Suppression of nuclear oscillations in Saccharomyces cerevisiae expressing Glu tubulin


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In most eukaryotic cells, the C-terminal amino acid of α-tubulin is aromatic (Tyr in mammals and Phe in Saccharomyces cerevisiae) and is preceded by two glutamate residues. In mammals, the C-terminal Tyr of α-tubulin is subject to cyclic removal from the peptide chain by a carboxypeptidase and readdition to the chain by a tubulin–Tyr ligase. There is evidence that tubulin–Tyr ligase suppression and the resulting accumulation of detyrosinated (Glu) tubulin favor tumor growth, both in animal models and in human cancers. However, the molecular basis for this apparent stimulatory effect of Glu tubulin accumulation on tumor progression is unknown. Here we have developed S. cerevisiae strains expressing only Glu tubulin and used them as a model to assess the consequences of Glu tubulin accumulation in cells. We find that Glu tubulin strains show defects in nuclear oscillations. These defects are linked to a markedly decreased association of the yeast ortholog of CLIP170, Bik1p, with microtubule plus-ends. These results indicate that the accumulation of Glu tubulin in cells affects microtubule tip complexes that are important for microtubule interactions with the cell cortex.

Microtubules are fibrous structures in the cytoplasm of eukaryotic cells that play a vital role in cell organization, motility, and division. Microtubule functions involve cell-cycle-dependent changes in polymer organization and dynamics that rely to a large extent on the biochemical properties of the microtubule building block, the α/β-tubulin heterodimer. Tubulin self-assembles to form different structures (including microtubule tip complexes) that are important for microtubule plus-end dynamics. We propose that similar perturbations in cancer cells may interfere with the cell physiology and favor tumor progression.

Materials and Methods

Yeast Strains and Media. The S. cerevisiae strains and plasmid used are listed in Table 1. The TUB1 and tub1-Glu strains were obtained by using the “plasmid shuffle” technique (5). pRB539Glu was obtained from pRB539 by integration of a stop codon in place of the C-terminal Phe codon in the TUB1 coding sequence.

Integrated strains (iTUB1 and itub1-Glu) were constructed by cloning pBR539 and pRB539Glu (Sac1 and Aat1I) in a pUC18 plasmid (Sac1 and Aat1I). pUC18-TUB1 and pUC18-tub1-Glu were cut with NsiI and used for integration in the TUB1 locus by using the plasmid shuffle technique (5).

Growing Conditions and Antibodies. Cells were grown at 30°C to midlogarithmic growth phase. For the temperature shift experiment, cells were either left at 30°C or shifted to 10°C for 4 days. Growth was monitored by cell counting. Benomyl was supplied by Aldrich.

For Western blotting, we used α-tubulin mAb YOL1/34 (Sera-Lab, Crawley Down, Sussex, U.K.). Intact α-tubulin (Phe tubulin) was detected with mAb YL1/2 (6), Glu tubulin was detected with polyclonal antibody NS8, which was obtained by injecting rabbits with the peptide STAEEEE linked to keyhole limpet hemocyanin (eight injections of 100 μg; Neurosystem, Strasbourg, France). The antibody was affinity-purified on immobilized peptide before use. Carboxypeptidase A comes from Sigma–Aldrich and is used for 10 min at 30°C at a concentration of 2 μg/ml.

Video Microscopy and Quantification. For time-lapse video microscopy examination, cells were mixed with a suspension containing 6 μm of latex beads (10% vol/vol; Polysciences) laid on coverslips and fenced with mineral oil. The coverslips were mounted in a rose chamber covered by another coverslip. Time-lapse Z sequences were collected either on a Leica (Deerfield, IL) or Zeiss microscope controlled by METAMORPH software (Universal Imaging, Media, PA). The acquisition time was 200–500 ms.

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Abbreviations: TTL, tubulin-Tyr ligase; TLP, TTL-like protein.

