tissue origins. Some of the tumors available to us had been passaged through nude mice, while others were directly obtained in surgery. For these analyses, we used the presence of Δ2-tubulin as a marker for the loss of TTL activity, as the appearance of a signal indicating loss of enzyme activity is much easier to detect than the diminution of enzyme concentration in a mixed cell population. Of the tumors

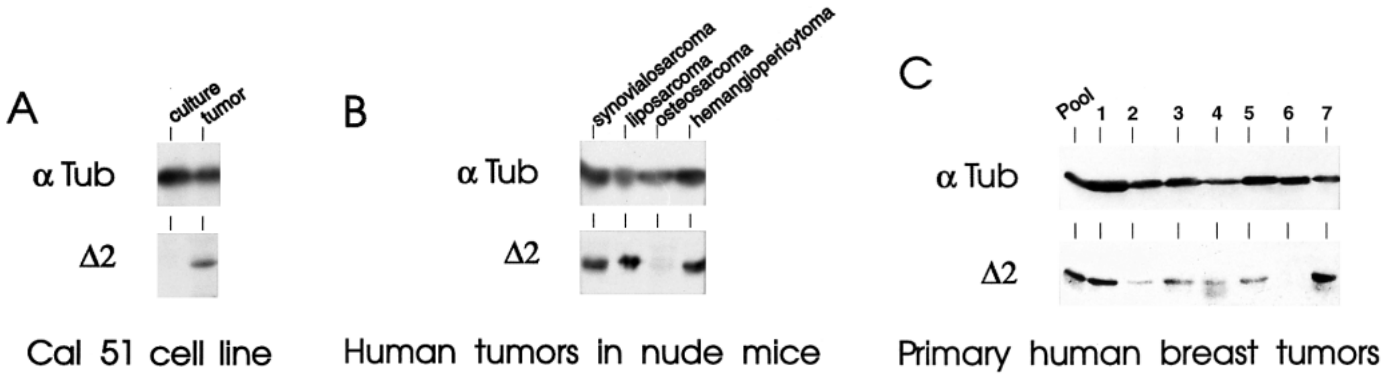


**Fig. 7.** Immunoblot analysis of the Δ2-tubulin content of different human tumors. (A) Cells from a human breast cancer cell line (Cal 51) were either grown in vitro ('culture'), or were injected into a nude mouse to elicit a tumor ('tumor'), then cells were extracted as described in Materials and Methods. 5 μl samples were analysed on western blots for total α-tubulin content and for Δ2-tubulin content, using monoclonal anti-α-tubulin antibody (DM1A) and polyclonal anti-Δ2-tubulin antibody (L7), respectively. (B) Small biopsies from several human tumors were placed in nude mice to elicit tumor growth. Tumor extracts were obtained as described in Materials and Methods, then analysed on western blots for total α-tubulin and for Δ2-tubulin content, as above. (C) Samples (15 μl) of either a pool of cytosols (left lane) or cytosols from individual human breast tumors (one sample in each lane), were analysed by western blot for Δ2-tubulin, as above.

assayed after passage in nude mice, a synovialosarcoma, a liposarcoma, an osteosarcoma and a hemangiopericytoma, all but the osteosarcoma had evident levels of Δ2-tubulin (Fig. 7B). The presence of Δ2-tubulin strongly indicates that a substantial proportion of tumor cells have lost TTL activity.

We have also assayed primary human breast tumors, directly obtained from surgery, for Δ2-tubulin content. We first assayed Δ2-tubulin in a pool of 100 human breast tumor biopsy extracts. Δ2-tubulin was clearly evident in the pool extract (Fig. 7C, left lane), suggesting that it occurs commonly in breast tumors. We have also assayed a panel of biopsies of individual primary human breast tumors for the presence of Δ2-tubulin. The results showed six of seven tumors to be positive. This confirmed that Δ2-tubulin is frequently evident in primary human breast tumors (Fig. 7C), and that TTL is therefore likely to have been suppressed, in at least a substantial subpopulation of the tumor cells.

## DISCUSSION

We have isolated several stable subclonal lines of NIH-3T3 cells that have no tubulin tyrosine ligase activity. While our data show that the loss of TTL activity has no dramatic consequences for cells in culture, our results suggest that TTL activity may be important for the growth of cells within an organism. Both TTL$^-$ cells and TTL$^-$ cells rescued with TTL cDNA form tumors in nude mice, but in the latter case there is a strong selection for cells that have lost TTL activity during tumorigenesis. We have therefore examined a variety of human tumors and have found that the loss of TTL activity is widespread during growth in situ. These results demonstrate that TTL activity is frequently suppressed during tumor growth.

### Δ2-tubulin as a marker of TTL$^-$ cells

In addition to the loss of the C-terminal α-tubulin tyrosine, another recently described post-translational modification of tubulin involves the loss of the two C-terminal amino acids of α-tubulin. Since Δ2-tubulin is absent from all cycling cells where the tubulin tyrosine ligase cycle is normal, and is absent again in TTL$^-$ cells after their rescue by TTL cDNA, we conclude that its presence is the direct result of the suppression of TTL activity. As Δ2-tubulin has come up abundantly in most tumor cells that we tested after growth in situ, the presence of Δ2-tubulin may offer an excellent marker for tumor cells in a background of normal tissue, and may therefore be useful in malignancy screens.

