STU1, a Suppressor of a β-Tubulin Mutation, Encodes a Novel and Essential Component of the Yeast Mitotic Spindle

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Abstract. We have isolated a cold-sensitive allele of *TUB2*, the sole gene encoding β-tubulin in *S. cerevisiae*, that confers a specific defect in spindle microtubule function. At 14°C, *tub2-406* cells lack a normal bipolar spindle but do assemble functional cytoplasmic microtubules. In an attempt to identify proteins that are important for spindle assembly, we screened for suppressors of the cold-sensitivity of *tub2-406* and obtained four alleles of a novel gene, *STU1*. Genetic interactions between *stu1* alleles and alleles of *TUB1* and *TUB2* suggest that Stu1p specifically interacts with microtubules. *STU1* is essential for growth and disruption of *STU1* causes defects in spindle assembly that are similar to those produced by the *tub2-406* mutation. The nucleotide sequence of the *STU1* gene predicts a protein product of 174 kD with no significant similarity to known proteins. An epitope-tagged Stu1p colocalizes with microtubules in the mitotic spindle of yeast. These results demonstrate that Stu1p is an essential component of the yeast mitotic spindle.

Chromosome segregation in eukaryotic cells is mediated by the mitotic spindle, a complex bipolar structure comprised of microtubules and associated proteins. Spindle microtubules fall into two classes: kinetochore microtubules attach chromosomes to the poles, and polar microtubules interdigitate with their counterparts from the opposite spindle pole. Astral microtubules radiate away from the mitotic spindle apparatus. Mitosis in the budding yeast *Saccharomyces cerevisiae* is similar to that in higher eukaryotes except in the former case, the nuclear envelope remains intact throughout mitosis, and the yeast spindle poles, called spindle pole bodies (SPBs),[1] are embedded in the nuclear envelope (Byers, 1991). Therefore, astral microtubules reside in the cytoplasm and remain compartmentally separated from spindle microtubules in the nucleus.

Genetic approaches have led to the identification of several microtubule-associated proteins in yeast. Six of these are microtubule motor proteins including five kinesins (Meluh and Rose, 1990; Hoyt et al., 1992; Lillie and Brown, 1992; Roof et al., 1992) and one dynein heavy chain (Eschel et al., 1993; Li et al., 1993). Three of the kinesins, Kar3p, Cin8p and Kip1p, are important for mitotic spindle function. Cin8p and Kip1p produce outward forces that operate on the spindle poles during spindle assembly (Hoyt et al., 1992; Roof et al., 1992). Kar3p is thought to exert a force that opposes the actions of Cin8p and Kip1p (Saunders and Hoyt, 1992; Hoyt et al., 1993). The roles of the other kinesins and dynein heavy chain are not yet clear. A few non-motor microtubule-associated proteins have also been found in yeast. Cik1p is tightly associated with Kar3p and may serve to regulate the cellular compartment in which Kar3p functions (Page and Snyder, 1992; Page et al., 1994). Bik1p localizes to the mitotic spindle and cells lacking Bik1p display defects in microtubule assembly and microtubule-mediated functions (Berlin et al., 1990). In addition, a 38- and a 50-kD protein, identified by their affinity for tubulin in vitro, have been shown to colocalize with both cytoplasmic and nuclear microtubules (Barnes et al., 1992). The precise functions of these proteins are as yet unknown.

Microtubules are polymers of the α-tubulin/β-tubulin dimer. In *S. cerevisiae*, α-tubulin is encoded by two genes, *TUB1* and *TUB3* (Schatz et al., 1986), and β-tubulin is encoded by a single gene, *TUB2* (Neff et al., 1983). A number of conditional-lethal alleles of the *TUB2* gene have been isolated, and some of these display specific defects in cytoplasmic or spindle microtubule function (Thomas et al., 1985; Huffaker et al., 1988; Sullivan and Huffaker, 1992; Reijo et al., 1994). *tub2* mutants that lack only cytoplasmic microtubule function fail to orient the spindle in the mother-bud neck (Sullivan and Huffaker, 1992). However, chromosome segregation is unimpeded and occurs entirely within the mother cell. Subsequent cytokinesis produces one cell with two nuclei and one anucleate cell. The opposite effect is observed in *tub2* mutants that lack only spindle microtubule function (Reijo et al., 1994). The spindle poles are moved to the mother-bud neck, but chromosome segregation does

1. *Abbreviations used in this paper*: DAPI, 4′6′-diamidino-2-phenylindole; HA, hemagglutinin; SPB, spindle pole body.

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not occur. These cells arrest with large buds and a single region of nuclear DNA at the bud neck.

In this report we describe a cold-sensitive allele of *TUB2*, *tub2-406*, that causes a specific defect in spindle microtubule function. To identify spindle-associated proteins, we screened for second-site mutations that are able to suppress the cold-sensitivity of *tub2-406*. This approach led to the discovery of a novel protein, Stu1p, that is an essential component of the yeast mitotic spindle.

Materials and Methods

Yeast Strains and Media

The yeast strains used for these experiments are listed in Table I and are essentially isogenic with the S288C background. YPD and SD media were prepared as described by Sherman (1991). Sporulation medium was 0.3% potassium acetate.

Isolation and Characterization of the tub2-406 Allele

A collection of 700 heat-sensitive and cold-sensitive yeast mutants generated by ethyl methane sulfonate mutagenesis was obtained from D. Botstein (Stanford University, Stanford, CA). Cells were grown in YPD medium at 26°C to early log phase and subsequently shifted to the restrictive temperature (4 h at 37°C for heat-sensitive strains, 16 h at 14°C for cold-sensitive strains). Microtubules were visualized by immunofluorescence microscopy (described below). One of the cold-sensitive mutants displayed defects in spindle assembly as described in the Results. This mutant was backcrossed twice to CUY10, and cold-sensitivity segregated as a single genetic locus. Complementation and linkage analysis revealed that cold-sensitivity was caused by a mutation in *TUB2*. We designated this allele *tub2-406*.

The method for recovery of the chromosomal *tub2-406* mutation on a plasmid has been described (Thomas et al., 1985). The *tub2-406* allele on this plasmid, pDP80, was completely sequenced on one strand using oligonucleotide primers and the USB Sequenase version 2.0 kit.

Isolation of Spontaneous Suppressors of tub2-406

100 individual colonies (~10^6 cells per colony) of strain CUY393 were each resuspended in 100 μl sterile water and spread onto separate YPD plates. After incubation at 16°C for 10 d, colonies that arose were tested for growth at 14°C for 5 d. To ensure independence of the suppressors, only one cold-resistant colony from each plate was pursued. Each was mated to CUY398 to determine if suppression was dominant or recessive at 14°C. The resulting diploids were then sporulated, and tetrads were dissected to determine if suppression segregated as a single locus, and if so, if the mutations were intragenic (linked to the *TUB2* locus) or extragenic. The extragenic mutations were placed into two linkage groups by crossing the suppressors in all pairwise combinations. To determine if either loci was *TUB1* or *TUB3*, one or two strains from each linkage group were crossed to DPY133 and DPY134. For all genetic crosses, we dissected at least 11 tetrads, and we defined genes as "tightly linked" if no recombinants (i.e., all parental ditypes) were observed.

Allele Specificity

The *stu1-1* and *stu1-2* alleles were crossed into 15 strains containing the following cold-sensitive *tub2* alleles: *tub2-104*, *tub2-120*, *tub2-209*, *tub2-401*, *tub2-402*, *tub2-403*, *tub2-404*, *tub2-407*, *tub2-412*, *tub2-418*, *tub2-423*, *tub2-429*, *tub2-434*, *tub2-445*, and *tub2-451* (Thomas et al., 1985; Huffaker et al., 1988; Reijo et al., 1994). For each diploid, either the *TUB2* or the closely linked *ACT1* chromosomal locus was marked with *URA3*. The diploids were sporulated and tetrads dissected. Haploid segregants were scored for cold-sensitivity and uracil auxotrophy.