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Typically, 18–20 sequential z-axis images were collected in 0.3-μm steps every 30 s. Spindle-tracking and fluorescent-counting was performed automatically by METAMORPH software in maximal intensity projections computed from the original three-dimensional datasets.

**Results**

**Analysis of the α-Tubulin C Terminus in Wild-Type and Mutant *S. cerevisiae* Strains.** We tested whether the *TUB1* and *tub1-Glu* clones faithfully expressed the tubulin variant encoded by the plasmid gene or whether tubulin composition was posttranslational modified by T complex protein or TTL activity in these clones. To perform this test, the presence of Phe tubulin and Glu tubulin was assessed in protein preparations from both strains by Western blotting (Fig. 1). As an additional control, a wild-type W303 strain was also analyzed. When the extracts were exposed or not to carboxypeptidase A (CPA) before immunoblotting, the Phe–tubulin signals were abolished, whereas strong signals were observed with Glu tubulin antibody (Fig. 1, row CPA). These results indicate that *TUB1* and *tub1-glu* clones express only Phe tubulin and Glu tubulin, respectively, and suggest that there is neither T complex protein nor TTL activity in *S. cerevisiae* in our experimental conditions. However, a putative TTL-like protein (TLP), YBR094W/TLP1, has been identified in *S. cerevisiae* (8). To test whether this TTL-related protein had TTL-like activity, we cloned the YBR094W/TLP1 gene in a high-copy number plasmid (2μ TLP1 construct). The overexpression of the corresponding protein was checked by Western blotting (data not shown). Such overexpression did not induce detectable amounts of Phe tubulin in *tub1-Glu* strains (Fig. 1, lane *tub1-Glu + 2μ TLP1*). Thus, despite structural homology with the mammalian TTL, the YBR094W/TLP1 protein is apparently devoid of tubulin–Phe ligase activity.

**tub1-Glu Strain Shows Benomyl Supersensitivity and Growth Defect.** Supersensitivity to the microtubule depolymerizing drug benomyl is a common occurrence in both α-tubulin and β-tubulin mutants (5). Sensitivity of the *TUB1* and *tub1-Glu* cells to benomyl was tested by spotting fixed numbers of cells on rich plates containing different concentrations of the drug (15–25 μg/ml) (Fig. 2A) and examining cell growth. In the presence of benomyl, cell growth impairment was markedly enhanced in *tub1-Glu* compared with *TUB1* cells, indicating supersensitivity to benomyl in cells expressing Glu tubulin. Supersensitivity to benomyl can be indicative of an α-tubulin cold-sensitive mutation (4). Therefore, we examined cell growth in *tub1-Glu* and *TUB1* strains at both warm and cold temperatures (Fig. 2B). The two strains showed similar growth kinetics at 30°C. In contrast, cell growth was substantially impaired in the *tub1-glu* strain at 10°C (Fig. 2B). The same cells also showed impaired viability at 10°C compared with controls (data not shown). These results indicate cold-sensitive impairment of cell growth and viability in *tub1-Glu* strain.

**Delay in Anaphase Onset in tub1-Glu Strains.** We used both direct cell observation and flow cytometry analysis to test whether the impaired cell growth observed in *tub1-Glu* strains at 10°C was related to defects at a particular stage of the cell cycle. Direct observation showed an accumulation of cells with a large bud in *tub1-glu* clones compared with controls (Fig. 2C) and an accumulation of cells with replicated DNA in flow cytometry analysis (data not shown), suggesting a mitotic block in *tub1-Glu* cells.