The presence of Δ2-tubulin, representing the specific loss of the C-terminal two amino acids of α-tubulin, is not the product of random proteolysis (Paturle-Lafanechère et al., 1994). It is always associated with tubulin that migrates at a normal mass on gels, and is never observed in cells in culture that contain TTL, nor in extracts from normal tissues, except brain. It therefore serves as a specific marker for the loss of TTL activity. Δ2-tubulin is quite useful as a marker, as it is much easier to see a signal arise in a null background than to directly determine the loss of TTL in a mixed cell population. Further, as Δ2-tubulin is the result of an enzymatic process, the signal representing loss of TTL is greatly amplified.

### Loss of tubulin tyrosine ligase activity during tumor growth

The tumors that arise when TTL$^-$ cells are injected into nude mice are composed of cells with glu- and Δ2-tubulin microtubule arrays, and they are devoid of detectable amounts of TTL. The result is similar for TTL$^-$ cells that have been rescued by TTL cDNA. Nude mouse tumors derived from rescued cells also contain, for the most part, glu- and Δ2-microtubule arrays, and have lower levels of TTL. Control experiments show that accumulation of glu- and Δ2-microtubule arrays is not due to plasmid instability. Further, TTL remains suppressed when tumor cells are explanted and grown in culture. Since the TTL gene is expressed ectopically and is inserted in different locations in the different clones, TTL suppression must be independent of the position of the gene in the genome and of specific promoter function. These results demonstrate that there is strong selection pressure to suppress TTL activity during tumor growth.

An important question that arises from our observations on NIH-3T3 cells concerns the generality of TTL suppression during tumor growth. It is therefore of interest that an established human tumor cell line, Cal 51, exhibited normal levels of TTL activity during growth in culture, but suppressed the tyrosine ligase during tumorigenesis in the nude mouse. Further, of several human tumors that had been amplified in nude mice, all but one of these tumors contained substantial Δ2-tubulin following growth in situ, indicating the absence of TTL activity. Most importantly, almost all human breast tumors surveyed (6/7) showed evidence of TTL suppression. Such data strongly suggest that TTL suppression is an important factor of tumor growth in a variety of situations. Such a possibility may of course open opportunities for tumor prognosis and treatment.

Typically, a tumor suppressor is a protein whose loss of function correlates with tumor induction or facilitates tumor growth (Levine, 1993; Haber and Harlow, 1997). Thus, cells expressing tumor suppressors are subject to strong negative selection during cell transformation and tumor growth. Strong clonal selection to suppress an ectopically expressed protein has been proposed recently as a means to distinguish putative tumor suppressors from other proteins, such as differentiation markers, whose expression is commonly lost in tumor cells (Harris et al., 1996). Such selection against TTL occurs during tumor formation in situ, even when expressed after plasmid transfection. According to this criterion, TTL appears to qualify as a potential tumor suppressor. However, if this is true, it remains to be explained why the initial TTL status does not strongly influence the rate of tumor growth from NIH-3T3 cells in nude mice.

Suppression of TTL may occur through any of several different mechanisms. Indeed, tumor suppressors such as pRb, p53 and keratin 1 can be suppressed by a combination of mechanisms, which may include mutation, transcriptional suppression or specific proteolysis (Levine, 1993; Pahl and Baeuerle, 1996). Whatever mechanisms underlie TTL suppression in tumors from TTL-rescued cells, they must include genetic changes, since we have observed that a high proportion of cells regrown in vitro from such tumors continue to maintain glu- and Δ2-tubulin microtubules.

### Tubulin modification and tumorigenesis

Why should a tubulin modification be implicated in tumorigenesis? One possible explanation comes from accumulating evidence that microtubules play a substantial role in progression through the $G_1$ phase of the cell cycle. For example, normal microtubule dynamics appear to be required for progression of non-transformed fibroblasts through $G_1$ (Trielli et al., 1996). Interference with normal microtubule dynamics by the drug taxol, which induces a microtubule hyperassembly state, causes a stable $G_1$ block. Cell transformation by SV40 large T antigen abolishes the taxol block on cell cycle progression (Trielli et al., 1996).

Additionally, the microtubule assembly state affects tyrosine phosphorylation of focal adhesion plaques (Bershadsky et al., 1996), and proteins central to the control of $G_1$ progression such as pRb, p53 and MAP kinase are associated with the microtubule network (Maxwell et al., 1991; Reszka et al., 1995; Thomas et al., 1996). Microtubules are also implicated in the function of APC, a β-catenin-associated tumor suppressor (Munemitsu et al., 1994; Smith et al., 1994) that is linked to the majority of colon cancers (Polakis, 1995). It is thus apparent that there are several ways that microtubules might play a critical role in $G_1$ progression. Such evidence lends weight to the possibility that elements that modulate microtubules, such as TTL, could play a role in tumor suppression.

Clearly, there is much that needs to be done to understand the capacity of $TTL^-$ cells to form sarcomas and to understand whether suppression of TTL activity is directly related to tumorigenesis. We have conducted a limited survey of primary human breast tumors and have found that almost all suppress TTL activity. A broad survey of human tumors to determine the frequency of TTL suppression according to tumor type and grade is now clearly warranted by our findings.

Evidence presented here clearly indicates a widespread suppression of TTL activity among tumors in situ. Such evidence suggests that TTL might therefore have a tumor suppressor function. Although this is a highly interesting possibility, a firm conclusion cannot be drawn without further work focused on this problem. We will now be most interested to determine if forced TTL expression might specifically inhibit tumor growth, and conversely, if TTL knockout might lead to a high incidence of tumors in transgenic mice.

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