Cloning and Sequencing STU1

The *STU1* gene was cloned by complementation of the *stu1-1* suppressor phenotype. CUY529 was transformed with a yeast genomic library constructed in the *URA3* YCp50 vector (Rose et al., 1987). One of the Ura^+ transformants failed to grow at 14°C. The plasmid recovered from this strain, pDP1, was able to complement both the *stu1-1* and *stu1-2* mutations; CUY529 and CUY535 containing pDP1 failed to grow at 14°C. Subclones and ExoIII nested deletions of the 11.5-kb insert localized the complementing activity to a 5-kb region. To confirm that the *STU1* gene had been cloned, a 3.5-kb EcoRI fragment outside the complementing region was

Table I. Yeast Strains

Strain	Genotype
CUY10	*MATa his4-539 lys2-801 ura3-52*
CUY392	*MATα tub2-406 his4 ura3-52*
CUY393	*MATa tub2-406 his4 ura3-52*
CUY398	*MATα tub2-406 ACT1::URA3::ΔACT1 his4 lys2-801 ura3-52*
CUY518	*MATa/MATα tub2-406/tub2-406 stu1-1/STU1 ACT1::URA3::ΔACT1/ACT his4/his4 lys2-801/LYS2 ura3-52/ura3-52*
CUY523	*MATa/MATα tub2-406/tub2-406 tub1-108/TUB1 ACT1::URA3::ΔACT1/ACT1 his4/his4 lys2-801/LYS2 ura3-52/ura3-52*
CUY527	*MATα tub2-406 stu1-3 ACT1::URA3::ΔACT1 his4 ura3-52*
CUY529	*MATa tub2-406 stu1-1 his4 ura3-52*
CUY530	*MATα tub2-406 stu1-1 ACT1::URA3::ΔACT1 his4 ura3-52*
CUY531	*MATa tub2-406 tub1-107 his4 lys2-801 ura3-52*
CUY533	*MATα tub2-406 stu1-4 ACT1::URA3::ΔACT1 his4 lys2-801 ura3-52*
CUY535	*MATa tub2-406 stu1-2 his4 ura3-52*
CUY536	*MATα tub2-406 stu1-2 ACT1::URA3::ΔACT1 his4 lys2-801 ura3-52*
CUY538	*MATα tub2-406 tub1-108 his4 ura3-52*
CUY540	*MATa/MATα tub2-406/tub2-406 stu1-1/STU1 tub1-108/TUB1 his4/his4 ura3-52/ura3-52*
CUY544	*MATa/MATα tub2-406/tub2-406 his4/his4 ura3-52/ura3-52*
CUY545	*MATa/MATα tub2-406/tub2-406 cdc16-1/cdc16-1 his4/his4 ura3-52/URA3*
CUY546	*MATa/MATα ade2/ADE2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52*
CUY547	*MATa/MATα stu1-Δ1::HIS3/STU1 ade2/ADE2 his3-Δ200/his3-Δ200 leu2-3,112/ leu2-3,112 ura3-52/ura3-52*
CUY548	*MATa stu1-Δ1::HIS3 ade2 his3-Δ200 leu2-3,112 ura3-52 (pDP66 - CEN URA3 STU1)*
CUY549	*MATa stu1-Δ1::HIS3 ade2 his3-Δ200 leu2-3,112 ura3-52 (pDP79 - CEN URA3 STU1::HA_3)*
CUY573	*MATα stu1-Δ1::HIS3 ade2 his3-Δ200 leu2-3,112 ura3-52 (pDP83 - 2 μm URA3 STU1::HA_3)*
CUY574	*MATα stu1-Δ1::HIS3 ade2 his3-Δ200 leu2-3,112 ura3-52 (pDP85 - 2 μm URA3 STU1)*
DPY133	*MATα tub2-406 TUB1::URA3::ΔTUB1 his3-Δ200 lys2-801*
DPY134	*MATα tub2-406 TUB3::URA3::TUB3 ade2 leu2-3,112 his3-200 ura3-52*

subcloned into the *URA3* integrating plasmid, pRS306 (Sikorski and Hieter, 1989), to make plasmid pDP2. BamHI was used to cut pDP2 once within the insert and once within the plasmid multiple cloning site. The resulting DNA fragment was transformed into CUY529 and CUY535. Ura$^+$ transformants of both strains were crossed to CUY392, and tetrads were dissected. In both cases, only parental genotypes were obtained; for each tetrad, two spores were Ura$^+$ and cold-resistant, and two spores were Ura$^-$ and cold-sensitive. To physically map the *STU1* gene, an 800-bp EcoRI fragment from the genomic insert of pDP1 was used to probe lambda phage containing overlapping clones of yeast genomic DNA (Riles et al., 1993). The probe hybridized to overlapping clones, ATCC numbers 70689 and 70851, corresponding to the left arm of chromosome II. The nearest mapped gene is *ILS1* on clone number 70323.

To sequence *STU1*, the following fragments (see Fig. 6) were subcloned from pDP1 into their corresponding sites in the CEN-based pRS416 vector (Sikorski and Hieter, 1989): the 4.4-kb ClaI fragment (pDP5), the 4.7-kb XbaI fragment (pDP6), the 8.0-kb KpnI fragment (pDP13), the 1.4-kb XbaI-SacI fragment (pDP67), and the 0.9-kb KpnI-ClaI fragment (pDP71). ExoIII nuclease was used to construct a series of nested deletions from either one or both ends of these subcloned fragments. Both strands of the *STU1* gene were sequenced using the USB Sequenase Version 2.0 kit and double-stranded templates. The nucleotide sequence and the predicted protein sequence were analyzed using the Genetics Computer Group (Devereux et al., 1984) software.

Disruption of STU1

The *STU1* gene was disrupted by the one-step gene replacement method (Rothstein, 1991). The 8-kb genomic BamHI fragment containing the *STU1* locus was cloned into the BamHI site of pRS416 to create pDP11 (see Fig. 6). Unidirectional ExoIII digestion from the downstream BamHI site of pDP11 was used to delete most of the 3′ noncoding region and remove the two downstream ClaI sites. The resulting plasmid, pDP21, contains the entire *STU1* gene and ~65 bp past the putative stop codon. The upstream multiple cloning site of pDP21 was modified by removing the BamHI, XbaI, SpeI and NotI sites; the plasmid was cleaved at the unique sites BamHI and NotI, treated with Klenow to generate blunt ends, and religated. The resulting plasmid, pDP65, was cleaved at the unique ClaI site within *STU1* and blunt ends were generated with Klenow. The plasmid was then cleaved at the unique XbaI site within *STU1* and an XbaI-blunt end fragment containing the *HIS3* gene was ligated therein. In the resulting plasmid, pDP69, a 2.5-kb internal *STU1* fragment is replaced with the 1.4-kb *HIS3* gene. This construct deletes *STU1* sequences encoding amino acids 175–997 and is referred to as *stu1-Δ1::HIS3*. A 2.9-kb fragment containing *stu1-Δ1::HIS3* was liberated from pDP69 by digestion with EcoRI and SacII, gel purified, and transformed into a wild-type diploid strain CUY546.

To analyze the phenotype of cells carrying the *stu1-Δ1::HIS3* deletion, CUY547 was sporulated in 0.3% potassium acetate for 3 d, at which point >80% sporulation was observed. To break the asci, cells were incubated in 100 μg/ml Zymolyase 100T (ICN Biomedicals, Inc., Costa Mesa, CA) in 23 mM β-mercaptoethanol for 1 h at 37°C, resuspended in 1.5% NP-40 and sonicated briefly. The cells were then resuspended in sterile water and sonicated again. Microscopic examination revealed that 86% of the cells were single, free spores; 7% were either associated as a diad, triad, or tetrad; and 7% were apparently unsporulated diploids that had resisted spheroplasting, detergent, and sonication. The cells were then added to YPD and incubated at 30°C with vigorous shaking for 7 h. At this point, the cells were fixed with formaldehyde and processed for immunofluorescence as described below.

