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The two α-tubulin isotypes in budding yeast have opposing effects on microtubule dynamics *in vitro*

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The yeast *Saccharomyces cerevisiae* has two genes for α-tubulin, *TUB1* and *TUB3*, and one β-tubulin gene, *TUB2*. The gene product of *TUB3*, Tub3, represents ~10% of α-tubulin in the cell. We determined the effects of the two α-tubulin isotypes on microtubule dynamics *in vitro*. Tubulin was purified from wild-type and deletion strains lacking either Tub1 or Tub3, and parameters of microtubule dynamics were examined. Microtubules containing Tub3 as the only α-tubulin isotype were less dynamic than wild-type microtubules, as shown by a shrinkage rate and catastrophe frequency that were about one-third of that for wild-type microtubules. Conversely, microtubules containing Tub1 as the only α-tubulin isotype were more dynamic than wild-type microtubules, as shown by a shrinkage rate that was 50% higher and a catastrophe frequency that was 30% higher than those of wild-type microtubules. The results suggest that a role of Tub3 in budding yeast is to control microtubule dynamics.

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INTRODUCTION

Although all microtubules are composed of α/β-tubulin heterodimers, mammalian microtubules are highly heterogeneous with regard to the α- and β-monomers. Mammalian species, for example, contain multiple genes encoding both α- and β-tubulin monomers, and each monomer can undergo several different post-translational modifications (for review see Ludueña, 1998). The mammalian β-tubulin isotype composition affects microtubule dynamics *in vitro* (Panda *et al.*, 1994) and *in vivo* (Gonçalves *et al.*, 2001). Similarly, the isotype composition affects the interaction of tubulin with antimitotic drugs (Banerjee & Ludueña, 1992; Derry *et al.*, 1997; Khan *et al.*, 2000) and the stability of the tubulin heterodimer (Schwarz *et al.*, 1998). In contrast to mammalian species, the budding yeast *Saccharomyces cerevisiae* has only two α-tubulin-encoding genes, *TUB1* and *TUB3* (Schatz *et al.*, 1986a), and a single β-tubulin encoding gene, *TUB2* (Neff *et al.*, 1983). Because of this genetic simplicity, yeast provides an opportunity for studying the influence of the α-tubulin isotypes on microtubule structure and function.

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The sequences of the two yeast α-tubulin isotypes exhibit ~90% identity (Schatz *et al.*, 1986a). In addition, both genes have a single intron sequence located at the same codon and are located on chromosome XIII (Schatz *et al.*, 1986a). With the use of green fluorescent protein (GFP) fusion constructs it has been shown that both gene products are incorporated into cytoplasmic and nuclear microtubules (Straight *et al.*, 1997; Carminati & Stearns, 1997). Yeast cells can tolerate a 30-fold overexpression of *TUB1* or *TUB3* without a significant effect on cellular phenotypes (Weinstein & Solomon, 1990). Deletion of *TUB1* is lethal because it provides ~90% of the cell's α-tubulin. *TUB3* can support viability in the absence of *TUB1* if it is expressed at a sufficiently high concentration (Schatz *et al.*, 1986b). Because increased expression of *TUB3* can compensate for the deleterious effects of *TUB1* disruption, it has been concluded that these two proteins function interchangeably (Schatz *et al.*, 1986b). A similar result has been reported for fission yeast, which also has two genes encoding α-tubulin (Adachi *et al.*, 1986). Although the disruption of *TUB3* has minimal cellular effects, *TUB3*-null cells do show increased sensitivity to the microtubule-destabilizing compound benomyl and decreased spore viability (Schatz *et al.*, 1986b; C.J.B., M.L.G. and R.H.H., unpublished observations).

To investigate the contribution of each α-tubulin isotype to yeast microtubule properties, we purified tubulin from a wild-type strain containing both α-tubulin isotypes (~90% Tub1 and ~10% Tub3) and from strains containing only a single α-tubulin isotype, either Tub1 or Tub3. We examined the dynamic behaviour of microtubules assembled from each of these tubulin preparations. Interestingly, Tub1 increased microtubule dynamics, whereas Tub3 decreased microtubule dynamics.

