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Identification of Novel Temperature-sensitive Lethal Alleles in Essential β-Tubulin and Nonessential α2-Tubulin Genes as Fission Yeast Polarity Mutants

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We have screened for temperature-sensitive (ts) fission yeast mutants with altered polarity (*alp1–15*). Genetic analysis indicates that *alp2* is allelic to *atb2* (one of two α-tubulin genes) and *alp12* to *nda3* (the single β-tubulin gene). *atb2*$^+$ is nonessential, and the ts *atb2* mutations we have isolated are dominant as expected. We sequenced two alleles of ts *atb2* and one allele of ts *nda3*. In the ts *atb2* mutants, the mutated residues (G246D and C356Y) are found at the longitudinal interface between α/β-heterodimers, whereas in ts *nda3* the mutated residue (Y422H) is situated in the domain located on the outer surface of the microtubule. The ts *nda3* mutant is highly sensitive to altered gene dosage of *atb2*$^+$; overexpression of *atb2*$^+$ lowers the restrictive temperature, and, conversely, deletion rescues ts. Phenotypic analysis shows that contrary to undergoing mitotic arrest with high viability via the spindle assembly checkpoint as expected, ts *nda3* mutants execute cytokinesis and septation and lose viability. Therefore, it appears that the ts *nda3* mutant becomes temperature lethal because of irreversible progression through the cell cycle in the absence of activating the spindle assembly checkpoint pathway.

INTRODUCTION

Microtubules are important and ubiquitous structures that play essential roles in various cellular processes, including motility, mitosis, transport of proteins and mRNAs, and cell morphogenesis (reviewed in Mitchison and Kirschner, 1986; Lehmann, 1995; Hyman and Karsenti, 1996). They assemble from heterodimers composed of α- and β-tubulin subunits, which are both evolutionarily highly conserved. Additional members of tubulin subfamilies including γ-tubulin exist in various species (Oakley, 1992; Burns, 1995). The crystallographic structure of the α/β-heterodimer has been solved recently (Nogales *et al.*, 1998); however, many fundamental aspects of microtubule function and dynamics are still not fully understood.

Genetically amenable organisms such as yeasts and fungi have proven to be ideal systems with which to investigate the cellular function of microtubules (Oakley and Morris, 1981; Neff *et al.*, 1983; Hiraoka *et al.*, 1984; Toda *et al.*, 1984; Schatz *et al.*, 1986a). *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast), although very divergent in evolution, have analogous genomic organization of tubulin-encoding genes (i.e., two α-tubulin genes and one β-tubulin gene) and show similar genetic properties in those genes (Neff *et al.*, 1983; Hiraoka *et al.*, 1984; Toda *et al.*, 1984; Adachi *et al.*, 1986; Schatz *et al.*, 1986a,b). In both yeasts, a single β-tubulin–encoding gene is essential for cell viability (*nda3*$^+$ in fission yeast and *TUB2* in budding yeast) (Neff *et al.*, 1983; Hiraoka *et al.*, 1984). In contrast, two homologous α-tubulin–encoding genes (*nda2*$^+$ and *atb2*$^+$ in fission yeast, and *TUB1* and *TUB3* in budding yeast) show different genetic behavior. One of the two homologues is essential for cell viability (*nda2*$^+$ and *TUB1*), whereas the other (*atb2*$^+$ and *TUB3*) is not; however, the nonessential α-tubulin can compensate for loss of the essential α-tubulin gene when overexpressed, indicating that these two pairs of α-tubulin molecules

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are functionally interchangeable (Adachi *et al.*, 1986; Schatz *et al.*, 1986b).

Determining the molecular basis for the temporal and spatial definition of cell polarity is a fundamental problem in cell biology. The shape of every eukaryotic cell is believed to be maintained by the cytoskeleton, which consists of three filamentous systems: actin-based microfilaments, tubulin-based microtubules, and intermediate filaments. The cytoskeleton does not act as a static structural scaffold of the cell. Instead, in virtually every system characterized to date, the cytoskeleton is highly dynamic, frequently changing its structure during cell cycle progression and developmental differentiation. This dynamic behavior is intimately related to growth control (Drubin and Nelson, 1996; Gönczy and Hyman, 1996).

Fission yeast cells are rod shaped and have defined growth polarity during both the vegetative cycle and developmental states (Mitchison and Nurse, 1985; Snell and Nurse, 1993). Previous work from our laboratory and other laboratories has clearly shown the importance of microtubule integrity in determining growth polarity. Mutations in the tubulin genes (Toda *et al.*, 1983; Umesono *et al.*, 1983b) or their regulators (Mata and Nurse, 1997; Hirata *et al.*, 1998) result in altered cell shapes such as bent or branched cells. The cortical actin-dependent pathway is also important for the maintenance of growth polarity as well as cytokinesis (Gould and Simanis, 1997). As in other organisms, the localization of these cytoskeletal molecules changes dramatically during cell cycle progression (Marks and Hyams, 1985; Marks *et al.*, 1986; Tanaka and Kanbe, 1986; Hagan and Hyams, 1988), and these molecules play key roles in specific cell cycle events, as demonstrated by the fact that genes encoding cytoskeletal components and regulators were first identified as cell division cycle (*cdc*) mutants (Nurse *et al.*, 1976; Gould and Simanis, 1997).

To understand in more detail the molecular pathways that regulate cell polarity, we have undertaken a large-scale screen of temperature-sensitive (ts) mutants to identify those with altered growth polarity (*alp*) (Hirata *et al.*, 1998). We have isolated a class of *alp* mutants that become bent or branched and lose microtubules after incubation at the restrictive temperature. Genetic as well as molecular analyses indicate that two of these *alp* loci are allelic to *atb2* and *nda3*, encoding α2-tubulin and β-tubulin, respectively. The identification of mutations in the *atb2* gene as ts polarity mutants is intriguing because previous studies have shown that cells in which *atb2*$^+$ is deleted are viable, with few defects (Adachi *et al.*, 1986), which suggests that the *alp2* mutants we have isolated here must be dominant in nature. The identification of *nda3* as a ts mutant is also interesting because despite extensive and systematic mutational analysis of the β-tubulin gene in the past, very few ts mutants have been isolated (Yamamoto, 1980; Oakley and Morris, 1981; Umesono *et al.*, 1983a,b; Thomas *et al.*, 1985; Huffaker *et al.*, 1988; Matsuzaki *et al.*, 1988; Stearns and Botstein, 1988; Reijo *et al.*, 1994; Sage *et al.*, 1995). We have determined, therefore, the mutation sites of the tubulin genes in these ts mutants. There is a single point mutation in each mutant, affecting codons that correspond to amino acids that are highly conserved through evolution. Furthermore, in contrast to previously isolated tubulin mutants, detailed phenotypic analyses suggest that the spindle assembly checkpoint control might not be operational in these ts tubulin mutants.

MATERIALS AND METHODS

Strains, Media, and Chemicals

All mutant strains were derived from HM123 (*h*$^-$*leu1*) (Table 1). JY6 (*h*$^+$*leu1his2*), and TP108-3D (*h*$^+$*leu1 ura4his2*) were used for backcrossing mutants. Complete medium, YPD (1% yeast extract, 2% polypeptone, and 2% dextrose), which contains 10 μg/ml Phloxine B (Sigma, St. Louis, MO) (called YPDP), YES (0.5% yeast extract, 3% dextrose, and 75 μg/ml adenine, histidine, leucine, and uracil), modified synthetic EMM2, and MES (3% malt extract, and 75 μg/ml adenine, histidine, leucine, and uracil) have been described previously (Moreno *et al.*, 1991). Plates contained 1.6% agar.

Genetic Techniques and Nomenclatures

Standard procedures for *S. pombe* genetics were followed as described (Moreno *et al.*, 1991). Cell number was measured using Sysmex F-800 (TOA Medical Electronics, Tokyo, Japan). *S. pombe* cells were transformed using the lithium method (Ito *et al.*, 1983). A temperature-sensitive phenotype is abbreviated to the lowercase letters ts, e.g., ts *atb2*. Proteins are designated by an uppercase first letter, e.g., Atb2. Gene disruptions are abbreviated as the gene preceded by Δ such as Δ*atb2*.

Isolation of ts Mutants with Polarity Defects

Wild-type HM123 cells were mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine as described previously (Uemura and Yanagida, 1984). Approximately 300 viable cells were spread per one YPDP plate. Plates were incubated at 29°C for 4 d, and colonies were then replica plated on YPDP and incubated at 36°C for 1 d. Strains that did not form colonies or that formed sick dark-red colonies were picked, and the morphology of these cells was examined by Calcofluor staining (Streiblová and Wolf, 1972). Approximately 1 of 50 colonies showed either no growth or retarded growth at the restrictive temperature. In total, 200,960 colonies were screened in this way, and 2822 ts mutants were isolated. Mutants that showed altered cell shape (bent or branched) at 36°C were selected for further analysis. These mutants were grown in liquid YPD and shifted to 36°C for 8 h, and the cell morphology was examined once again by Calcofluor staining. In total, 22 ts mutants that showed bent, curved, or branched morphologies were isolated and designated *alp* (Table 2).