Epitope Tagging Stu1p

DNA encoding three copies of the influenza hemagglutinin (HA) epitope was cloned into the *STU1* gene to generate the *STU1::HA$_3$* construct. The plasmid GTEPI (from B. Futcher, Cold Spring Harbor Laboratory, NY) contains a DNA fragment encoding three adjacent copies of the HA epitope cloned into the NotI site in the polylinker of pBluescript II SK- (Stratagene, La Jolla, CA). To isolate this fragment, GTEPI was digested with SacI and the 3′ overhang was converted to a blunt-end with T4 DNA polymerase. A 10-bp linker containing the XbaI recognition site was ligated to the blunt end. The plasmid was then digested with XbaI to cleave in the linker and at the XbaI site of the Bluescript polylinker, liberating a 138-bp fragment containing the HA$_3$ tag. This fragment was then ligated in frame into the XbaI site of pDP65, corresponding to amino acid residue 997 of Stu1p. Plasmids from *E. coli* transformants were screened for correct orientation of the insert using appropriate restriction enzymes. One such plasmid, pDP79, was transformed into yeast diploid strain CUY547 (*stu1-Δ1::HIS3/STU1*). Transformants were sporulated and tetrads were dissected. In tetrads with four viable spores, the two His$^+$ spores were always Ura$^+$, indicating that the plasmid containing *STU1::HA$_3$* complements the *stu1-Δ1::HIS3* disruption. Furthermore, His$^+$Ura$^+$ spores were unable to grow on media containing 5-fluoroorotic acid (Boeke et al., 1984), indicating that maintenance of plasmid pDP79 is essential for viability. To express the *STU1::HA$_3$* gene from a 2-μm plasmid, the 5.5-kb KpnI fragment from pDP79 that contains the entire *STU1::HA$_3$* construct was subcloned into the KpnI site of pRS426 (Christianson et al., 1992). The resulting plasmid, pDP83, was transformed into CUY547. Tetrad analysis demonstrated that *STU1::HA$_3$* on a 2-μm plasmid also complements the *stu1-Δ1::HIS3* disruption, and maintenance of pDP83 is essential for viability.

Immunofluorescence Microscopy

To visualize microtubules, cells were fixed at their last growth temperature by adding formaldehyde to a final concentration of 3.7% and incubating at this temperature for ~2 h. To visualize SPBs, cells were fixed as described for only 20 min as the SPB antigen is sensitive to formaldehyde fixation. To visualize both microtubules and the HA epitope, cells were fixed for 25 min at 30°C to maintain the integrity of the formaldehyde-sensitive HA epitope and to satisfactorily preserve microtubule structures. Fixed cells were then spheroplasted with 25 μg/ml Zymolyase 100T in 1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5, and 20 mM β-mercaptoethanol at 37°C for 30 min. Spheroplasts were labeled for immunofluoresence as described previously (Kilmartin and Adams, 1984). Rat monoclonal anti-yeast-α-tubulin antibody, YOL1/34 (Kilmartin et al., 1982), and a pool of mouse monoclonal antibodies against the 90-kD component of the yeast SPB (Rout and Kilmartin, 1990) were gifts from John Kilmartin (Medical Research Council, Cambridge, UK). Rabbit anti-β-tubulin polyclonal antibody 206 (Bond et al., 1986) was a gift from F. Solomon (Massachusetts Institute of Technology, Cambridge, MA). Mouse monoclonal antibody 12CA5 which recognizes the HA epitope (Wilson et al., 1984) was obtained from Berkeley Antibody Co. (Berkeley, CA). Fluorescein-conjugated goat anti-rat, rabbit anti-mouse, and goat anti-mouse antibodies and rhodamine-conjugated goat anti-rabbit antibodies were obtained from Cappel Research Products (Durham, NC). Before labeling the HA tag, both mAb 12CA5 and fluorescein goat anti-mouse antibodies were preadsorbed with wild-type fixed yeast spheroplasts overnight at 4°C. Cellular DNA was visualized by incubating spheroplasts in 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) for 5 min. For quantitation of phenotypes observed by indirect immunofluorescence, at least 200 cells were counted.

Electron Microscopy

Electron microscopy was performed as described by Byers and Goetsch (1991).

Results

tub2-406 Cells Are Defective in Spindle Assembly

We screened a collection of 700 conditional-lethal mutants by immunofluorescence microscopy for defects in mitotic spindle formation (see Materials and Methods). This screen identified one new cold-sensitive allele of *TUB2* that we designated *tub2-406*. This allele contains a single point mutation, a guanine to adenine transition in the first position of codon 100, that results in a valine to methionine amino acid substitution. At 30°C, the doubling time of *tub2-406* cells is the same as that of wild-type cells. However, *tub2-406* cells fail to grow at temperatures below 18°C. Like many other cold-sensitive *tub2* mutants (Thomas et al., 1985; Huffaker et al., 1988; Reijo et al., 1994), *tub2-406* cells display a mitotic cell cycle arrest at the nonpermissive temperature. When asynchronous cultures of *tub2-406* homozygous diploid cells were shifted to 14°C for 15 h (about two generation times for wild-type cells at this temperature), 98% of

the cells arrested uniformly with large buds and unsegregated DNA located at the mother-bud neck (Fig. 1 *c*). Thus, the mutation blocks chromosome separation, but not nuclear migration.

We visualized microtubules in *tub2-406* cells by immunofluorescence microscopy. At the permissive temperature, *tub2-406* cells assembled metaphase microtubule arrays that resembled those observed in wild-type cells (Kilmartin and Adams, 1984). These cells contained brightly staining short intranuclear spindles with weaker staining cytoplasmic microtubules extending from each spindle pole (Fig. 1 *b*, cell at left). At 14°C, however, *tub2-406* cells lacked a typical short spindle (Fig. 1 *d*). Instead, these cells contained either two closely apposed bright dots or a single elongated bright spot of staining coincident with nuclear DNA. The intensity and location of these bright staining structures suggested that they represented nuclear microtubules. However, the length of these structures was less than that of a normal short spindle. All cells possessed cytoplasmic microtubules that extended from the area of bright staining into both the mother cell and bud. The cytoplasmic microtubules were longer and more numerous than those in wild-type cells. Thus, *tub2-406* cells failed to assemble normal short spindles but were capable of assembling extensive cytoplasmic microtubules at the nonpermissive temperature.

We observed two discrete dots of microtubule staining in some of the *tub2-406* cells at 14°C, suggesting that these cells contained duplicated and separated SPBs. To determine if this was indeed the case, we stained cells with an antibody specific for SPBs. In 98% of the cells, we could distinguish two separated dots of staining, confirming that the SPBs had separated to some extent (Fig. 1, *e* and *f*). However, the distance between SPBs in *tub2-406* cells appeared to be significantly less than the length of a normal short spindle. To more accurately determine this distance, we used electron microscopy and examined seven diploid *tub2-406* cells for which we could observe both SPBs in a single thin section. In four of these cells, the SPBs were located on opposite sides of the nuclear membrane (Fig. 2, *b* and *c*); the average distance between the SPB midpoints was 673 nm (SEM = 61 nm). This value is about 60% of the distance between separated SPBs in diploid cells with normal short spindles (Jacobs et al., 1988; see data on *cdc16-1* arrested diploids below). Microtubules extended from these SPBs into the nucleus, but neither the microtubules nor SPBs were organized into the antiparallel bipolar array characteristic of short spindles. Instead the microtubules splayed outward from the SPBs, and the SPBs did not directly face one another. In the other three cells, the SPBs were separated but resided on the same side of the nuclear membrane (Fig. 2 *a*). The average distance between these SPB midpoints was 821 nm (SEM = 84 nm). These cells also contained splayed