RESULTS

Purification and assembly of yeast tubulin

Using Ni-affinity chromatography (Gupta *et al.*, 2002), we obtained tubulin that was homogeneous on Coomassie-stained SDS–polyacrylamide gel electrophoresis (PAGE) gels (Fig. 1A). Interestingly, the α- and β-subunits separated on the gels when 99% pure SDS was used but not when impure SDS was used (compare lanes 1 and 5). In contrast, brain tubulin shows good resolution of the two subunits on SDS–PAGE gels only when the SDS is contaminated with sodium tetradecyl and hexadecyl sulphates (Best *et al.*, 1981) (compare lanes 4 and 6 in Fig. 1A). In SDS–PAGE gels of yeast tubulin the α-subunits have slightly greater mobilities than the β-subunit. This is opposite to

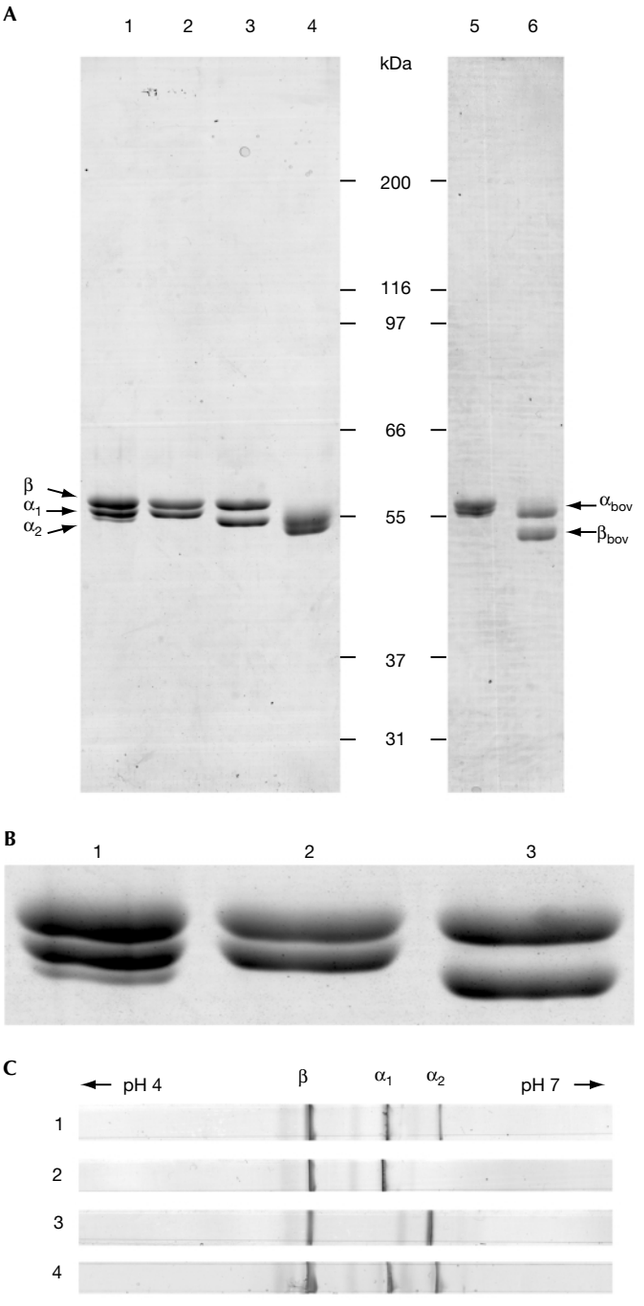

Fig. 1 | SDS–PAGE and isoelectric focusing of yeast tubulin. (**A**) Coomassie-blue-stained SDS–PAGE gels. Tubulin (2 μg) was loaded onto each lane. Lanes 1–4 contained 99% pure SDS (cat. no. L3771; Sigma-Aldrich, St Louis, MO) and lanes 5 and 6 contained impure SDS (cat. no. L5750; Sigma-Aldrich). Lanes 1 and 5, wild-type tubulin; lane 2, Tub1/Tub2 tubulin; lane 3, Tub3/Tub2 tubulin; lanes 4 and 6, bovine brain tubulin. In lane 1 α_1 refers to Tub1 and α_2 refers to Tub3. (**B**) Expanded view of lanes 1–3 from (**A**). (**C**) Isoelectric focusing. Lane 1, wild-type tubulin (15 μg); lane 2, Tub1/Tub2 tubulin (15 μg); lane 3, Tub3/Tub2 tubulin (15 μg); lane 4, wild-type (15 μg) and Tub3/Tub2 (7.5 μg) tubulin.

what is observed with brain tubulin but is similar to what is observed with tubulin from *Tetrahymena thermophila* (Suprenant *et al.*, 1985). Figure 1A and B shows that Tub1 and Tub3 have slightly different mobilities (compare lanes 1 and 3), an observation also made with tubulin from fission yeast (Adachi *et al.*, 1986).