Complementation Analysis

h$^+$ mutant strains were isolated after mating of the mutant strains to JY6 (*h*$^+$*leu1his2*). Crisscross mating was performed, and free spores were plated on two YPDP plates, one of which was incubated at 29°C, the other at 36°C. If the difference in the number of colonies formed between these two plates was $>10^4$-fold, the two mutants were assigned as allelic. Strain 1146 (*alp8*) (Table 2) contained two

mutations: one is responsible for the morphological defects, which is allelic to *tea1* (Mata and Nurse, 1997), and the other is responsible for the ts growth phenotype.

Dominance–Recessive Test

A stable prototrophic diploid heterozygous for the *atb2* locus was constructed between the ts *mei1-B102atb2-996* strain (TPR19A) (Table 1) and a mater strain (TP71-7C), and temperature sensitivity was examined.

DNA Preparation and Manipulation

Standard molecular biology techniques were followed as described (Sambrook *et al.*, 1989). Enzymes were used as recommended by the suppliers (New England Biolabs, Beverly, MA, and Boehringer Mannheim, Indianapolis, IN). Nucleotide sequencing was performed by the dideoxy method (Sanger *et al.*, 1977).

Identification of the alp2 and alp12 Loci as atb2 and nda3, Respectively

S. pombe genomic libraries constructed in the vectors pDB248 (Beach and Nurse, 1981; Hirano *et al.*, 1988) and pUR19 (Barbet *et al.*, 1992) were used for the isolation of plasmids that complemented the ts *alp2* or *alp12* mutant (DH1-7C: h^-*leu1alp2* [*atb2*]-*996*; DH1-2D: h^-*leu1 ura4alp2* [*atb2*]-*996*; DH1-2B: h^+*leu1ura4his2alp2* [*atb2*]-*996*; and PR7: h^-*leu1ura4alp12* [*nda3*]-*1828*) (Table 1). For the *atb2* mutant, 4 of 36,000 colonies transformed with the pDB248-based library were capable of growing at 36°C, whereas for *alp12*, 5 of 20,000 colonies transformed with the pUR19-based library grew at 36°C. Segregation analysis indicated that the Ts^+ phenotype was plasmid dependent. Plasmid DNAs were recovered from these transformants. Four different plasmids (pALA200, pALB200, pALC200, and pALD200) and three different plasmids (pCR8 [isolated three times], pCR9, and pCR10) were recovered from the *alp2* and *alp12* transformants, respectively. Restriction mapping indicated that three (pALA200, pALC200, and pALD200) contained overlapping inserts and carried the $atb2^+$ gene, whereas pALB200 contained the $nda2^+$ gene. pCR8, 9, and 10 all contained overlapping inserts and carried the $nda3^+$ gene.

Identification of the *alp2* loci as *atb2* has been described previously (Yaffe *et al.*, 1996). Furthermore, tetrad analysis between ts *atb2-996* and an *atb2* disruptant (Δatb2) (Table 1) showed a tight linkage; 23 tetrads were dissected, and all showed parental ditypes. Allelism between *alp12* and *nda3* was determined as follows. pCR10 was transformed into the ts *alp12* (*nda3*)-*1828* mutant. At 36°C, a number of Ts^+Ura^+ colonies appeared that stained less intensely on YPDP (at an approximate frequency of 10^{-2}). Segregation analysis indicated that the Ura^+ phenotype was stable, showing that pCR10 had integrated stably in the genome via homologous recombination. Free spore analysis between these integrants and a mater strain (TP108-3D) (Table 1) showed that no ts segregant appeared from 10^3 colonies, indicating that *alp12* is allelic to *nda3*.

Cloning of the ts atb2 and nda3 Genes and Determination of the Mutation Sites

To determine the mutation sites in the ts *atb2* mutants, 0.6-kb (corresponding to the first 166 residues of Atb2) and 1.3-kb (residues 167–449) *Hin*dIII fragments that comprise the entire $atb2^+$ gene (Toda *et al.*, 1984) were cloned into an integration vector and used to transform ts *atb2-996* and *-1212* strains (Yaffe *et al.*, 1996). Both of the ts mutants were suppressed by the plasmid containing the 1.3-kb fragment but not by that containing the 0.6-kb fragment, indicating that the mutation sites of *atb2-996* and *-1212* are located in the 1.3-kb *Hin*dIII fragment. A DNA fragment corresponding to amino acids 167–449 was amplified from ts *atb2-996* and *-1212* strains by PCR using the following oligonucleotides as primers: Alp2-N1, AAAAAAGCTTCAATTTTCTATGTATCC; and Alp2-C1, AAAAGGATCCTTAGTACTCTTCTTCCA (underlined are *Hin*dIII and *Bam*HI sites, respectively). The 858-bp amplified fragment was subcloned into pUC19 (Vieira and Messing, 1982). In each case, two independent PCRs were performed, amplified fragments were cloned separately, and the nucleotide sequences were determined. The same sequence was obtained from the independent clones and contained a single point mutation (see RESULTS), indicating that the base changes were not due to errors during PCR.

To determine the mutation site of the ts *nda3* mutant, the entire ORF was cloned into pUC19 from the ts *nda3-1828* mutant by PCR using the following two oligonucleotides: Nda3-N3P, TATGCTGCAGCTAACGAAACTCACCTAC; and Nda3-C1B, TATGGATCCAACGTAGATAAACACT (underlined are *Pst*I and *Bam*HI sites, respectively). Sequencing of the two clones derived from independent PCRs showed nine putative deviations (leading to amino acid substitutions at six places) in the ts *nda3-1828* strain from the published nucleotide sequence of the wild-type $nda3^+$ gene (Hiraoka *et al.*, 1984). To determine which site is mutated in ts *nda3-1828*, the wild-type $nda3^+$ gene was cloned in a similar manner, and its nucleotide sequence was determined. Comparison of the nucleotide sequences of ts *nda3-1828* and wild-type $nda3^+$ revealed that the published data contain sequencing errors in eight positions. In ts *nda3-1828*, nucleotide 1566 (A of initiator methionine is denoted as +1) is mutated from T to C, which results in substitution of Tyr by His at residue 422 (see RESULTS). These sequence data are available from EMBL/GenBank/DDBJ under accession numbers AF042827 ($nda3^+$) and AF042828 (*nda3–1828*).

Immunochemical Assays

For indirect immunofluorescence microscopy, the methanol fixation method was used (Alfa *et al.*, 1993). TAT-1 antibody (provided by Dr. Keith Gull, University of Manchester, Manchester, United Kingdom) and Cy3-conjugated sheep anti-mouse immunoglobulin G (Sigma) were used to visualize microtubules, and DAPI was used for chromosomal DNA.

Cell extracts were prepared as described (Matsusaka *et al.*, 1995), except that HB buffer (Moreno *et al.*, 1991) was used in the disruption of cells. Standard procedures for immunoblotting were followed (Harlow and Lane, 1988). Monoclonal anti-β-tubulin antibody (Sigma), anti-α-tubulin (TAT-1, gift from Dr. Keith Gull), and anti-Cdc2 (Y100, gift from Dr. Hiroyuki Yamano, ICRF) were used as primary antibodies. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad, Richmond, CA) and a chemiluminescence system (ECL, Amersham, Arlington Heights, IL) were used to detect bound antibody.

RESULTS

Isolation of Mutants That Are Defective in Growth Polarity

We performed a large-scale visual screen for mutants with defects in growth polarity. We first isolated ts mutants and then examined the cell morphology of these mutants after incubation at the restrictive temperature using Calcofluor, which stains septa and growing ends of the cell (Streiblová and Wolf, 1972; Mitchison and Nurse, 1985). Mutants with bent, curved, or branched morphology were selected. In total, 22 different mutants were obtained, and complementation tests indicated that these represent 15 loci, designated *alp1–15* (Table 2). Some examples of the cell morphology and DAPI staining of *alp2* and *12* mutants are shown in Figure 1.