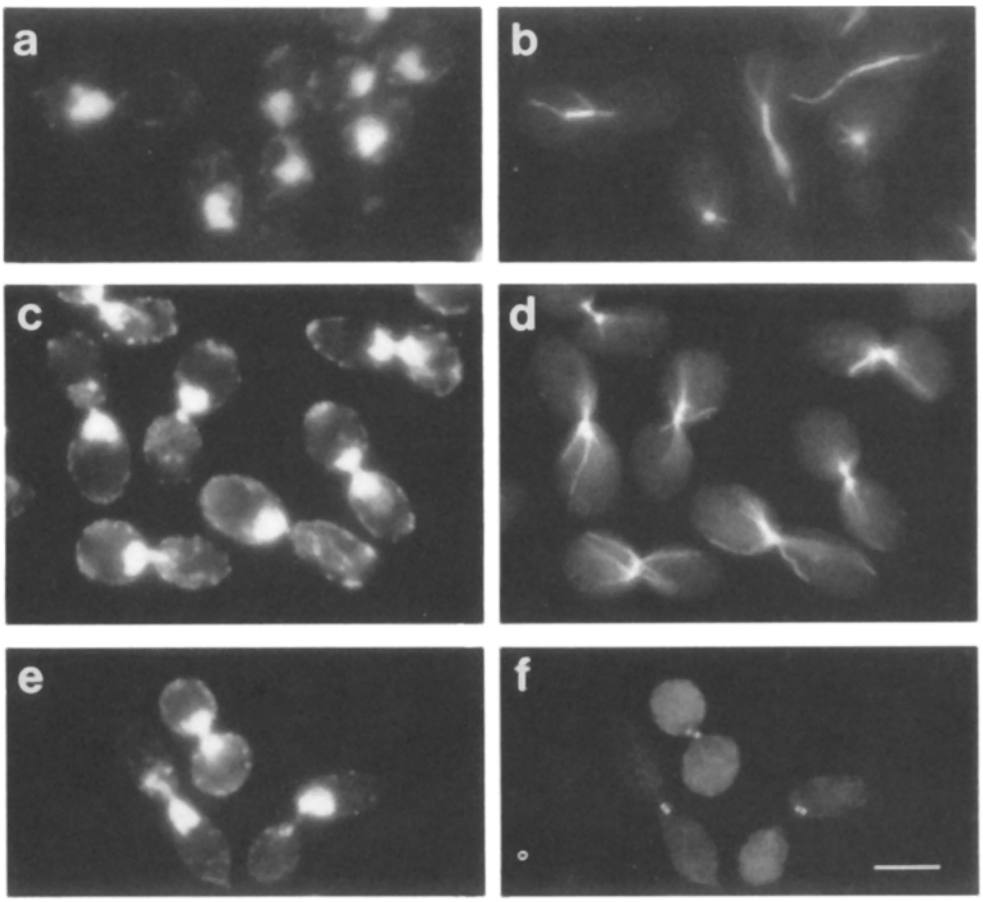

Figure 1. Phenotype of *tub2-406* cells at the permissive and nonpermissive temperatures. *tub2-406* homozygous diploid cells (CUY544) were grown at 30°C (*a* and *b*) and shifted to 14°C for 15 h (*c–f*). DAPI staining of cellular DNA (*a*, *c*, and *e*). Immunofluorescence microscopy of cells labeled with α-tubulin-specific antibody (*b* and *d*) and a SPB-specific antibody (*f*). Bar, 5 μm.

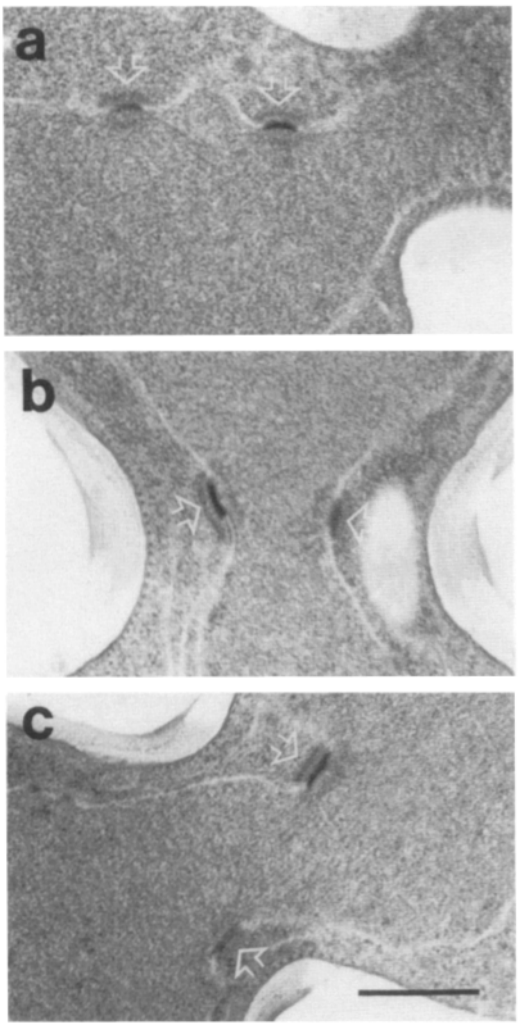

Figure 2. Electron micrographs of *tub2-406* cells at the nonpermissive temperature. *tub2-406* homozygous diploid cells (CUY544) were grown at 30°C and shifted to 14°C for 16 h. Arrows point to the SPBs in each cell. Bar, 0.5 μm.

microtubules that did not align with microtubules from the opposite pole. We did not observe side-by-side SPBs in this mutant.

The tub2-406 Mutation Causes Collapse of Short Spindles

We used a *tub2-406 cdc16-1* homozygous diploid to examine the effect of the *tub2-406* mutation on the stability of short spindles. Cells containing the *cdc-16-1* mutation are heat-sensitive and arrest prior to anaphase at 37°C; a *tub2-406 cdc16-1* double mutant is both cold and heat sensitive. When an asynchronous population of *tub2-406 cdc16-1* cells was shifted from 26 to 37°C for 4 h, the cells arrested uniformly with short spindles characteristic of the *cdc16-1* phenotype (Fig. 3, *a* and *b*). The average length of spindles in three of these cells was 1.10 μm (SEM = 0.16 μm) as determined by electron microscopy (Fig. 4 *a*). When these 37°C-arrested cells were subsequently shifted to 14°C for 30 min, the spindles collapsed into the abnormally short length characteristic of *tub2-406* single mutants, and likewise, the cytoplasmic microtubules lengthened (Fig. 3, *c* and *d*). We examined 14 of these latter cells by electron microscopy. In all cells, the SPBs remained on opposite sides of the nuclear membrane but were drawn into close proximity in the region of the mother-bud neck (Fig. 4 *b*). The average distance between the SPB midpoints was 628 nm (SEM = 52 nm), similar to the distance between SPBs on opposite sides of the nuclear membrane in the *tub2-406* single mutant. Side-by-side SPB configurations were not observed, nor were separated SPBs on the same side of the nuclear membrane. Taken together, these observations indicate that the *tub2-406* mutation causes partial collapse of preformed mitotic spindles.

Identification and Genetic Characterization of STU1

To isolate spontaneous suppressors to *tub2-406*, we plated CUY393 cells at 16°C. Cold-resistant colonies arose at a frequency of $\sim 10^{-6}$. Twenty-six independent mutants grew at wild-type rates at 14°C and were further characterized. For 11 of these mutants, suppression segregated as a single gene

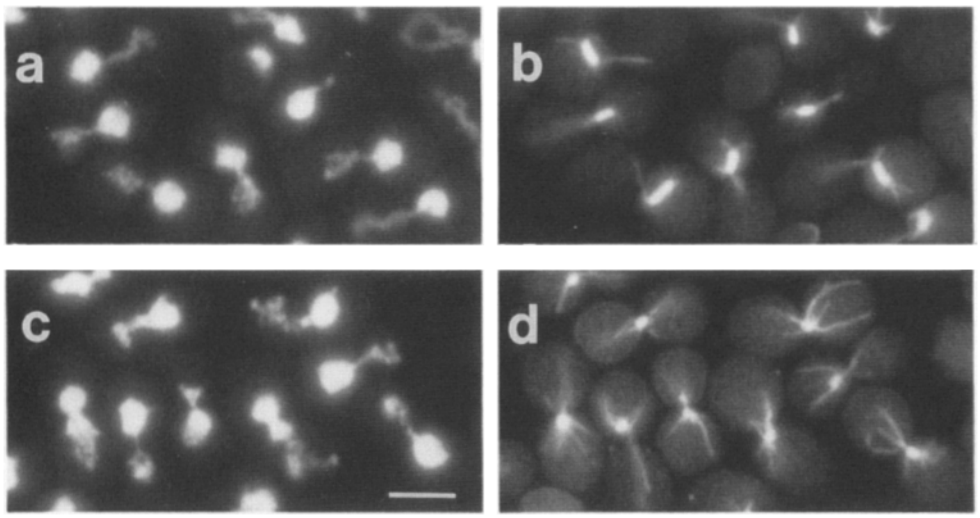

Figure 3. Microtubules in the *tub2-406 cdc16-1* double mutant. *tub2-406 cdc16-1* homozygous diploid cells (CUY545) were grown at 30°C, shifted to 37°C for 4 h (*a* and *b*), and subsequently shifted to 14°C for 30 min (*c* and *d*). Cells were stained with DAPI (*a* and *c*) and α-tubulin-specific antibody (*b* and *d*). Bar, 5 μm.