To confirm that purified protein obtained from each strain contained the correct α-tubulin isotypes, we performed isoelectric focusing (Fig. 1C). Previously, Barnes *et al.* (1992) identified the position of each tubulin monomer in the isoelectrically focused gel on the basis of theoretical pI values and amino-terminal sequencing. Consistent with their findings was our observation of two prominent bands and one minor band when wild-type tubulin was focused (Fig. 1C, lane 1). The minor band in wild-type tubulin with the highest pI value corresponds to Tub3. In contrast, there were only two bands when tubulin from the *TUB1*-null and *TUB3*-null strains were focused (Fig. 1C, lanes 2 and 3). The Tub3 bands in lanes 1 and 3 do not align exactly; this could be due to experimental artefacts or to differences in post-translational modifications. To differentiate between these two possibilities we focused a mixture of tubulin from the wild-type and *TUB1*-null strains (Fig. 1C, lane 4). In this case only one Tub3 band was observed, indicating that there are no differences in Tub3 between the two strains.

The critical tubulin concentration for assembly refers to the concentration below which tubulin assembly does not occur. The value reflects the efficiency of the protein to self-assemble into polymers. The critical concentration of tubulin from each strain was determined by measuring the amount of polymer assembled from increasing concentrations of tubulin. Previously, Davis *et al.* (1993) reported that yeast tubulin has a critical concentration that is ~20% of that for bovine brain tubulin. Consistent with these results was our finding of a critical concentration of 0.18 mg ml^{-1} for wild-type tubulin, 0.21 mg ml^{-1} for Tub1/Tub2 tubulin, and 0.13 mg ml^{-1} for Tub3/Tub2 tubulin (Table 1). Although DMSO stimulates the assembly of brain tubulin by lowering the critical concentration, it had little or no effect on the assembly of yeast tubulin. Electron microscopy confirmed that the three different tubulins assembled into microtubules (data not shown).

Effects of α-isotypes on microtubule dynamics *in vitro*

The term microtubule dynamics describes the ability of these polymers to grow, shorten or pause (attenuation), while maintaining constant total polymer mass (Walker *et al.*, 1988). Microtubules at steady state of polymer mass undergo transitions from growth or attenuation to shortening (a catastrophe) and from shortening to attenuation or growth (a rescue). The dynamics of yeast microtubules nucleated from *Chlamydomonas* axonemes were monitored in real time by video-enhanced differential interference contrast microscopy. (Nucleation from axonemes allows one to identify the plus (growing) end of the microtubule.) Microtubules containing exclusively either Tub1/Tub2 or Tub3/Tub2 heterodimers were compared with wild-type microtubules. There were similarities in the dynamic properties of the three types of microtubule (Table 1). For example, the average growth rates for the microtubules were indistinguishable. In all cases individual microtubules spent most of the time growing (99% of the total time) until experiencing rapid and catastrophic disassembly. The percentage of the time spent shrinking was equally brief for all classes of microtubules (0.4–0.7%). In addition, the microtubules did not exhibit attenuated events (periods in which neither growth nor shortening was observed).

Table 1 | Microtubule dynamics data

Yeast strains	MGY1[a]	BGY1	MGY3
Tubulin composition			
α isotype present	Tub1, Tub3	Tub1	Tub3
β isotype present	Tub2-His_6	Tub2-His_6	Tub2-His_6
C_i^b (mg ml^{-1})	0.18	0.21	0.13
Growth rate (μm h^{-1})	10.6 ± 1.9 (32)[c]	10.8 ± 1.2 (20)	11.2 ± 1.9 (21)
Shrinkage rate (μm min^{-1})	103 ± 18 (22)[d]	159 ± 15 (20)	36 ± 6 (14)
Catastrophe (min^{-1})	0.046 ± 0.010 (20)	0.060 ± 0.014 (19)	0.021 ± 0.007 (9)
Rescue (min^{-1})	<0.0023[e] (0)	<0.0032[e] (0)	0.0024 ± 0.002 (1)
Dynamicity (dimer s^{-1})	16.6	23.0	12.3
Percentage of time spent in			
growth phase	99.6	99.6	99.3
shrinkage phase	0.40	0.40	0.70
attenuated phase	0.00	0.00	0.00
Total time (min)	431	315	420