We tested whether the block occurred before or after nuclear division. At permissive temperature, ∼90% of the cells with large buds had two nuclei (one in the mother cell and one in the bud) in *TUB1* and *tub1-Glu* cells (Fig. 2D). In contrast, at restrictive temperature, the two clones differed strikingly, with

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**Table 1. Yeast strains and plasmids**

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<th>Strains</th>
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<td><em>tub1-Glu</em></td>
<td><em>TUB1</em> except pRB539Glu</td>
<td>This study</td>
</tr>
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<td><em>iTUB1</em></td>
<td>MATa/α ade4/ADE4, his3-200/his3-200, leu2-3.112/leu2-3.112, ura3-52/ura3-52, lys2-801, <em>tub1</em>:HIS3/tub1*:HIS3, promtub1*:TUB1-LEU2/promtub1*:TUB1-LEU2, tub3*:TRP1/tub3*:TRP1</td>
<td>This study</td>
</tr>
<tr>
<td><em>itub1-Glu</em></td>
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<td>This study</td>
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<td><em>W303</em></td>
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<td><em>TUB1</em> LEU2 CEN</td>
<td>Ref. 5</td>
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<tr>
<td>pRB539Glu</td>
<td>tub1-Glu LEU2 CEN</td>
<td>This study</td>
</tr>
<tr>
<td>p81225</td>
<td>BIM1-GFP URA3 CEN</td>
<td>Ref. 6</td>
</tr>
<tr>
<td>p8681</td>
<td>BIK1-GFP URA3 2μ</td>
<td>Ref. 7</td>
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**Fig. 1.** Analysis of the α-tubulin C terminus in control and mutant strains. Western blotting of whole-cell extracts from *TUB1* and *tub1-Glu*, expressing or not the Tlp1p protein (2μ TLP1) and W303 strains. Extracts were exposed or not to carboxypeptidase A (CPA) before immunoblotting. Primary antibodies were directed against total α-tubulin (YOL34), Phe tubulin (YLT1/2), or Glu tubulin (NDB) as indicated.
80% of the cells with two nuclei in control cells, compared with only 30% in the tub1-Glu cells. The remaining tub1-Glu cells contained a single nucleus located in the mother cell. These results indicate a delay in anaphase onset in a large proportion of tub1-Glu cells at restrictive temperature.

Inhibition of Nuclear Oscillations in Glu Tubulin Strains. Blockage in mitosis before nuclear division has been observed in situations in which the nucleus never enters the bud in anaphase because of abnormal nucleus positioning or motion (9–11). These data prompted us to examine nuclear behavior in cells expressing either Phe tubulin or Glu tubulin. In the experiments reported in the following sections, the spindle and the microtubule plus-ends were labeled by expressing either GFP-Bim1p or Bik1p fusion protein in yeast cells (12, 13). Nuclear oscillation and microtubule end-labeling were examined with similar results in strains with plasmid (TUB1 and tub1-Glu) and integrated (iTUB1 and itub1-Glu) tubulin genes.

In pilot experiments using cells expressing GFP-Bim1p before and during mitosis, we found no obvious difference between Phe tubulin or Glu tubulin. In the experiments reported in the following sections, the spindle and the microtubule plus-ends were labeled by expressing either GFP-Bim1p or Bik1p fusion protein in yeast cells (12, 13). Nuclear oscillation and microtubule end-labeling were examined with similar results in strains with plasmid (TUB1 and tub1-Glu) and integrated (iTUB1 and itub1-Glu) tubulin genes.

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expressing either GFP-Bim1p or GFP-Bik1p, suggesting that differences between Phe tubulin and Glu tubulin cells did not depend on the microtubule end-marker used for analysis (Table 2).

Microtubule Behavior in Anaphase Phe Tubulin and Glu Tubulin Cells.

Nuclear oscillations involve the guidance of microtubules toward the bud cortex, followed by the capture of clustered microtubule plus-ends at the cortex, with subsequent microtubule sliding and spindle pulling. Such events were observed in Phe tubulin clones expressing GFP-Bim1p (Fig. 4 Top) (14). By contrast, in GFP-Bim1p expressing Glu tubulin cells, microtubule orientation often remained apparently random during the period of observation (Fig. 4 Middle). The spindle seemed subject to noncoordinated forces resulting in no significant movement (Fig. 4 Bottom). In some cells, a preferential orientation to the bud was observed, but microtubule plus-ends did not display clear clustering as in Phe tubulin cells (Fig. 4 Bottom). Although microtubule plus-ends could reach the bud cortex, microtubule sliding and spindle migration in the bud through the neck was a rare event.