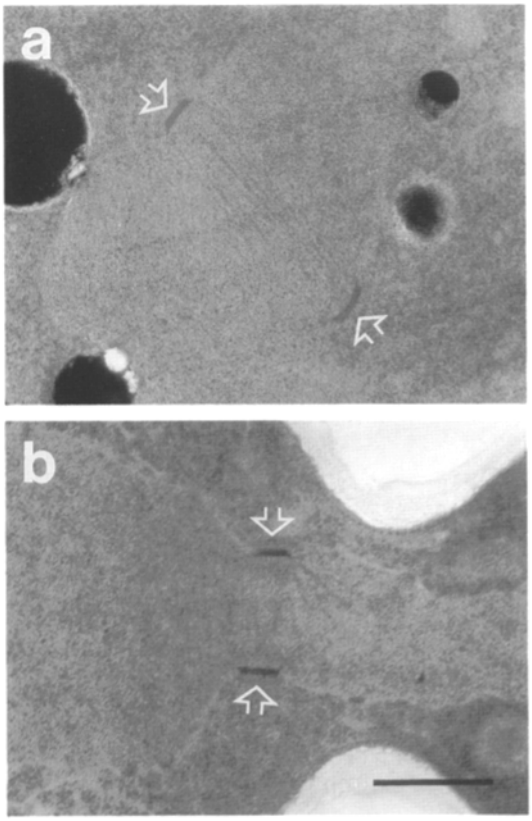

Figure 4. Electron micrographs of *tub2-406 cdc16-1* double mutant cells. *tub2-406 cdc16-1* homozygous diploid cells (CUY545) were grown at 30°C, shifted to 37°C for 5 h (*a*), and subsequently shifted to 14°C for 30 min (*b*). Arrows point to the SPBs in each cell. Bar, 0.5 μm.

mutation in backcrosses to a *tub2-406* strain. Five of these mutations were tightly linked to the *TUB2* locus and assumed to be intragenic suppressors. Two of the mutations were tightly linked to *TUB1*, the gene that encodes one of the two yeast α-tubulins. The remaining four mutations were tightly linked to one another. These latter mutations were recessive for suppression of *tub2-406* and failed to complement one other for this phenotype. We conclude that these four mutations identify a single gene that we have named *STU1* (for *s*uppressor of *tu*bulin). None of the *stu1* alleles conferred a conditional-lethal phenotype in either a *tub2-406* or *TUB2* background.

To test for genetic interactions between *stu1*^{sup} and other *tub2* alleles, we constructed haploid yeast strains containing either *stu1-1* or *stu1-2* and one of 15 other cold-sensitive *tub2* alleles (see Materials and Methods). *stu1-1* and *stu1-2* suppressed the cold-sensitivity of *tub2-423*, and *stu1-1* suppressed the cold-sensitivity of *tub2-434*. None of the other 13 *tub2* alleles were suppressed by either *stu1*^{sup} allele. Thus, suppression by *stu1-1* and *stu1-2* was allele specific. In addition, the double mutant yeast strains, *stu1-2 tub2-401*, *stu1-2 tub2-403*, and *stu1-2 tub2-104* were inviable at 30°C. At this temperature, yeast strains carrying any one of these alleles grew at wild-type rates. Thus, lethality results from the combination of two unlinked alleles, a phenomenon referred to as "synthetic lethality."

We have also observed genetic interactions between

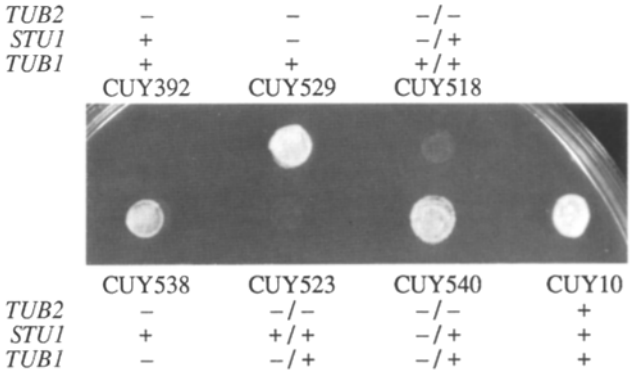

Figure 5. Unlinked noncomplementation of the *stu1-1* and *tub1-108* alleles. Strains were spotted at low density onto YPD plates and incubated at 14°C for 5.5 days. "+" indicates the *TUB2*, *STU1*, or *TUB1* wild-type alleles; "–" indicates the *tub2-406*, *stu1-1*, or *tub1-108* mutant alleles. Note that strain CUY540 can grow at 14°C, indicating that the *stu1-1* and *tub1-108* suppressor alleles fail to complement each other.

stu1^{sup} and *tub1*^{sup} alleles. All of the *stu1*^{sup} alleles and one of *tub1*^{sup} alleles, *tub1-108*, are recessive suppressors. In other words, *stu1*^{sup}*/STU1 tub2-406/tub2-406* and *tub1-108/TUB1 tub2-406/tub2-406* diploids are cold-sensitive. Nonetheless, each of the *stu1*^{sup} alleles failed to complement the *tub1-108* allele for suppression of *tub2-406*. For example, a diploid strain made by mating a *tub2-406 stu1-1* haploid and a *tub2-406 tub1-108* haploid grew at 14°C although the diploid contained a wild-type copy of both *STU1* and *TUB1* (Fig. 5). Thus, these alleles display unlinked noncomplementation.

STU1 Encodes a Novel Gene

The wild-type *STU1* gene was cloned by complementation of the *stu1-1* suppression phenotype. A yeast genomic library was transformed into CUY529 (*stu1-1*, *tub2-406*) and transformants were screened for plasmid dependent cold-sensitivity. One plasmid, pDP1, with an 11.5-kb insert was recovered. Genomic DNA from this insert directed integration of a *URA3* marker to the *STU1* locus, confirming that the clone contains the actual *STU1* gene. The *STU1* gene maps to the left arm of chromosome II, near *ILS1* (see Materials and Methods).

The complementing activity of pDP1 was localized to a 5-kb region (Fig. 6). This fragment contains a single 4,539-bp open reading frame that potentially encodes a 1,513–amino acid, 174-kD protein (Fig. 7). The predicted protein sequence showed no significant homology to any known proteins using either the BLAST (Altschul et al., 1990) or FASTA (Pearson and Lipman, 1988) database searches. We also divided the protein sequence into six 250–amino acid segments but none of these segments showed any significant similarly to known proteins. In addition, no matches were found with any protein motif patterns in the PROSITE database (Bairoch, 1992).