Table 1. Strain list

Strains	Genotypes	Derivations
HM123	*h⁻leu1*	Our stock
JY6	*h⁺leu1his2*	Our stock
TP108-3D	*h⁺leu1ura4his2*	Our stock
TP71-7C	*h⁻arg3ura2*	Our stock
Δatb2	*h⁻leu1atb2::LEU2*	Adachi et al. (1986)
KM52-110	*h⁻leu1nda2-KM52*	Toda et al. (1984)
DH1-7C	*h⁻leu1atb2-996*	This study
DH1-2D	*h⁻leu1ura4atb2-996*	This study
DH1-2B	*h⁺leu1ura4his2atb2-996*	This study
1212	*h⁻leu1atb2-1212*	This study
DH2-8D	*h⁻leu1alp1-1315*	Hirata et al. (1998)
DH12	*h⁻leu1nda3-1828*	This study
PR7	*h⁻leu1ura4nda3-1828*	This study
PR11	*h⁻leu1nda3-1828atb2::LEU2*	This study
TPR19A	*mei1-B102leu1 arg1atb2-996*	This study

Classification of Polarity Mutants with Microtubule Staining

It has become clear that microtubules are important for the determination of cell shape and for growth polarity of fission yeast (Toda *et al.*, 1983; Yaffe *et al.*, 1996; Mata and Nurse, 1997; Hirata *et al.*, 1998). We therefore used indirect immunofluorescence microscopy by using anti-tubulin antibody (TAT-1, kindly provided by Dr. Keith Gull) to examine the microtubule cytoskeleton of the *alp* mutants. It was clear from these analyses that at least four (*alp1*, *2*, *11*, and *12*) of the genes identified in our screen were required for the maintenance of microtubule structures, because the microtubules in these mutants became fragile and disappeared when the temperature shifted. In this study, we have characterized *alp2* and *alp12* at the molecular level. Three alleles of *alp2* (*-996*, *-1212*, *-1377*) (Table 2) and one allele of *alp12* (*-1828*) were identified. Microtubule staining of *alp2* and *alp12* mutants is shown in Figure 2. Cytoplasmic microtubules became unstable and disappeared after 6 h incubation at 35.5°C, although some short nuclear spindle microtubules still remained (Figure 2, B and C, bottom panels).

In addition to the loss of microtubule structures, *alp2* and *alp12* mutants showed various defective phenotypes in cell cycle and cell shape control. These include displacement of the nucleus (24% for *alp2-996* and 17% for *alp12-1828* after 8 h at 35.5°C) (Figure 1) and accumulation of septated cells. Also, asymmetrical rather than medial septation was often observed in many cells (~50% of septated cells), resulting in anucleate cells following cytokinesis (8% for *alp2-996* and 2% for *alp12-1828*).

Identification of the alp2 and alp12 Mutations as Novel Alleles in Mutant Tubulin Genes

We cloned the *alp2⁺* and *alp12⁺* genes by complementation using a fission yeast genomic library constructed with a multicopy vector. Four plasmids were isolated that suppressed ts *alp2-996*, and three suppressed *alp12-1828*. Restriction mapping of these plasmids indicated that the four plasmids suppressing the

Table 2. Complementation groups and defective phenotypes of the *alp* mutants

Loci	Alleles	Morphology[a]	Microtubules	Genes/product
alp 1	1315, 1356, 1584	Bent/branched	No	Cofactor D[b]
2	996, 1212, 1377	Bent/branched	No	*atb2⁺*/α2-tubulin
3	149, 601	Curved/cut		
4	225, 1891	Bent/cut	Few	
5	1134	Bent		
6	719	Bent/cut		
7	738	Bent/cut		
8	1146[c]	Bent/branched		*tea1⁺*
9	1437	Bent		
10	103	Curved		
11	924	Bent/branched	No	Cofactor B[d]
12	1828	Bent/branched	No	*nda3⁺*/β-tubulin
13	1837	Bent/cut		
14	1270	Bent/branched	Few	
15	1218	Bent/cut		

[a] Mutants that showed >1% of cells with branched morphology or 10% of "cut" at 36°C for 8 h are indicated as "branched" or "cut" (Hirano et al., 1986), respectively.

[b] Hirata et al., 1998.

[c] The original isolate (1146) contained two mutations; one in *tea1* (Mata and Nurse, 1997), which leads to branched morphology at 36°C, and the other in an unknown locus, which causes temperature-sensitive growth.

[d] Radcliffe and Toda, unpublished observations.

alp2 mutation consist of two previously identified genes, *nda2*$^+$ and *atb2*$^+$, which encode the two α-tubulins (α1- and α2-tubulin, respectively) (Toda *et al.*, 1984; Adachi *et al.*, 1986), and all three *alp12*-complementing plasmids contained the *nda3*$^+$ gene, which encodes β-tubulin (Hiraoka *et al.*, 1984). Genetic analysis indicated that *alp2* was allelic to *atb2*, and *alp12* was allelic to *nda3* (see MATERIALS AND METHODS). Therefore, we shall hereafter use *atb2* for *alp2* and *nda3* for *alp12* preceded by ts, such as ts *atb2*.

Mutations in tubulin genes, or genes involved in microtubular pathways, often result in supersensitivity to antimicrotubule drugs such as members of the benzimidazole family of compounds (Umesono *et al.*, 1983b; Adachi *et al.*, 1986; Hirata *et al.*, 1998). Consistent with this, ts *atb2* and *nda3* strains were supersensitive to the benzimidazole compound thiabendazole. They could not form colonies on rich media plates containing 10 μg/ml at 20°C, whereas wild-type cells could (Table 3).

Genetic Interaction of ts atb2 and nda3 Mutations with Other Tubulin Genes

It was curious that the nonessential *atb2* gene was identified in this study as a ts mutation. The mutant Atb2 proteins (α2-tubulin) must have a dominantly harmful effect on the maintenance of microtubules because deletion of *atb2*$^+$ does not show a lethal or ts phenotype (Adachi *et al.*, 1986). In line with this notion, genetic analysis demonstrated that the ts *atb2* mutant is dominant because a heterozygous diploid between *atb2-996* and *atb2*$^+$ showed the ts phenotype, although some weak growth was observed compared with a haploid ts *alp2-996* strain (Figure 3A). If the mutant Atb2 protein were to inhibit the function of β-tubulin by abortive binding, effectively taking the β-tubulin out of the pool of available subunits, it might be expected that an increased dosage of β-tubulin gene would suppress ts *atb2* mutations; however, a multicopy plasmid containing *nda3*$^+$ was incapable of suppressing ts *atb2* (Figure 3B, top right plate). This result suggests that the phenotypic dominance of ts *atb2* (α2-tubulin) over *nda2*$^+$ (α1-tubulin) is not ascribable to a loss of β-tubulin function.

Crossing ts *atb2-996* and cs *nda2-KM52* (Umesono *et al.*, 1983b) indicates that the double mutants are synthetically lethal. Among 12 tetrads dissected, no double mutants (14 spores predicted) were obtained, whereas 14 wild-type and 10 of each single mutant strain formed colonies at 29°C, a temperature permissive for both mutants. Thus for both ts *atb2* and cs *nda2* mutants, functional wild-type proteins of the other α-tubulin homologue are required for viability at the permissive temperature.

In the case of ts *nda3*, a different effect from increased dosage of α-tubulin-encoding genes was observed. Overexpression of *atb2*$^+$ led to enhanced temperature sensitivity in the ts *nda3* mutant (Figure 3B, bottom left plate). Incubation of ts *nda3* on minimal plates at 34.5°C allowed colony formation in the ts *nda3* mutant containing an empty vector, whereas mutant cells containing a multicopy plasmid carrying *atb2*$^+$ were incapable of forming colonies at this temperature. This suggested that the ts *nda3* mutant is highly sensitive to altered gene dosage of *atb2*$^+$. To examine the effect of *atb2*$^+$ gene dosage in more detail, the *atb2*$^+$ gene was disrupted in the ts *nda3* strain. In reverse parallel with the toxic effect of overexpression, the deletion of *atb2*$^+$ partially rescued the temperature sensitivity of *nda3-1828* (Figure 3C). This result indicates that the defective phenotypes observed in the ts *nda3* mutant can be ascribed, at least in part, to the existence of

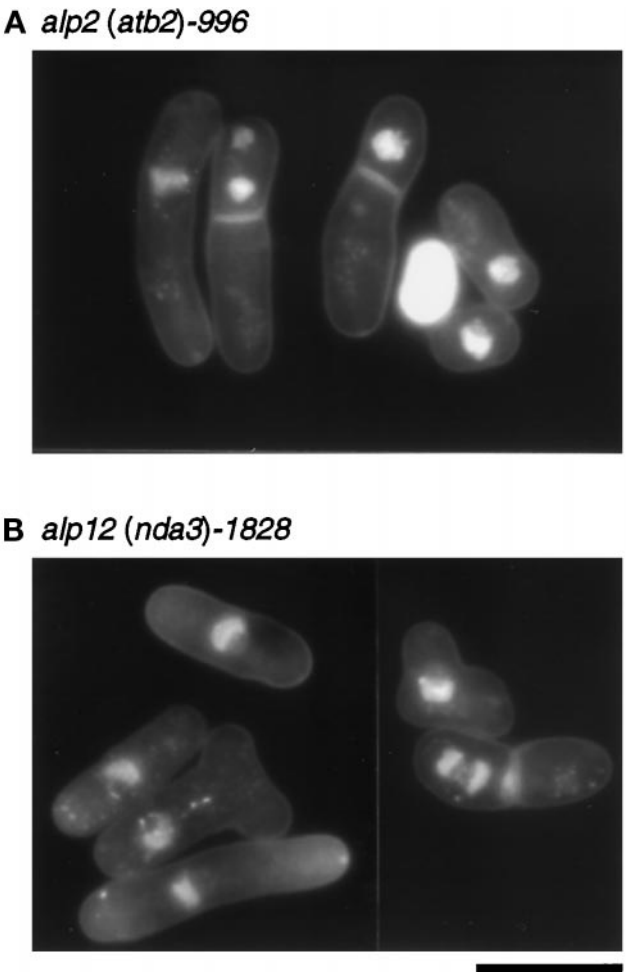


Figure 1. Nuclear staining of ts *alp2* (*atb2*) and *alp12* (*nda3*) cells. *alp2* (*atb2*)*-996* (A, DH1–7C; Table 1) or *alp12* (*nda3*)*-1828* cells (B, DH12) were grown exponentially at 26°C, shifted to 35.5°C, and incubated for 6 h. Cells were fixed and stained with DAPI. Bar, 10 μm.