Qualitatively similar observations were made with GFP-Bik1p-expressing cells (Fig. 5). However, in addition to reduced spindle motion, major differences in the distribution of GFP-Bik1p were observed between Phe tubulin and Glu tubulin cells. In Phe tubulin cells, microtubule ends were intensely labeled with GFP-Bik1p, which features bundled microtubule ends and long-trailing comets (Fig. 5 Top). In contrast, even in the rare cells showing significant spindle motion, the labeling of microtubule ends with GFP-Bik1p was barely detectable in Glu tubulin cells, and comets were absent or evanescent (Fig. 5 Middle and Bottom).

In quantitative analysis, spindle labeling was similar in Glu

Table 2. Quantitative analysis of spindle motion

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum speed, ( \mu m/min )</th>
<th>Minimum speed, ( \mu m/min )</th>
<th>Average speed, ( \mu m/min )</th>
<th>Maximum amplitude, ( \mu m )</th>
<th>Minimum amplitude, ( \mu m )</th>
<th>Average amplitude, ( \mu m )</th>
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<tbody>
<tr>
<td>iTUB1 GFP-Bim1p</td>
<td>0.8 ± 0.16</td>
<td>0.014 ± 0.004</td>
<td>0.220 ± 0.05</td>
<td>3.2 ± 0.76</td>
<td>0.14 ± 0.06</td>
<td>1.34 ± 0.2</td>
</tr>
<tr>
<td>iTUB1 GFP-Bik1p</td>
<td>0.7 ± 0.05</td>
<td>0.014 ± 0.003</td>
<td>0.211 ± 0.001</td>
<td>2.9 ± 0.27</td>
<td>0.24 ± 0.05</td>
<td>1.45 ± 0.2</td>
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<tr>
<td>itub1-Glu GFP-Bim1p</td>
<td>0.4 ± 0.05</td>
<td>0.006 ± 0.001</td>
<td>0.095 ± 0.009</td>
<td>1.3 ± 0.27</td>
<td>0.06 ± 0.01</td>
<td>0.63 ± 0.17</td>
</tr>
<tr>
<td>itub1-Glu GFP-Bik1p</td>
<td>0.4 ± 0.06</td>
<td>0.006 ± 0.001</td>
<td>0.111 ± 0.001</td>
<td>1.9 ± 0.40</td>
<td>0.07 ± 0.01</td>
<td>0.56 ± 0.2</td>
</tr>
</tbody>
</table>

Spindle position for all filmed cells was determined at 30-s intervals by using METAMORPH software. The speed and amplitude of spindle motions were derived from positional data by using the same software. The spindle speed was calculated between two successive time points as the ratio of displacement/elapsed time. Amplitudes were measured at each time point as the distance of the spindle center from its original position at \( t = 0 \). Data are shown ± SEM.