[a]MGY1 data previously reported in Gupta *et al.* (2002).
[b]Critical protein concentration.
[c]Results are reported as means ± s.d.; numbers of events are in parenthesis.
[d]Shrinkage events exceed catastrophes owing to brief pauses during disassembly.
[e]No rescue events were observed for MGY1 and BGY1.

However, there were also significant differences between the three types of microtubule (Table 1). The shrinkage rate was 1.5-fold greater for Tub1/Tub2 microtubules than for wild-type microtubules ($P < 0.0001$) and 4.4-fold greater than for Tub3/Tub2 microtubules (Table 1). The shrinkage rate for Tub3/Tub2 microtubules was about one-third of that for wild-type microtubules (Table 1). Data for the shrinkage phase of representative microtubules are presented in Fig. 2. The frequency of catastrophe was 1.3-fold greater for Tub1/Tub2 microtubules than for wild-type microtubules ($P < 0.001$) and 3-fold greater than for Tub3/Tub2 microtubules. Wild-type and Tub1/Tub2 microtubules did not display rescue events. The absence of rescue events in yeast microtubules *in vitro* has been observed previously (Davis *et al.*, 1993; Gupta *et al.*, 2002). However, we did observe one rescue event with Tub3/Tub2 microtubules. Although this is obviously a low frequency, it does seem that the lower shrinkage rate of these microtubules might increase the opportunity for rescue to occur. The effects of the α-tubulin isotype are apparent in the dynamicity term, a parameter that reflects the overall rate of tubulin exchange at the end of the microtubules (Toso *et al.*, 1993). Dynamicity for Tub1/Tub2 microtubules was 1.9-fold greater than that for Tub3/Tub2 microtubules (23 dimers s^{-1} compared to 12.3 dimers s^{-1}).

DISCUSSION

The effect of Tub3 on the dynamics of wild-type microtubules indicates that a small proportion of a tubulin isotype in a microtubule is sufficient to affect dynamic parameters. The results indicate that the minor α-tubulin isotype Tub3 contributes to the intrinsic stability of yeast microtubules *in vitro* by decreasing the rate of depolymerization and by decreasing the frequency of catastrophe. Similar observations have been made with bovine β-tubulin. Panda *et al.* (1994) analysed the dynamic properties of microtubules composed of a mixture of heterogeneous α-tubulin isotypes combined with specific β-tubulin isotypes, and found that microtubules composed of the $\alpha\beta_{III}$ isotype were twice as dynamic as those composed of either the $\alpha\beta_{II}$ or the $\alpha\beta_{IV}$ isotype. Unfortunately, the contribution of the several mammalian α-tubulin isotypes has not been examined. Because yeast microtubules contain only one β-subunit, the contributions of specific α-isotypes can be determined.

The observation that such a small content of Tub3 (~10% of α-tubulin) can have pronounced effects on microtubule dynamic instability is similar to the effects of anti-mitotic agents on microtubule dynamics. For example, at substoichiometric concentrations, Taxol has been shown to dampen microtubule dynamics (Derry *et al.*, 1995). Additionally, agents that normally promote microtubule disassembly, including colchicine and vinblastine, have been shown to decrease microtubule dynamics at very low concentrations (Jordan, 2002). In fact, it has also been suggested