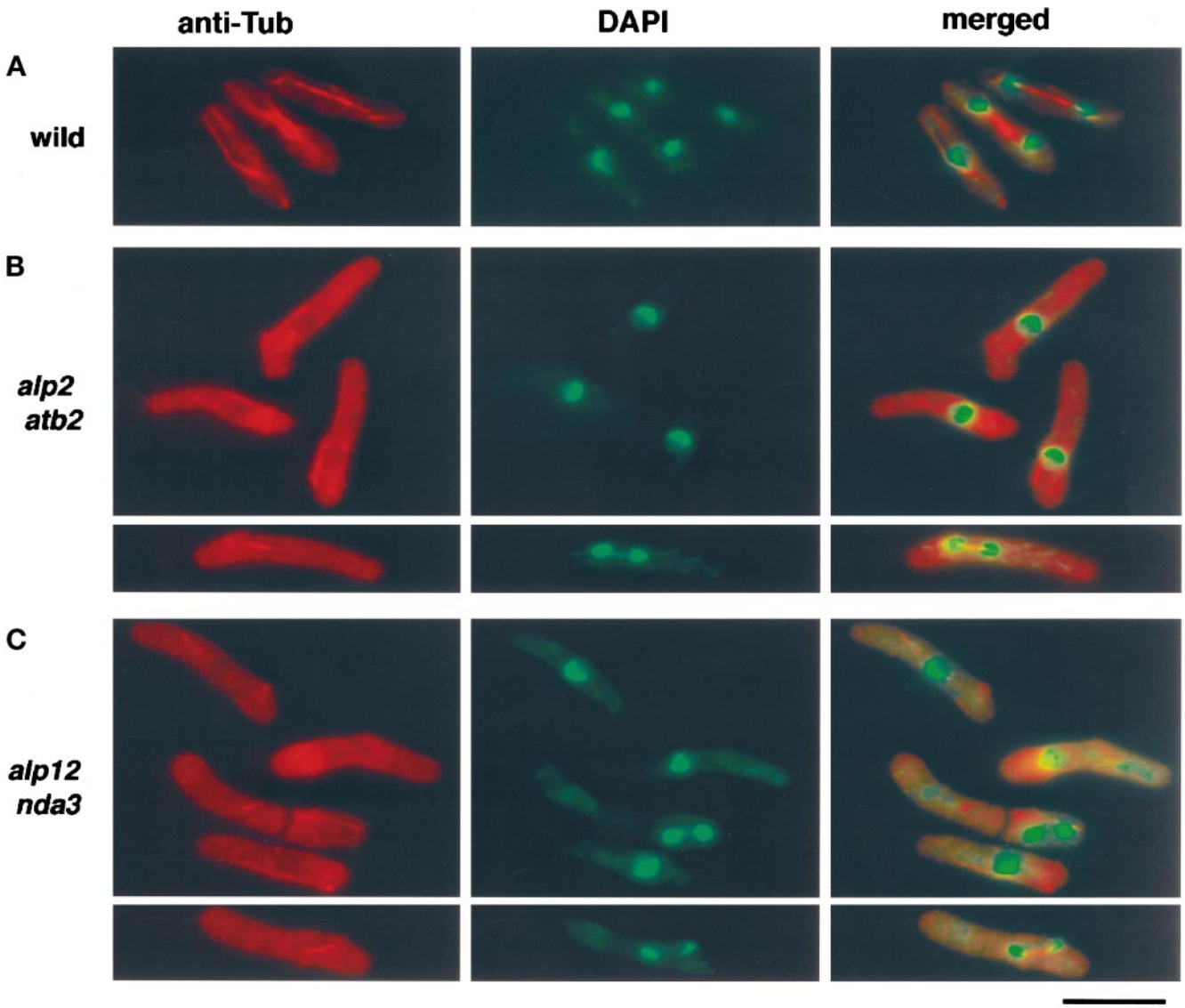


Figure 2. Microtubule staining in ts *alp2* (*atb2*) and *alp12* (*nda3*) cells. Mutant and wild-type cells were prepared as in Figure 1, fixed in methanol, and stained with anti-tubulin antibody (TAT-1, left panel) or DAPI (middle panel). Merged figures are shown in the right panel. Representative figures are shown for wild-type (top row), ts *alp2* (*atb2*) (second and third rows), and *alp12* (*nda3*) (fourth and fifth rows).

the Atb2 protein. It is noteworthy that the toxic effect is α2-tubulin specific, because ts *nda3* cells carrying the *nda2*$^+$ gene encoding α1-tubulin on an equivalent plasmid were capable of forming colonies, although of a slightly smaller size compared with those containing vector alone (Figure 3B, bottom left plate). Multicopy plasmids containing γ-tubulin (Horio *et al.*, 1991) do not suppress either ts *atb2* or *nda3*, nor do they enhance the lethality conferred by these mutations (Hirata and Toda, unpublished observations), suggesting that the interactions we report are specific.

Determination of Mutation Sites in the ts atb2 and nda3 Mutants

We determined the mutation sites of the ts *atb2* and *nda3* mutants (*atb2-996* and *-1212* and *nda3-1828*; see MATERIALS AND METHODS). In each case, a single point mutation was found, which resulted in alteration of an amino acid residue in Atb2 and Nda3. In ts *atb2-996*, nucleotide 1065, guanine (A of the initiator methionine is denoted as +1), was mutated to adenine, which resulted in the substitution of cysteine 356 (T<u>G</u>T) with tyrosine (T<u>A</u>T; mutated nucleotides are underlined). In the case of ts *atb2-1212*, nucleotide 737,

guanine, was mutated to adenine, which resulted in the substitution of glycine 246 (GGC) with aspartate (GAC). A comparison of amino acid sequences around the mutated residues is shown in Figure 4A. Both of the mutated residues have been highly conserved throughout evolution. In particular, glycine 246 is of interest because this residue is invariant not only in fission yeast α1-tubulin (Nda2) and α-tubulins from other species, but also in β- and γ-tubulins in virtually every species (Figure 4A).

The mutation site of ts *nda3-1828* is located near the C terminus of the Nda3 protein. Nucleotide 1566 was mutated from thymine to cytosine, which results in the substitution of tyrosine 422 (TAT) with histidine (CAT) (Figure 4B). Like glycine 246, this tyrosine is also conserved in all members of tubulins. It is noteworthy that the region around tyrosine 422 is rich in acidic amino acid residues, which are believed to be important for interactions with other proteins. Extensive mutational analysis of the budding yeast β-tubulin gene *TUB2* has shown the C terminus of β-tubulin to be essential for the normal function of microtubules (Reijo *et al.*, 1994).

ts atb2 Alters the Cellular Ratio of α1- to α2-Tubulin

Why do the *atb2* mutations that we isolated show a dominant ts growth defect? As shown above, it is not due to the absorption of β-tubulin. One possible explanation might be an altered cellular ratio of α1- and α2-tubulins. To examine this possibility, immunoblotting using anti-α-tubulin antibody was performed with protein extracts from a ts *atb2* mutant. As described previously (Adachi *et al.*, 1986), the two closely migrating bands of 55 and 57 kDa in wild-type extracts correspond to Atb2 and Nda2, respectively (Figure 5A, lane 1) (note that $p57^{nda2}$ is more abundant than $p55^{atb2}$). In an *atb2*-deleted strain, the $p55^{atb2}$ band is missing (Δatb2; Figure 5A, lane 2). In *atb2-1212* cells grown at the permissive temperature (Figure 5A, lane 3), the ratio of Atb2 to Nda2 is similar to that seen in wild-type extracts, because the level of $p57^{nda2}$ is much higher than that of $p55^{atb2}$. After shifting to the restrictive temperature of 35.5°C, the relative level of $p55^{atb2}$ increased (Figure 5A, lanes 4–6). Although the increase was modest, it was reproducible. Thus it is possible that the dominance of *atb2-1212* is ascribable to the higher relative abundance of mutant Atb2 protein over Nda2. Suppression by high dosage of the $nda2^{+}$ gene supports this notion, although how ts Atb2 protein interferes with microtubule function still remains to be determined (see DISCUSSION).