Fig. 5. Video microscopy analysis of spindle and microtubule behavior in Phe tubulin and Glu tubulin strains expressing GFP-Bik1p. (Top) Phe tubulin GFP-Bik1p strain during a nuclear oscillation. Arrows indicate spindle. Arrowheads indicate microtubules plus-ends. With GFP-Bik1p, long comets were observed on the bundled microtubules. Such comets remained conspicuous during microtubule capture and sliding at the cell cortex. (Middle) One aspect observed in Glu microtubule GFP-Bik1p strains. Arrows indicate the spindle. Microtubules apparently lack directional cues. Microtubule plus-ends are poorly labeled with GFP-Bik1p, and no comet is visible. (Bottom) Glu tubulin GFP-Bik1p strain during a nuclear oscillation. Arrows indicate spindle with a translocation event. Arrowheads indicate microtubules plus-ends. Nuclear oscillations were rarely observed in Glu tubulin strains (Fig. 3B). During such oscillations, the GFP-Bik1p labeling remained conspicuously different from controls, with barely apparent and evanescent GFP-Bik1p comets and poorly labeled microtubule ends. Times when images are captured are indicated. (Bar, 2 \( \mu m \)).
tubulin and Phe tubulin cells, whether GFP-Bim1p or GFP-Bik1p was used as a marker (Fig. 6A). The ratio of GFP-Bim1p fluorescence in the microtubule ends to GFP-Bim1p in the spindle was slightly higher in Glu tubulin cells compared with Phe tubulin cells. A similar analysis with GFP-Bik1p showed a dramatic 3-fold decrease of the microtubule ends/spindle fluorescence ratio in Glu tubulin cells compared with Phe tubulin cells (Fig. 6B). Taken together, these results indicate major and specific defects in the interaction of Bik1p with microtubule ends in Glu tubulin cells.

**Discussion**

**Absence of Cyclic Modification of the C-Terminal α-Tubulin Amino Acid in S. cerevisiae.** The tyrosination cycle is highly conserved among eukaryotes and has hitherto been found in most cells where it has been searched for, with the exception of the fission yeast, *Schizosaccharomyces pombe* (15). In *S. cerevisiae*, the C-terminal amino acid of α-tubulin is a Phe, not a Tyr, but the two amino acids are similar, and both can be added to the tubulin molecule by the mammalian TTL (7). Additionally, *S. cerevisiae* contains a TTL-related protein that has been identified based on sequence similarities and structural homology with the mammalian TTL (8). The existence of a phenylalanination–dephenylalanination cycle in *S. cerevisiae* was therefore a definite possibility. However, in this study, we found that tubulin remains fully Phe in TUB1 cells and fully Glu in tub1-Glu cells, in the presence or absence of overexpressed TTL-related protein. This finding strongly indicates that there is no turnover of the α-tubulin C-terminal Phe in budding yeast. The absence of TTL activity allowed a direct assay of the role of the C-terminal Phe of α-tubulin, by comparing strains containing either only Phe tubulin or only Glu tubulin.

**Nuclear Oscillation Defects in Glu Tubulin Strains.** In our study, expression of Glu tubulin instead of Phe tubulin in *S. cerevisiae* cells resulted in a severe impairment of nuclear motion through the neck into the bud, probably explaining the delay in anaphase onset observed at restrictive temperature in Glu tubulin cells. Other cold-sensitive tubulin mutations, including *tub2–401*, induce a defect in anaphase onset (16, 17). In *tub2–401* clones the mitotic block affects all cells uniformly. In contrast, a mitotic block is observed in only a proportion of *tub1–Glu* cells, may be because nuclear motion through the neck is impaired but not completely impossible in such cells.

Nuclear movements in yeast involve nuclear migration toward the bud, alignment and retention of the spindle at the neck, and nuclear oscillations through the aperture of the budded cell at anaphase onset. Motor proteins play a key role in this process by means of the regulation of microtubule dynamics and generation of pushing or pulling forces along astral microtubules (dynein, ref. 18; Kip2p, ref. 19; Kip3p, ref. 20; and Kar3p, ref. 21).