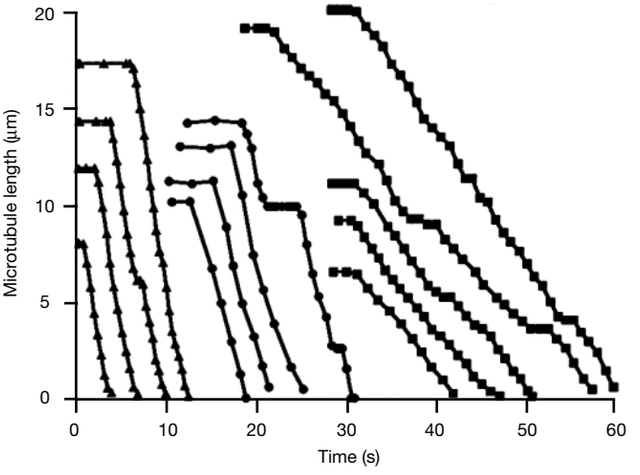

Fig. 2 | Catastrophic disassembly of representative microtubules. Only the disassembly portions of microtubule lifetimes are shown. Data from individual Tub1/Tub2 microtubules (triangles), wild-type microtubules (circles); and Tub3/Tub2 microtubules (squares) are shown.

```
          1                                                                  70
Tubl      MREVISINVGQAGCQIGNACWELYSLEHGIKPDGHLEDGLSKPKGGEEGFSTFFHETGYGKFVPRAIYVD
Tub3      MREVISINVGQAGCQIGNACWELYSLEHGIKEDGHLEDGLSKPKGGEEGFSTFFHETGYGKFVPRAIYVD
         71                                                                 140
Tubl      LEPNVIDEVRNGPYKDLFHPEQLISGKEDAANNYARGHYTVGREILGDVLDRIRKLADQCDGLQGFLFTH
Tub3      LEPNVIDEVRTGRFKELFHPEQLINGKEDAANNYARGHYTVGREIVDEVEERIRKMADQCDGLQGFLFTH
        141                                                                 210
Tubl      SLGGGTGSGLGSLLLEELSAEYGKKSKLEFAVYPAPQVSTSVVEPYNTVLTTHTTLEHADCTFMVDNEAI
Tub3      SLGGGTGSGLGSLLLENLSYEYGKKSKLEFAVYPAPQLSTSVVEPYNTVLTTHTTLEHADCTFMVDNEAI
        211                                                                 280
Tubl      YDMCKRNLDIPRPSFANLNNLIAQVVSSVTASLRFDGSLNVDLNEFQTNLVPYPRIHFPLVSYSPVLSKS
Tub3      YDICKRNLGISRPSFSNLNGLIAQVLSSVTASLRFDGSLNVDLNEFQTNLVPYPRIHFPLVSYAPILSKK
        281                                                                 350
Tubl      KAFHESNSVSEITNACFEPGNQMVKCDPRDGKYMATCLLYRGDVVTRDVQRAVEQVKNKKTVQLVDWCPT
Tub3      RAIHESNSVSEITNACFEPGNQMVKCDPTKGKYMANCLLYRGDVVTRDVQRAVEQVKNKKTVQMVDWCPT
        351                                                                 420
Tubl      GFKIGICYEPPTATPNSQLATVDRAVCMLSNTTSIAEAWKRIDRKFDLMYAKRAFVHWYVGEGMEEGEFT
Tub3      GFKIGICYEPPSVIPSSELANVDRAVCMLSNTTAIADAWKRIDQKFDLMYAKRAFVHWYVGEGMEEGEFT
        421                                 447
Tubl      EAREDLAALERDYIEVGADSYAEEEEF
Tub3      EAREDLAALERDYIEVGADSYAEE--F
```

Fig. 3 | Sequence alignment of the two α-tubulin yeast proteins, Tub1 and Tub3. Differences are indicated by the boxes.

that changes in β-tubulin isotype composition might affect drug resistance in cancer cells by altering the dynamic properties of microtubules (Gonçalves *et al.*, 2001).

Tub3 has over 90% amino acid sequence identity to Tub1. What then is unique about Tub3 that results in increased microtubule stability? A sequence alignment of Tub1 and Tub3 is shown in Fig. 3. In addition to two extra residues at the carboxy terminus, 39 amino acid residues in Tub1 are different from those in Tub3. The residues in Tub1 that are different in Tub3 were mapped onto a three-dimensional model of the Tub1/Tub2 tubulin dimer. As shown in Fig. 4, most of the 39 residues are found in clusters and are distributed on the surface of α-tubulin. Clusters are located at each lateral side of the molecule, specifically on the M-loop and helix 3, both of which are proposed to be involved in lateral interactions between protofilaments (Nogales *et al.*, 1999). Other residue changes localize to the intradimer and inter-dimer interfaces, which might affect longitudinal interactions within a protofilament, and on the inside of the microtubule. Finally, a cluster is located on the exterior side of the monomer oriented towards the outside surface of the microtubule. The position of these residues is illustrated in Fig. 5A, which depicts Tub1 as viewed from the minus end of a microtubule.