STU1 Is an Essential Gene Required for Spindle Assembly

To determine if the *STU1* gene product performs an essential function, we replaced the *STU1* sequence encoding amino acids 175–997 with a DNA fragment containing the *HIS3*

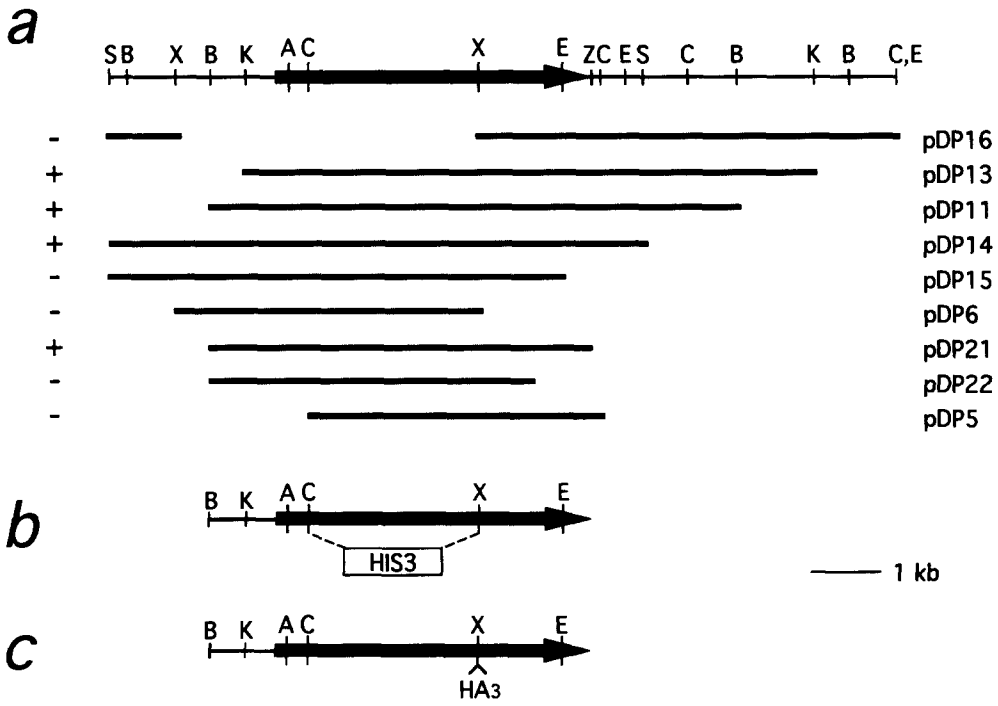

Figure 6. STU1 subclones and plasmid constructs. (*a*) The top bar represents the insert of pDP1 which complements the *stu1-1* allele. The *STU1* open reading frame is depicted by the solid arrow. Plasmids were constructed by subcloning restriction fragments into CEN-based shuttle vectors and by subjecting subclones to ExoIII nuclease digestion. Plasmid names are shown on the right (see Materials and Methods). Left, indicates whether each plasmid could (+) or could not (−) complement the *stu1-1* allele. Restriction sites used for subcloning are shown: *A*, SacII; *B*, BamHI; *C*, ClaI; *E*, EcoRI; *K*, KpnI; *S*, SalI; *X*, XbaI; *Z*, SacI. (*b*) The *STU1* gene was disrupted by removing the 2.5-kb ClaI-XbaI fragment and replacing it with a 1.4-kb fragment containing the *HIS3* gene. (*c*) A 138-bp fragment encoding the triple HA tag was inserted into the XbaI site; the reading frame was maintained.

gene. This construct was transformed into wild-type His⁻ diploids. Southern blotting of DNA from two independent His⁺ transformants confirmed that one copy of the *STU1* gene was disrupted by *HIS3* in each case (data not shown). These transformants were sporulated and 22 tetrads were dissected. All tetrads contained two His⁻ spores that were able to form colonies and two spores that were unable to form colonies, demonstrating that *STU1* is an essential gene (Fig. 8 *a*). Cells arising from the *stu1-Δ1::HIS3* spores were separated and examined under a dissecting microscope. In each case, we observed two to four large-budded cells, indicating that the *stu1-Δ1::HIS3* spores were able to germinate and undergo one to two cell divisions. The ability of *stu1-Δ1::HIS3* cells to proceed through one or two cell divisions may be attributable to the presence of wild-type Stu1p that the spores inherited from their heterozygous diploid parent. Only after this supply of Stu1p has been degraded or diluted during cell growth do the cells arrest. The observation that all spores ultimately arrested growth with large buds is consistent with Stu1p having an essential role in mitosis.

We further examined the arrest phenotype of cells containing the *STU1* disruption by microtubule immunofluorescence. *stu1-Δ1::HIS3/STU1* (CUY547) diploids were sporulated. The spores, which were an equal mixture of wild-type and *stu1-Δ1::HIS3* spores, were isolated and incubated at 30°C (see Materials and Methods). Preliminary experiments indicated that 7 h was sufficient time for most wild-type spores to germinate and complete one cell cycle. At this time, cells were fixed and processed for immunofluorescence. About 35% of the cells were large-budded, and of these, 60% were phenotypically wild type. The remaining 40% contained undivided DNA at the mother-bud neck, suggesting a cell cycle arrest. The microtubule arrays in these latter cells appeared identical to those observed by immunofluorescence in *tub2-406* cells (Fig. 8, *b–d*). The cells contained a short bright staining structure coincident with the nuclear DNA but lacked a typical bipolar spindle. Extending from this bright structure were unusually long and numerous cytoplasmic microtubules. This phenotype must be due to the *stu1-Δ1::HIS3* disruption because similar microtubule arrays were never observed in wild-type cells.

Although 40% of the large-budded cells displayed the mutant phenotype, this figure represented only 15% of the total cell population. Because *stu1-Δ1::HIS3* spores comprised half of the population before germination, one would expect up to 50% of the cells to show the mutant phenotype if the spores had germinated synchronously and the *stu1-Δ1::HIS3* cells had arrested uniformly in the first cell cycle. However, the spores did not germinate synchronously, as indicated by the fact that only 35% of the cells were large-budded. Moreover, tetrad analysis (see above) showed that *stu1-Δ1::HIS3* spores generally undergo one or two cell divisions before arresting. Therefore, we anticipated that a significant fraction of *stu1-Δ1::HIS3* cells would not display the arrest phenotype in this experiment.

Stu1p Colocalizes with the Mitotic Spindle

We used immunofluorescence microscopy to investigate the cellular location of Stu1p. We tagged Stu1p with three copies of the influenza HA epitope which is recognized by the monoclonal antibody 12CA5 (Wilson et al., 1984). The DNA fragment encoding the triple HA epitope was inserted within the coding region of *STU1* such that the open reading frame