It should be noted that despite the altered ratio of α1- to α2-tubulin, the total amount of α-tubulin proteins remained almost constant, suggesting that regulatory mechanisms maintain a steady-state level of α-tubulin protein, as has been reported in other organisms (May *et al.*, 1990; Gonzalez-Garay and Cabral, 1996). This supports previous data that were suggestive of this form of regulation (Adachi *et al.*, 1986). In contrast to the increase of Atb2 protein in the ts *atb2* mutant, the level of β-tubulin remained unchanged in the ts *nda3* mutant (Figure 5B). Thus mutation in the C-terminal amino acid (Y422H, Figure 4B) did not affect the stability of the β-tubulin molecule.

Table 3. Sensitivity of the *atb2* and *nda3* mutants to thiabendazole (TBZ)

	TBZ (μg/ml)[b]	
Strains[a]	28°C	20°C
ts *atb2*	+	−
ts *nda3*	++	−
alp1	+	−
Δ*atb2*	+	+
cs *nda2*	−	NA[c]
Wild type	++	++

[a] Strains used are as follows: ts *atb2* (DH1-7C; see Table 1); ts *nda3* (DH12); *alp1* (DH2-8D; Hirata et al., 1998), and Δ*atb2* (*atb2::LEU2*; Adachi et al., 1986); cs *nda2* (*h⁻leu1nda2-KM52*; Toda et al., 1984); and wild type (HM123).

[b] Mutant cells were streaked on rich YPD plates containing 10 μg/ml of TBZ and incubated at 28° or 20°C for 7 d. ++, Colony size was indistinguishable from that on plates without TBZ: +, tiny colonies were formed. −, no colonies were formed.

[c] NA, Not applicable.

The Spindle Assembly Checkpoint Is Not Operational in ts Tubulin Mutants

The temporal order within the cell cycle is maintained by surveillance mechanisms called checkpoints (Hartwell and Weinert, 1989). The spindle assembly checkpoint prevents cells from proceeding into mitosis when the bipolar spindle function is compromised (Murray, 1995; Wells, 1996). In fission yeast, it has been shown that a cs *nda3* mutation (*nda3-KM311*) activates the spindle assembly checkpoint at the restrictive temperature so that cells arrest in midmitosis with condensed chromosomes and maintain high viability (Hiraoka *et al.*, 1984; Moreno *et al.*, 1989). Upon release from the mitotic block, *nda3-KM311* cells reinitiate anaphase in a highly synchronized manner (Hiraoka *et al.*, 1984). We therefore were interested in determining whether the spindle assembly checkpoint is also intact in the newly isolated ts *nda3* mutant.

Contrary to our initial expectations, the spindle assembly checkpoint appeared not to be operational in the ts *nda3* mutant cells. As shown in Figure 6, A and B, the septation index increased 3 h after temperature shift-up and was accompanied by a sharp drop in viability. More than 60% of the septated cells had

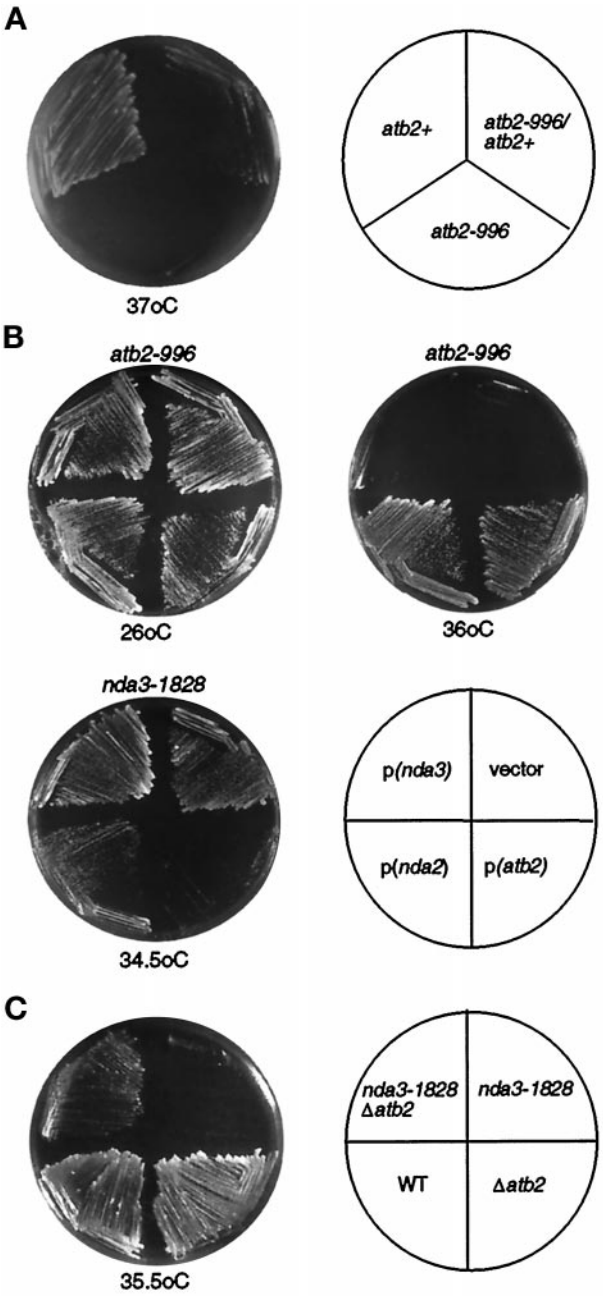


Figure 3. Genetic interaction between ts tubulin mutants and other tubulins. (A) Dominance–recessive test of ts *atb2* mutation. Haploid wild-type (*atb2*$^+$, TP71–7C; Table 1), ts *atb2* (*atb2-996*, TPR19A), and a heterozygous diploid constructed by crossing these two strains (*atb2-996*/*atb2*$^+$) were streaked on rich YPD plates and incubated at 37°C for 2 d. (B) *atb2-996* mutant cells (top two plates) or *nda3-1828* (bottom left plate) were transformed with an empty vector (vector), a multicopy plasmid containing *atb2*$^+$ [pALA200, p(*atb2*)], *nda2*$^+$ [pALB200, p(*nda2*)], or *nda3*$^+$ [pCR9, p(*nda3*)]. Transformants were incubated at either 26°C (B, top left), 36°C (B, top right) or 34.5°C (bottom left) for 3 d. (C) Four strains (top left, *nda3-1828*Δ*atb2*; top right, *nda3-1828*; bottom right, Δ*atb2*; bottom left, wild type) were streaked on YPD plates and incubated at 35.5°C for 2 d.

mislocalized septa (Figure 6B). Also, nuclear displacement was evident, and in some cells partial chromosome segregation occurred (Figure 1B). Polarity defects such as bent or branched cell morphology were apparent at 2 h. Consistent with continued cell cycle progression in these mutants, interphase-like hemispherical chromosomes, rather than condensed chromosomes, were seen after 6 h at 35.5°C (Figure 1B). This contrasts with the cs allele of *nda3* (*-KM311*), which results in accumulation of increasing numbers of cells with condensed chromosomes with prolonged incubation at the restrictive temperature (Umesono *et al.*, 1983a; Hiraoka *et al.*, 1984).

To examine the reversibility of the ts *nda3* mutant incubated at the restrictive temperature, the mutant cells were incubated for various periods (2, 4, and 6 h) at 35.5°C before being returned to 26°C, and the percentage of mitotic or septated cells was measured. No synchronous anaphase or septation was observed (Figure 6C). These results show that contrary to the cell cycle arrest phenotype of cs *nda3* mutants, in the ts *nda3* mutant the spindle assembly checkpoint is not functional, and mutant cells proceed into mitosis and subsequent events such as septation and cytokinesis at the restrictive temperature. A similar result was obtained with a ts *atb2* mutant, namely mitosis, and septation occurred at the restrictive temperature, although viability remained high (Figure 1A) (Radcliffe and Toda, unpublished observations).

If the ts *nda3* mutant becomes lethal because of cell cycle progression in the absence of activation of the spindle checkpoint, it would be expected that the lethality of the mutant would be rescued by blockage of the cell cycle before entry into mitosis. That is indeed the case. The ts *nda3* mutant arrested in early S phase by hydroxyurea (HU) treatment did not lose viability for up to 4 h incubation at the restrictive temperature, whereas the control culture in the absence of the drug lost viability sharply (Figure 6D). Further incubation at the restrictive temperature in the presence of HU (>4 h) led to a loss of viability of the ts *nda3* mutant, probably because of progression of the cell cycle by prolonged exposure to HU (21% after 6 h) (Sazer and Nurse, 1994). We therefore conclude that the loss of viability in the ts *nda3* mutant is ascribable to irreversible progression of the cell cycle without normal function of spindles, in which the spindle assembly checkpoint is not activated.