Eleven of the 39 residues that are different between Tub1 and Tub3 result in changes in charge. These 11 residues are located on the exterior surface of α-tubulin, positioned on each lateral side of the monomer and on the outside and inside surfaces of the microtubule, as shown in Fig. 5B. These alterations could drastically affect microtubule stability by affecting interactions between protofilaments and interactions with proteins that bind to and regulate microtubule stability. There are also three proline residues in Tub1 that have been altered in Tub3, two of which are charged substitutions (P32E, P83R and P221S) (Fig. 5B). These changes could certainly result in conformational changes in the dimer. In addition, it is feasible that the lack of the two negatively charged glutamate residues at the C terminus of Tub3 might affect interactions with the positively charged regions of microtubule-associated proteins.

Are the observed effects of Tub3 on microtubule dynamics *in vitro* relevant to its role *in vivo*? It is likely that one of the functions of Tub3 is to dampen dynamics for efficient microtubule function in yeast. For example, *TUB3*-null yeast cells are more sensitive to benomyl, suggesting less stable and more dynamic microtubules (Schatz *et al.*, 1986b; C.J.B., M.L.G. and R.H.H., unpublished observations). In addition, *TUB3*-null diploid cells exhibit decreased spore viability (Schatz *et al.*, 1986b; C.J.B., M.L.G. and R.H.H., unpublished observations, unpublished observations), a

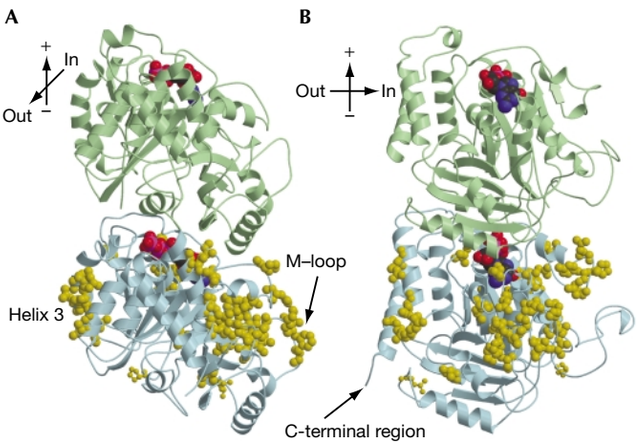

Fig. 4 | Locations of the residues in Tub1 that are different in Tub3. (**A**) View from the outside of the microtubule. (**B**) View from the M-loop lateral side of a protofilament. The side chains of amino-acid residues that are different are highlighted in yellow. The lateral elements helix 3 and M-loop are labelled. Orientation axes: + and – represent microtubule orientation; in and out represent the inside and outside of the microtubule, respectively. Structures were drawn as described previously (Gupta *et al.*, 2001).