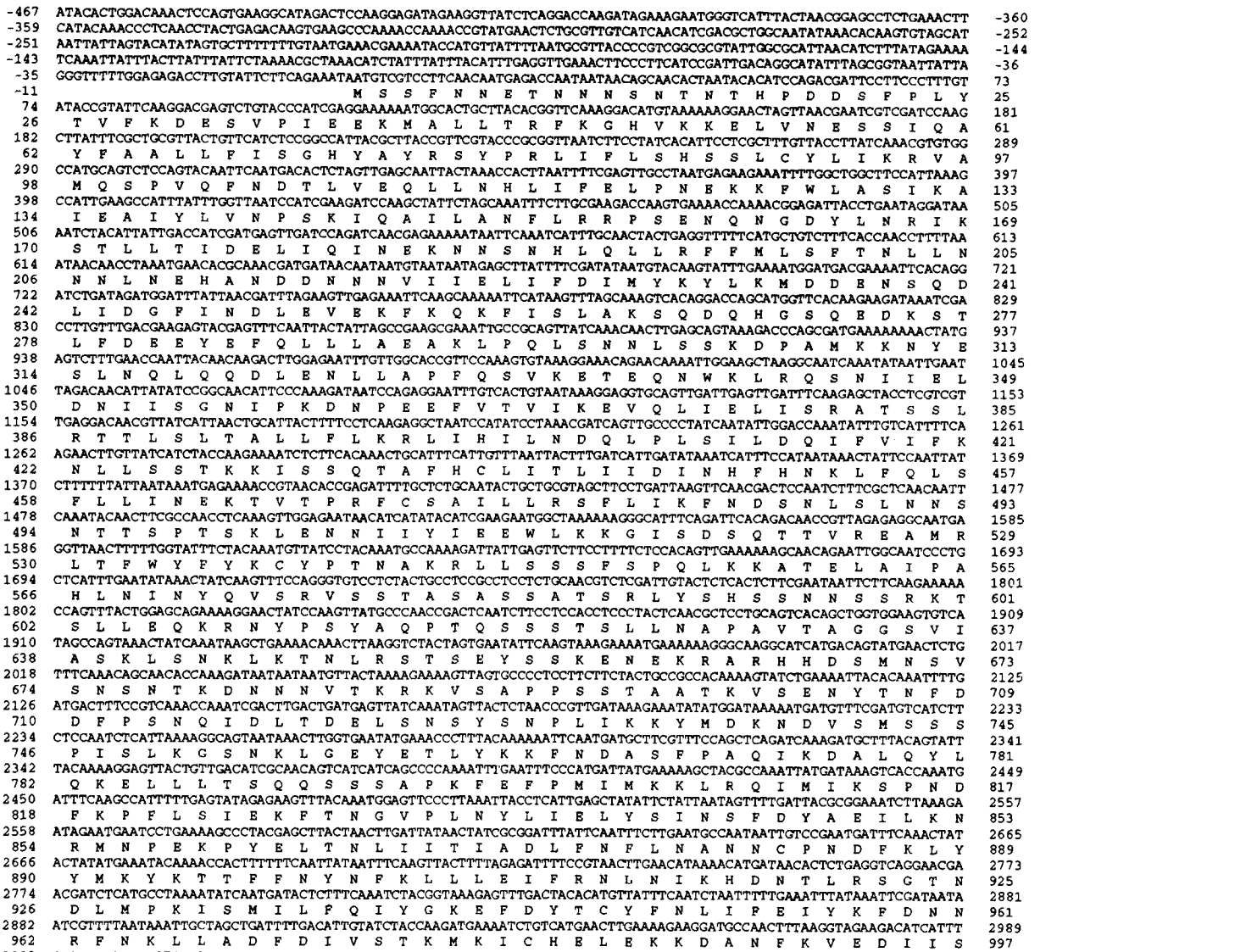

Figure 7. Sequence of the 5-kb region which complements *stul-1*. These sequence data are available from EMBL/GenBank under accession number Z35655.

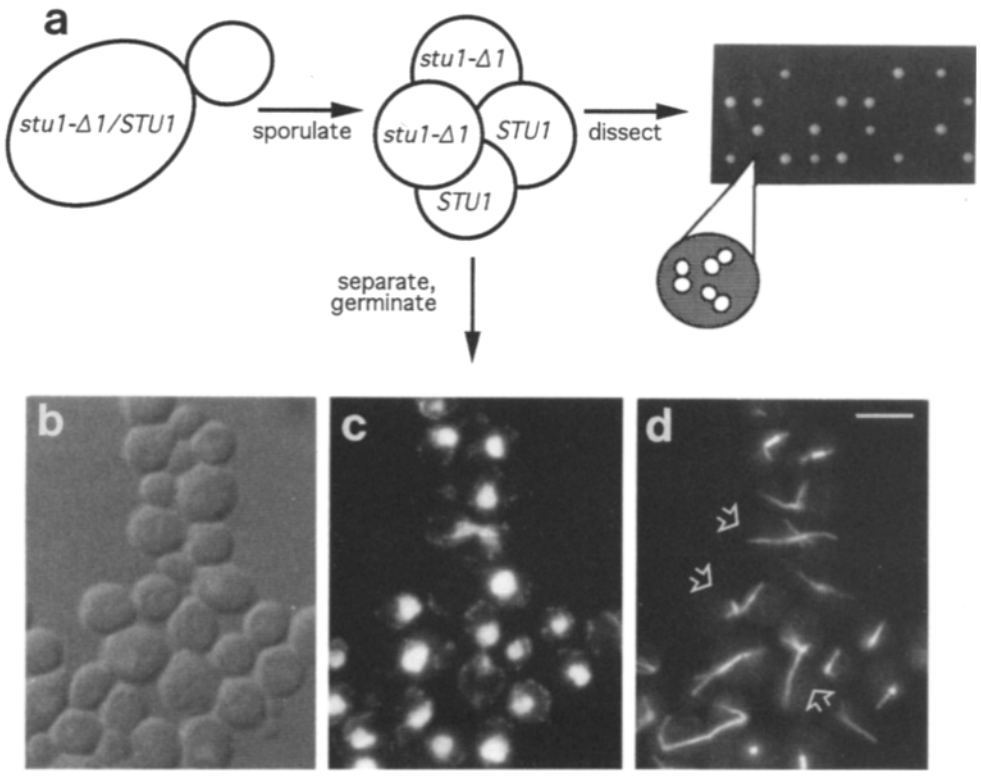

Figure 8. STU1 is essential for the assembly of the mitotic spindle. (*a*) One copy of *STU1* was disrupted in wild-type diploid cells. These cells (CUY547) were sporulated, and tetrad analysis revealed that spores containing the disruption were unable to form colonies. Microscopic analysis and manipulation of cells with a dissecting needle showed that these spores germinated but arrested as large-budded cells after one or two cell divisions. (*b–d*) Diploids heterozygous for the disruption were sporulated, and the spores were isolated and grown for 7 h at 30°C. The cells were then examined by Nomarski optics (*b*), DAPI staining (*c*), and anti-α-tubulin immunofluorescence (*d*). The open arrows in (*d*) indicate large-budded cells with the mutant phenotype. These cells presumably contain the *stu1-Δ1* disruption. Bar, 5 μm.

of the entire *STU1* gene was maintained. Thus, an additional 46 amino acids were introduced into the full length Stu1p at amino acid position 997. This construct, designated *STU1::HA$_3$*, carried on either a low-copy number CEN plasmid or a high-copy number 2 μm plasmid, complemented the *stu1-Δ1::HIS3* disruption.

To determine the cellular location of the epitope-tagged Stu1p, we examined haploid yeast cells with the *stu1-Δ1::HIS3* disruption carrying either the *STU1::HA$_3$*/CEN plasmid or the *STU1::HA$_3$*/2 μm plasmid. Staining of cells with the 12CA5 antibody was generally weaker when the *STU1::HA$_3$*/CEN plasmid was used, but a similar pattern of staining was observed for both plasmids (Fig. 9). In cells that contained short spindles, the epitope-tagged Stu1p colocalized with anti-β-tubulin staining uniformly along the length of the spindle. Less intense, diffuse staining was also seen within the nucleus surrounding the spindle. In cells carrying the CEN construct, 60% of short spindles showed coincident staining for Stu1p. The remaining 40% did not show staining probably because of the low abundance and formaldehyde sensitivity of the epitope-tagged Stu1p. In cells carrying the 2 μm construct, 80% of short spindles showed coincident staining for Stu1p.

Stu1p staining was also observed on long anaphase spindles, although the staining was weaker than that observed on short spindles. It was unclear if Stu1p was present at the ends of long spindles, because diffuse nucleoplasmic staining obscured this region. In cells carrying either the CEN or 2 μm plasmid, half of the long spindles showed coincident staining for Stu1p. Cells without spindles did not show significant staining either on microtubules or within the nucleus. Colocalization of Stu1p with cytoplasmic microtubules was never observed.

Similar patterns of Stu1p localization were observed using only the 12CA5 antibody (Fig. 9, *g* and *h*) indicating that the Stu1p staining we observed was not due to spill-over of the microtubule staining. When cells carrying the *STU1* gene without the epitope tag were stained with 12CA5 and anti-β-tubulin antibodies, no Stu1p staining was observed (not shown). Thus, the 12CA5 antibody was specific for the epitope-tagged Stu1p.

Discussion

The tub2-406 Mutation Affects Spindle Assembly

We report the isolation and characterization of a novel conditional-lethal allele of the yeast β-tubulin gene, *tub2-406*. Because yeast contain only one gene encoding β-tubulin, the mutant protein must be present in all microtubules in *tub2-406* cells. Nonetheless, this allele primarily affects the intranuclear microtubules that form the mitotic spindle. *tub2-406* cells fail to assemble a normal short spindle and are unable to segregate chromosomes, a process that requires nuclear but not cytoplasmic microtubules. On the other hand, these cells contain cytoplasmic microtubules and are able to move the nucleus to the bud neck, a process that requires cytoplasmic microtubule function. The cytoplasmic microtubules in *tub2-406* cells are longer and more numerous than those in wild-type cells. This phenotype could be a direct consequence of the mutation or an indirect consequence of the defect in spindle assembly. If tubulin subunits are not incorporated into the mitotic spindle, they may instead be incorporated into microtubules in the cytoplasm.