DISCUSSION

Cell Polarity and Microtubules

Microtubules have been shown to be indispensable in the execution of diverse cellular processes in many systems, including motility, mitosis, protein and mRNA transport, and cell morphogenesis. In fission

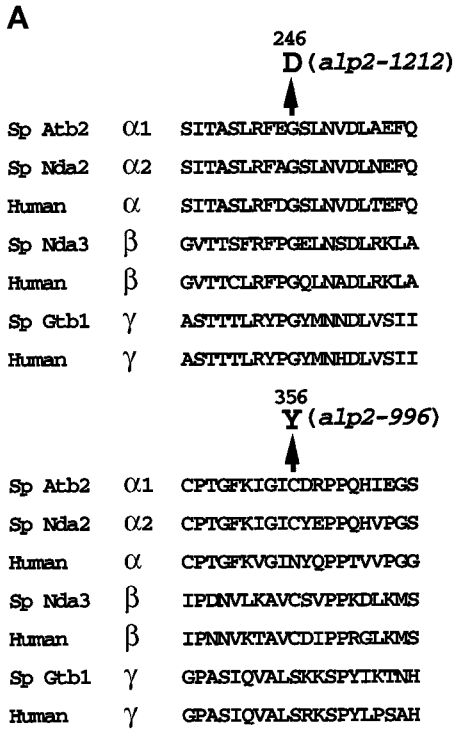
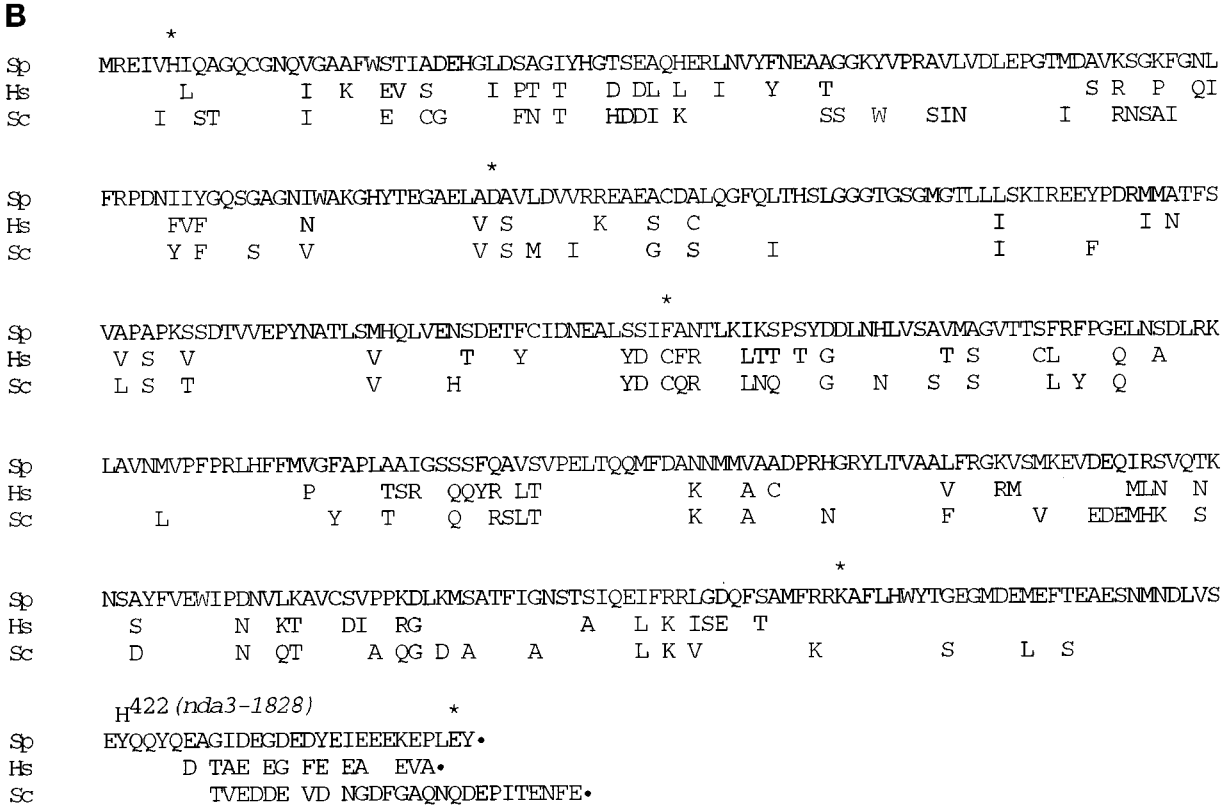


Figure 4. Determination of the mutation sites in the ts *atb2* (*alp2*) and *nda3* (*alp12*) mutants. Amino acid comparisons of α-tubulin (A) and β-tubulin (B) together with the amino acid substitution caused by a point mutation in each ts mutant are shown. Sp stands for fission yeast (*S. pombe*), Sc for budding yeast (*S. cerevisiae*), and Hs for human (*H. sapiens*). *atb2-996* contains a single base change at nucleotide number 1065 (G to A, A of the initiator methionine is denoted as +1), resulting in an amino acid substitution of cysteine 356 with tyrosine. *atb2-1212* contains a single base change at nucleotide 737 (G to A), which results in an amino acid substitution of glycine 246 with aspartate. *nda3-1828* contains a single base change at position 1566 (from T to C), resulting in substitution of tyrosine 422 with histidine. Only nonconserved amino acid residues are shown in B for Hs and Sc. Asterisks show amino acids where the previously published data (Hiraoka *et al.*, 1984) are incorrect because of sequencing errors.



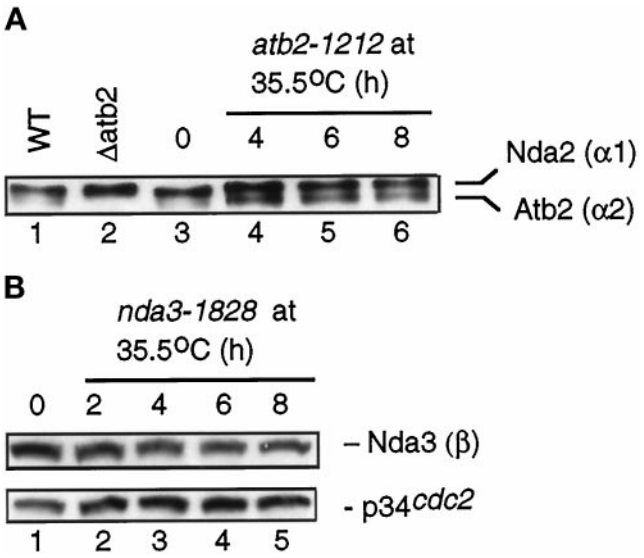


Figure 5. The level of tubulin proteins in ts *atb2* and *nda3* mutants. (A) Total cell extracts were prepared from wild type (HM123, lane 1; Table 1), deleted *atb2* (Δatb2, lane 2), or ts *atb2-1212* (1212, lanes 3–6). Cells were cultured at 26°C in lanes 1 and 2. *atb2-1212* cells were first grown at 26°C (lane 3) and shifted to 35.5°C. Samples were collected at 4 (lane 4), 6 (lane 5), and 8 (lane 6) h. Protein (2 μg) from cell extracts was run in SDS-PAGE, and immunoblotting was performed with anti-α-tubulin antibody (TAT-1). (B) ts *nda3-1828* cells (DH12) were grown as described in A, and samples were taken every 2 h after the shift. Cell extracts (20 μg) were run in SDS-PAGE, and immunoblotting was performed with mouse anti-β-tubulin antibody (Sigma) (top) or anti-Cdc2 antibody as a loading control (bottom).

yeast, microtubules also execute several distinct functions, including chromosome segregation (Umesono *et al.*, 1983a,b), distribution of organelles, in particular mitochondria and Golgi (Ayscough *et al.*, 1993; Yaffe *et al.*, 1996), and cell polarity and morphogenesis (Toda *et al.*, 1983; Mata and Nurse, 1997; Hirata *et al.*, 1998). Fission yeast tubulin mutants were originally isolated on the basis of cell cycle defective phenotypes in mitosis (nuclear division arrest [*nda*]) (Toda *et al.*, 1983). In this study we have screened for ts mutants with altered polarity of cell growth (*alp* mutant) and have identified new mutant alleles in tubulin genes. This clearly demonstrates that, as in other eukaryotes, microtubules are crucial elements in the determination of cell polarity in fission yeast. How do microtubules regulate cell polarity? A recent study has identified a "marker" molecule that translocates into the cell tips via microtubules and determines the orientation of cell tip growth (Mata and Nurse, 1997). There may be several molecules that mark the growing tips in a microtubule-dependent manner, and other alp^+ genes may encode such molecules.