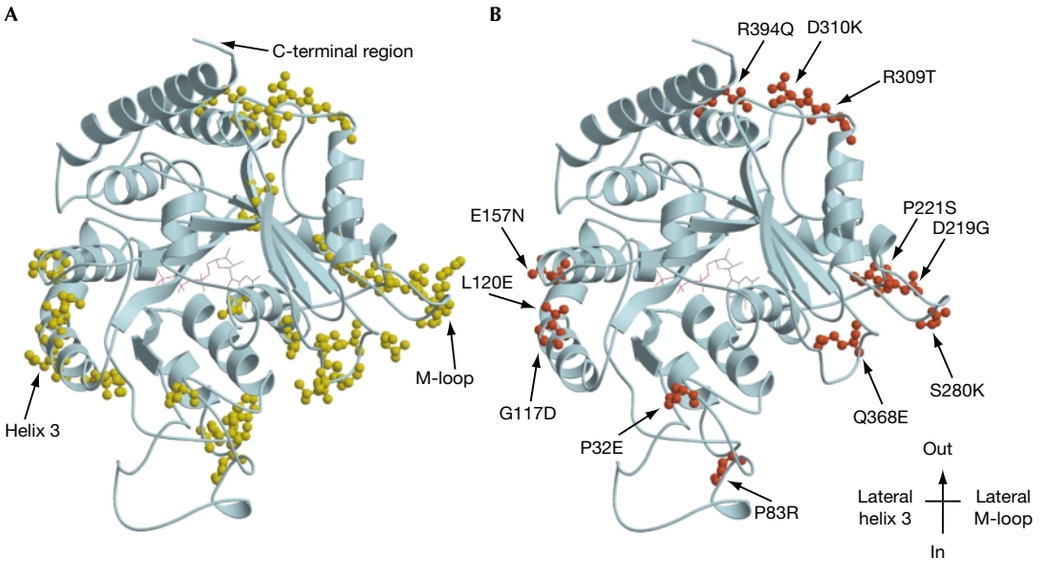

Fig. 5 | Residues in Tub1 that are different in Tub3 are located primarily on the outer surface of α-tubulin. The view is from the minus-end of a microtubule. (**A**) The side chains of the 39 amino-acid residues that are different are highlighted in yellow. (**B**) The side chains of 12 residues that result in changes of charge and/or involve proline residues are highlighted in red and labelled. Helix 3, the M-loop and the C-terminal region are labelled in (**A**) and also apply to (**B**). The C-terminal region is not included in the model.

process that requires microtubules. It has also been shown in fission yeast that mutations in the minor α-isotype can be dominant over the major isotype in causing temperature sensitivity and polarity defects (Radcliffe *et al.*, 1998).

MATERIALS AND METHODS

Yeast strains and purification of yeast tubulin. Yeast tubulin was purified from three haploid strains: MGY1 (Gupta *et al.*, 2001), BGY1 (Bode *et al.*, 2002) and MGY3 (this study). All three strains have a $(CAC)_6$ sequence just before the stop codon of *TUB2*, resulting in a His_6 tag on the C terminus of β-tubulin. BGY1 has the *TUB3* gene disrupted with a *LEU2* marker gene, and MGY3 has the *TUB1* gene deleted with a KanMX module (geneticin G418 resistance). The episomal plasmid pMG4 was created by cloning the coding region of *TUB3* along with 441 base pairs (bp) of upstream promoting DNA and 22 bp of downstream flanking DNA from MGY1 genomic DNA into the *Sac*I–*Bam*HI restriction sites of pRS424, which contains a selectable *TRP1* marker gene (Christianson *et al.*, 1992). The entire *TUB3* coding region on pMG4 was verified by DNA sequencing. Maintained at 10–40 copies per cell, pMG4 provides sufficient concentrations of Tub3 to support cell viability after *TUB1* deletion. To create MGY3, the strain MGY1 was transformed with pMG4 followed by deletion of *TUB1*. *TUB1* deletion was accomplished by homologous recombination with a transplacement fragment containing the selectable KanMX module encased by ~1,000 bp of *TUB1*-flanking genomic DNA. This ~3,500-bp fragment was amplified from the heterozygous *TUB1* deletion strain 26955 (Invitrogen, Carlsbad, CA) and gel-purified before transformation. The presence of pMG4 in strain MGY3 was verified by plasmid rescue followed by analytical digestion. *TUB1* deletion was verified by analytical polymerase chain reaction. Tubulin from the three strains was purified to apparent homogeneity with a His_6-based affinity purification procedure (Gupta *et al.*, 2002).

Assembly conditions for yeast tubulin *in vitro*. Tubulin was assembled at 30 °C for 30 min in volumes of 40–50 μl with 0.1–1.5 mg ml^{-1} tubulin in PEM (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9) supplemented with 1 mM GTP. After reaching steady state, samples were fixed in 0.25% glutaraldehyde and negatively stained for electron microscopy. The amount of polymerized tubulin was determined by sedimentation assay as described previously (Bode *et al.*, 2002).

Gel electrophoresis. Tubulin samples were analysed by SDS–PAGE. Isoelectric focusing was performed with 17 cm ReadyStrip IPG pH 4–7 strips (Bio-Rad, Hercules, CA) in accordance with the instructions provided. After focusing, the strips were stained with 0.04% Coomassie Brilliant Blue R-250.

***In vitro* microtubule dynamics.** Steady-state dynamics of individual microtubules were monitored in real time by video-enhanced differential interference contrast light microscopy with a modified protocol of Walker *et al.* (1988) as described previously (Gupta *et al.*, 2002).

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