Several spindle polarity determinants, including septin ring, Kar9p, Bni1p, and Kip3p, provide positional cues for directing the nucleus to the neck (18, 22–24). A cytoplasmic dynein-dependent pathway is responsible for microtubule pulling at the cell cortex during the pronounced spindle oscillations at the neck of a budded cell and contributes to the forces required to pull the nucleus through the aperture between mother cell and bud (18, 25, 26). In *tub1–Glu* and *tub1–Glu* cells, nuclear positioning to the neck did not show obvious alterations, whereas microtubule pulling, nuclear oscillations, and nucleus migration into the bud were drastically reduced. These reduced spindle movements were associated with a dramatic decrease of Bik1p association with microtubule ends. Such a reduction could have several origins, including (i) a reduced rate of microtubule polymerization (27) or a decrease in microtubule number (27) in Glu tubulin cells, (ii) detection bias linked to differences in microtubule bundling between Phe tubulin and Glu tubulin cells, or (iii) intrinsic anomalies in the Bik1p interaction with Glu microtubule ends. Differences in microtubule growth rates or number and detection bias would, however, similarly affect the Bim1p and Bik1p signals at microtubule ends. The comparable Bim1p signals observed between Phe microtubule ends and Glu microtubule ends are strong indications that the decrease of Bik1p fluorescence at microtubule ends in Glu tubulin strains corresponds to a bona fide depletion of Bik1p at Glu microtubule tips. Bik1p is the yeast ortholog of CLIP170, a protein known to be a key factor for dynein-dependent microtubule capture and pulling at the cell cortex (28–30). Therefore, it seems logical that a perturbation of Bik1p association with microtubule ends perturbs nuclear oscillations, known to be dynein-dependent. In addition to apparent perturbation of microtubule pulling at the cortex, microtubules in Glu tubulin cells spend most of the time in disorganized arrays, lacking a preferential orientation. Such aster-like organization can be observed in Phe tubulin strains but is generally short-lived. CLIP170 is known to be required for polarization of microtubule array in epithelial cells (31). Bik1p may be similarly required for the directional control of microtubules in yeast.

Whereas Bik1p is depleted at microtubule ends in Glu tubulin cells, its association with spindle microtubules is apparently normal in the same cells, suggesting that the absence of the C-terminal Phe residue does not directly impair Bik1p interaction with microtubules. Apparently, the mammalian ortholog of Bik1p, CLIP170, has several modes of interaction with microtubules. *In vitro*, CLIP170 behaves as a classical microtubule-associated protein interacting with the microtubule lattice (32, 33). It seems likely that Bik1p has similar microtubule-associated protein-like behavior and that this accounts for the strong staining of spindle microtubules with Bik1p. The microtubule end-tracking behavior of CLIP170 *in vivo* is not fully understood: it apparently requires recognition of a specific feature of the microtubule distal end and subsequent release from an older, more proximal part of the microtubule (27). Such a complex
sequence of binding and dissociation events has not been observed in vitro as yet, indicating that it may require cell regulators. Microtubule tip complexes contain signaling proteins, such as Glu proteins or G protein exchange factors (31, 34). The composition of the tip complex may be regulated by a signaling cascade, affecting the intrinsic microtubule binding activity of tip components. Our results may reflect an involvement of the α-tubulin C terminus in such signaling cascades.

From Yeast to Cancer? In mammalian cells, CLIP170 is apparently crucial for microtubule interactions with the dynein–dynactin cortical complexes and for resulting correct spindle orientation (35). CLIP170 is also associated with kinetochores and is probably central for the dynein-dependent morphogenesis of the spindle (36, 37). If our observations in yeast apply to mammalian tubulin, several dynein-dependent functions may be affected by tubulin detyrosination in mammalian cells, and this would obviously increase the probability of genomic instability. This study identifies dynein complexes as possible molecular targets affected by the accumulation of Glu tubulin and opens new clues to elucidate the mechanisms through which the tyrosination cycle may interfere with the cell physiology and favor tumor growth.

We thank Dr. Frank Solomon for his generous gift of plasmids and strains, Dr. Jennifer Tirnauer for providing the GFP-Bim1p plasmid, Dr. David Pellman for providing the GFP-Bik1p plasmids, and Dr. Yasmina Saoudi for help in imaging. This work was supported in part by a grant (to D.J.) from la Ligue Nationale contre le Cancer.