Work from budding yeast, on the other hand, suggests that in this organism the main role of microtubules is in mitosis and meiosis, namely nuclear migration and chromosome segregation (Huffaker *et al.*, 1988; Jacobs *et al.*, 1988). This suggests that despite the similarity in genomic organization and genetic properties of tubulin genes in these two yeasts (Neff *et al.*, 1983; Hiraoka *et al.*, 1984; Toda *et al.*, 1984; Adachi *et al.*, 1986; Schatz *et al.*, 1986a,b), their biological roles have diverged considerably during evolution. This may be reflected in the different distribution of cytoplasmic microtubules. Fission yeast has long cytoplasmic arrays that run along the cell axis, whereas budding yeast lacks this kind of network structure; instead, cytoplasmic microtubules emanate from the spindle pole body and form bundles (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Hagan and Hyams, 1988).

Why Do the atb2 Mutants That Are Isolated Become Temperature Sensitive?

How does ts α2-tubulin (Atb2) protein act in a dominant manner over α1-tubulin (Nda2) at the restrictive temperature? There are several possibilities. The first is that ts Atb2 protein forms an abortive complex with some other protein(s), which usually execute a function that is essential for microtubule biogenesis and cell viability (Figure 7A). This scenario is not unique: a similar mutant has been reported and analyzed previously. Fission yeast contains two genes encoding type I protein phosphatases, $dis2^+$ and $sds21^+$ (Ohkura *et al.*, 1989). Unlike $nda2^+$ and $atb2^+$, neither $dis2^+$ nor $sds21^+$ is essential by itself, but simultaneous disruption leads to lethality. The *dis2-11* mutation, however, shows a dominant cs phenotype. In this case, the reason that *dis2-11* shows cs appears to be due to the absorption, by cs Dis2, of a protein called Sds22, which is a regulatory subunit of both type I protein phosphatases and is essential for cell viability (Ohkura and Yanagida, 1991; Stone *et al.*, 1993). Multicopy plasmids containing $dis2^+$, $sds21^+$, or $sds22^+$ genes are capable of suppressing cs *dis2-11*. These interactions are strikingly similar to the interactions between the tubulin genes that we present here. The observation that in the ts *atb2* mutant the relative ratio of Nda2 and ts Atb2 protein alters in a way that ts *atb2* cells appear to contain more ts Atb2 protein is intriguing. It has been reported that free α-tubulin molecules become highly unstable (Tian *et al.*, 1997). It is possible that in the ts *atb2* mutant interaction between Nda2 and other essential proteins is compromised, which may lead to an apparent reduction of the relative ratio of Nda2 and ts Atb2 protein. The rescue of ts *atb2* by high-dosage $nda2^+$ gene supports this assumption.

β-Tubulin is a strong candidate for a protein that is absorbed by the ts Atb2 protein. The data, however, show that this is probably not the case, because an increased dosage of the β-tubulin gene did not sup-

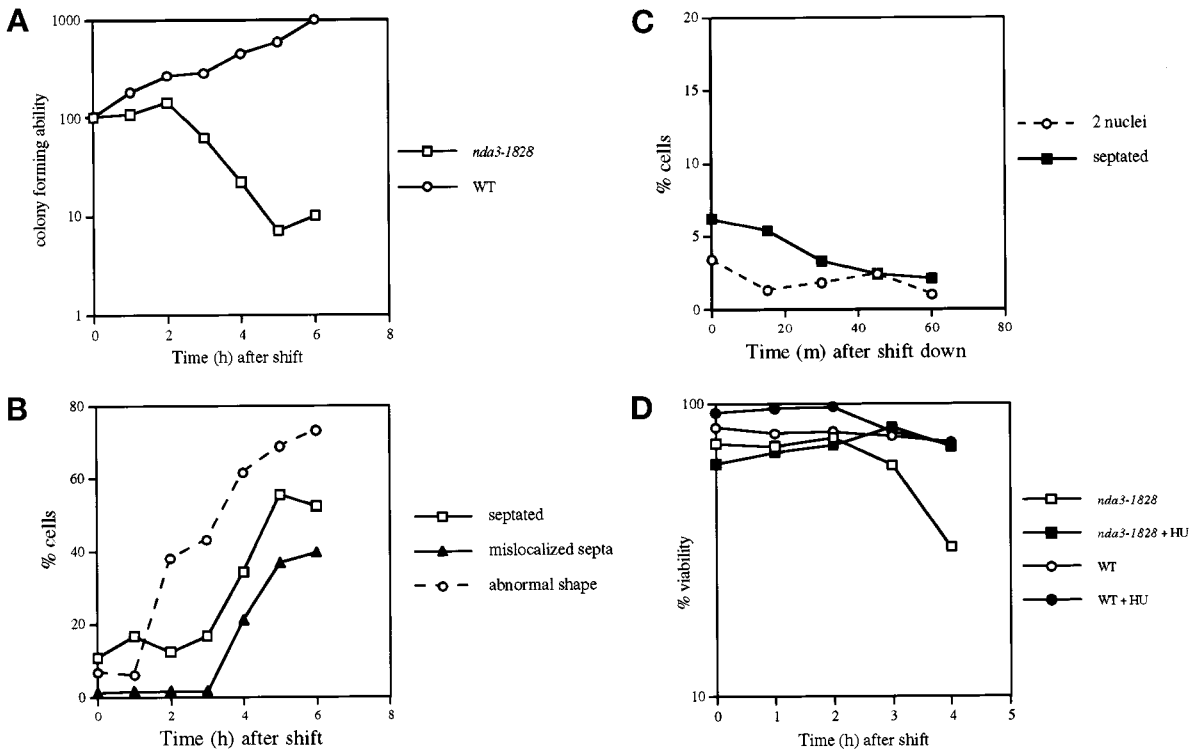


Figure 6. ts *nda3* mutants proceed through the cell cycle and lose viability at the restrictive temperature. (A and B) ts *nda3* mutant (DH12; Table 1) and wild-type (HM123) cells were grown at 26°C and shifted to 35.5°C, and aliquots were collected hourly. After appropriate dilution (10^{-4}), cells were plated on rich YES plates to examine colony-forming ability per culture (A, the value at time 0 was taken as 100%; ○, wild type; □, ts *nda3*). Vertical axis (%) is shown logarithmically. Septation index and percentage of cells with abnormal cell morphology in the ts *nda3* mutant (B, □ and ○, respectively) were observed with Calcofluor staining, and the percentage of cells that showed septa displaced from the center of the cell is also shown (▲). In wild-type cells, these abnormal cells were never observed (<0.1%). (C) For the reversibility experiments, ts *nda3* cells incubated at 35.5°C for 2 h (note that viability is still high at this time point; see A) were shifted down to 26°C, and septation index (using Calcofluor, ■) and the percentage of cells with two nuclei (using DAPI, ○) were scored at 15-min intervals for 1 h. (D) The same strains as in A (wild type shown as circles and ts *nda3* as squares) were grown in the presence (closed symbols) or absence (open symbols) of 10 mM hydroxyurea in rich YPD for 3 h at 27°C and shifted to 35.5°C. Cell number was measured at each time point, and viability was examined by plating cells (10^{-4} dilution) on rich YES plates. After plates were incubated at 27°C, the number of colonies was counted, and viability at each time point was calculated by dividing the number of viable cells by the cell number. Vertical axis (%) is shown logarithmically.

press the ts *atb2* mutant. Other candidates for interacting proteins are the cofactors that are required for the correct folding of tubulin molecules (Tian *et al.*, 1996). Cofactors B, D, and E are proposed to interact with α-tubulin to produce assembly-competent α/β-tubulin heterodimers (Tian *et al.*, 1997). All of these molecules are essential for cell viability in fission yeast (cofactor D/Alp1 [Hirata *et al.*, 1998; Radcliffe and Toda, unpublished observations]). Preliminary analysis from our laboratory, however, makes this possibility also unlikely because none of the fission yeast homologues of these cofactors suppress the ts *atb2* mutant when introduced on multicopy plasmids (Radcliffe and Toda, unpublished observations). There may, of course, be other α-tubulin binding proteins, such as microtubule-associated proteins (MAPs), that await identification.

The Implications of the Mutation Sites in ts Atb2 Protein

The second possibility is that ts Atb2 protein dominantly interferes with the assembly of newly synthesized α/β-tubulin heterodimers (Figure 7A). The electron crystallographic structure of the α/β heterodimer was recently solved (Nogales *et al.*, 1998). The tubulin molecule is divided into three functional domains, namely the N-terminal domain (1–205), which is responsible for GTP binding, the intermediate domain (206–381), which is required for heterodimer and/or intradimer formation, and the remaining C-terminal domain, which is thought to be important for interactions with various MAPs and motors. Assignment of the mutation sites of ts *atb2* (*-996* and *-1212*), which reside in the central domain, to the three-dimensional structure of α/β-heterodimers has proved illuminat-

ing. Cysteine 356 (mutated to tyrosine in *atb2-996*) is located at the longitudinal interface between the α- and β-tubulin monomers (Nogales *et al.*, 1998). It is also the residue whose analogous position in β-tubulin is the binding site for the tubulin-depolymerizing drug colchicine (Bai *et al.*, 1996). Interestingly, glycine 246 (mutated to aspartate in *atb2-1212*) is spatially adjacent to the β-sheet in which cysteine 356 is included, and furthermore, both of these amino acid residues are in close contact with the GTP/GDP exchangeable site of β-tubulin (Nogales *et al.*, 1998). It is also noteworthy that the region adjacent to glycine 246 (242LRFEG246) shows homology to regions that possess ribose-binding activity within various ATPases (Burns and Farrell, 1996). All of these facts raise the interesting possibility that the ts *atb2* mutation alters the physical interaction between α- and β-tubulin, or between α/β-heterodimers, and is accompanied by an alteration in the level of GTP/GDP exchange or hydrolysis of β-tubulin.

In the worm *Caenorhabditis elegans*, the mutation analogous to G246D was isolated in β-tubulin–encoding *mec-7* (G244S, called *u129*) (Savage *et al.*, 1994). Consistent with the fission yeast *atb2-1212* mutant, the *u129* mutant exhibits a dominant phenotype. Thus it appears that for either α- or β- tubulin, this conserved glycine has an essential role in microtubule biogenesis and/or assembly, and that mutation of this residue results in a dominant phenotype. Given the high degree of conservation in both sequence (invariant glycine) and function (dominant mutations) in higher eukaryotic systems in which mutants are not readily available, inducible expression of this mutant form of tubulin might be a useful approach for conditionally disrupting microtubules. This hypothesis is currently being tested.

ts Mutation in the β-Tubulin–encoding nda3$^+$ Gene

The toxicity of the high-dosage *atb2*$^+$ gene and suppression by its deletion in the ts *nda3* mutant suggest an altered affinity of ts Nda3 toward the Atb2 protein. It is possible that heterodimers of Atb2/ts Nda3 might specifically interfere with microtubule assembly and/or maintenance in this mutant. However, because *nda2*$^+$ is an essential gene, we are unable to similarly test whether its deletion rescues ts of *nda3-1828*. Further analysis will be required to establish any distinction between the two α-tubulin molecules in terms of their specific interaction with ts Nda3.

The mutation site in *nda3-1828* resides in the C-terminal region of the molecule Y422H. In animal cells, the C-terminal region is responsible for interactions with MAPs (MAP-1 and MAP-2) (Rivas *et al.*, 1988; Cross *et al.*, 1991), and the crystallographic analysis shows that the C-terminal region resides on the outer surface of the microtubule (Nogales *et al.*, 1998).

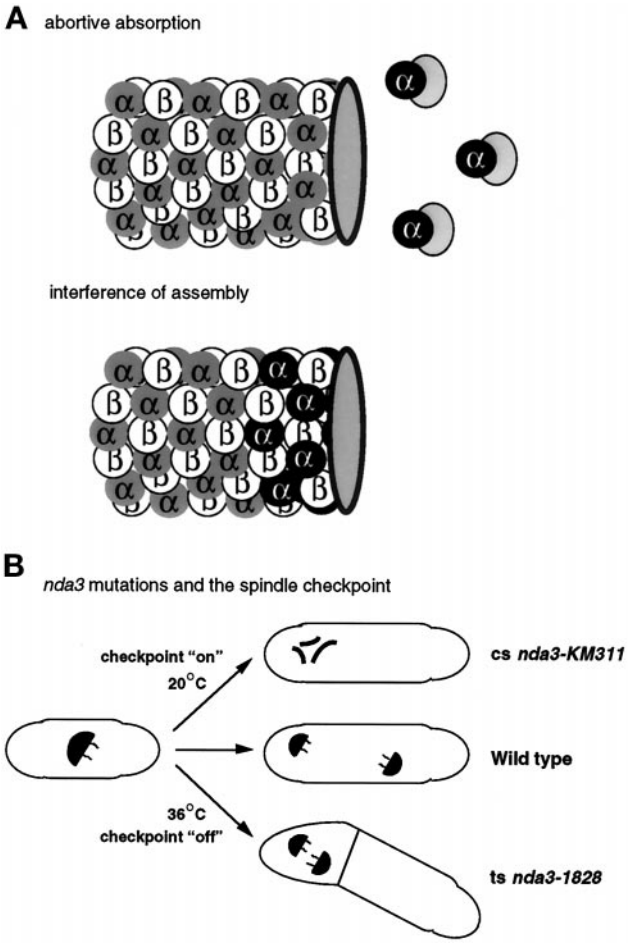


Figure 7. Possible models for novel ts mutations in tubulin genes. (A) ts Atb2 protein irreversibly absorbs proteins essential for microtubule biogenesis and assembly, which results in the disappearance of microtubules at the restrictive temperature (abortive absorption). ts Atb2 protein (α2-tubulin) is incorporated into microtubule structures, but interferes with the assembly of microtubules, which also results in microtubule disassembly (interference of assembly). ts Atb2 protein is shown as black circles with α in white letters, Nda2 (α1-tubulin) as gray circles with α in black letters. Hypothetical proteins that are essential for microtubule structures are shown in dotted circles bound to ts Atb2 proteins. (B) Differences in the defective phenotypes of cs *nda3-KM311* and ts *nda3-1828* mutants are shown. *nda3* mutants arrest at mitosis with condensed chromosomes in which the spindle assembly checkpoint is activated (on), whereas ts *nda3* mutants proceed through mitosis without activating the checkpoint (off), form septa, and eventually partial chromosome segregation occurs.

Systematic mutational analysis of budding yeast β-tubulin also suggests that regions near the C terminus are essential for microtubular function (Reijo *et al.*, 1994), and a truncation from glutamate 431 results in a ts growth defective phenotype (Matsuzaki *et al.*, 1988). Recently an essential protein that shows properties similar to mammalian MAPs has been identified in budding yeast (Irminger-Finger *et al.*, 1996). It is there-

fore possible that the C-terminal region is also required for an interaction with MAPs in fission yeast and that this interaction is perturbed in the ts *nda3* mutant.

Despite extensive mutational analysis of β-tubulin genes, only a few ts mutants have been successfully isolated to date. In contrast, many cs mutations have been isolated (Oakley and Morris, 1981; Thomas *et al.*, 1985; Huffaker *et al.*, 1988; Matsuzaki *et al.*, 1988; Stearns and Botstein, 1988; Davis *et al.*, 1994; Reijo *et al.*, 1994; Savage *et al.*, 1994; Sage *et al.*, 1995). In view of this, the *nda3-1828* mutant is of interest and may be a useful tool with which to obtain further insight into the structure and function of microtubules.

Mitotic Spindle Assembly Checkpoint and ts Tubulin Mutants

A surprising observation arising from this study is that in both ts *atb2* and *nda3* mutants, it appears that the spindle assembly checkpoint is not operational (Figure 7B). In these mutants, cell cycle events such as septation, which usually occur after bipolar spindle function, continue to take place at the restrictive temperature despite defects in mitosis. In contrast, previously identified cs *nda3-KM311* mutant cells arrested uniformly in midmitosis with condensed chromosomes and no septa (Umesono *et al.*, 1983b; Hiraoka *et al.*, 1984). This is the typical terminal phenotype when the spindle assembly checkpoint is functional. One possible explanation of this phenotypic difference is that in the ts *nda3* mutant, microtubular function, especially spindle function, is insufficiently defective to activate the spindle assembly checkpoint. As shown in Figure 2, we sometimes observe short residual spindle microtubules in ts *nda3* cells incubated at the restrictive temperature. A similar phenotype has been observed in ts *atb2* and cs *nda2* mutants (Umesono *et al.*, 1983b; this study). Because fission yeast has two α-tubulin–encoding genes, a single mutation may be unable to completely abrogate the function of the other gene, and as a result partial microtubular function remains. It is therefore possible that the temperature lethality of *nda3-1828* mutant cells arises from abortive cell cycle progression attributable to residual spindle function, preventing activation of the checkpoint machinery rather than cell cycle arrest caused by the loss of microtubules.

It is worth pointing out that the phenotype of the ts *nda3* mutant, which loses viability at the restrictive temperature, is similar to that of spindle assembly checkpoint mutants such as cs *nda3-KM311 mad2*, *nda3-KM311cdc16*, and *nda3-KM311dma1* double mutants in which components of the spindle assembly checkpoints are missing (Fankhauser *et al.*, 1993; Murone and Simanis, 1996; He *et al.*, 1997). The *nda3-1828* mutant could prove useful in the genetic dissection of the transduction mechanisms that monitor spindle defects via the checkpoint machinery.

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