The *tub2-406* mutation affects both the assembly and stability of mitotic spindles. When asynchronously growing

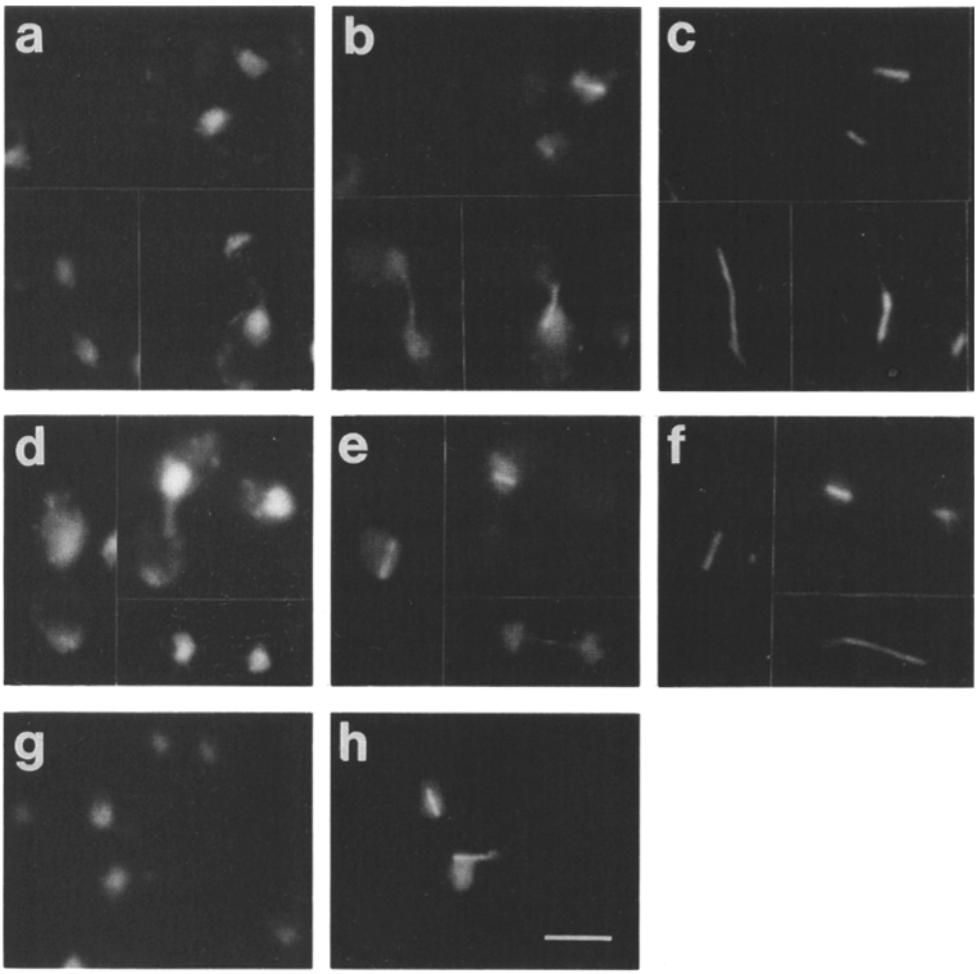

Figure 9. Stu1p colocalizes with microtubules in the mitotic spindle. CUY549 and CUY573 cells, deleted for *STU1* and carrying the triple HA-tagged *STU1* on plasmids, were labeled with DAPI (*a*, *d*, and *g*), anti-HA antibody (*b*, *e*, and *h*), and anti-β-tubulin antibody (*c* and *f*). The cells in *a–c* carry the tagged *STU1* gene on a CEN plasmid, whereas the cells in *d–h* carry the tagged *STU1* on a 2 μm plasmid. The cells in *g* and *h* were not double-labeled for β-tubulin. Bar, 5 μm.

tub2-406 cells were shifted to 14°C, we could distinguish by immunofluorescence two separated SPBs in almost all cells. When we examined these cells by electron microscopy, we observed two classes of SPBs separation. In the first class, SPBs were located on opposite sides of the nuclear envelope but were separated by only 60% the length of a normal short spindle. The SPBs did not face each other, suggesting that the microtubule connections between these structures were defective. In the second class, SPBs were separated but still resided on the same side of the nuclear envelope. When we synchronized cells with short spindles and then shifted them to 14°C, we observed that all cells displayed the first class of SPB separation. Therefore, in asynchronous populations, the first SPB class likely resulted from the collapse of spindles that had formed prior to the shift to the restrictive temperature. We assume that the second class arose from cells that had not separated SPBs prior to the temperature shift. In wild-type cells, SPB separation is rapid, and intermediates in this process have not been observed (Byers, 1981). The second class of SPB separation we observed in *tub2-406* cells may represent an intermediate in bipolar spindle formation.

The defects in spindle assembly and stability in *tub2-406* cells are less severe than those in cells that have lost all microtubules. Treatment of cells with the microtubule-destabilizing drug nocodazole completely blocks SPB separation (Jacobs et al., 1988). If SPBs have not separated before treatment with nocodazole, they remain in the side-by-side configuration after treatment. When cells containing short spindles are treated with nocodazole, the spindles collapse to ~20% their original length, but the SPBs remain on opposite sides of the nuclear membrane. Thus, *tub2-406* cells retain partial capacity to separate SPBs and to prevent the complete collapse of spindles. This conclusion is consistent with the presence of nuclear microtubules in *tub2-406* cells.

It is possible that the *tub2-406* mutation affects the function of only one class of spindle microtubules. The *tub2-406* phenotype is most consistent with a defect in polar microtubules. For example, the mutant β-tubulin encoded by *tub2-406* may be unable to interact appropriately with a protein that mediates the interaction of polar microtubules from each half spindle. Such "cross-linking" of polar microtubules may be required to maintain spindle integrity. The *tub2-406* phenotype is not consistent with a specific defect in kinetochore microtubule function. Temperature-sensitive mutations in *NDC10*, a gene that encodes a component of the yeast kinetochore, prevent attachment of chromosomes to the mitotic spindle (Goh and Kilmartin, 1993). Nonetheless, this muta-

tion does not block the formation or elongation of the mitotic spindle.

Genetic Evidence for Interaction of Stu1p with Microtubules

The *tub2-406* allele confers a specific defect in spindle microtubule function. One explanation for this phenotype is that the mutation interferes with the interaction between microtubules and a particular protein whose action is required for spindle assembly. This situation is ideally suited for genetic analysis because a compensating mutation that alters the microtubule-binding protein will suppress the original microtubule defect. By screening for suppressors of *tub2-406*, we isolated four alleles of a single gene, *STU1*. Genetic interactions between *stu1^{sup}* and tubulin alleles suggest that Stu1p does interact with microtubules.

We observed that suppression of *tub2-406* by *stu1-1* and *stu1-2* is allele specific. A *stu1* mutation that compensates for a particular *tub2* mutation should not suppress many other *tub2* mutations. Consistent with this model, the *stu1-1* and *stu1-2* alleles suppress only two out of 15 other cold-sensitive *tub2* alleles. These alleles, *tub2-423* and *tub2-434*, also confer spindle-specific defects (Reijo et al., 1994) and could also disrupt the interaction of microtubules with Stu1p. Five of the other *tub2* alleles tested confer specific defects in spindle assembly as well but are not suppressed by either *stu1-1* or *stu1-2*. Although these latter *tub2* mutants are phenotypically similar, the molecular mechanisms underlying their defects may differ from those in *tub2-406* cells.

In contrast to its ability to suppress some *tub2* mutations, the *stu1-2* allele is synthetically lethal in combination with three other *tub2* alleles. Synthetic lethality often occurs between alleles whose gene products participate in a common process. Examples of synthetic phenotypes between pairs of mutant tubulin alleles in *Saccharomyces* have been previously reported (Huffaker et al., 1987). Furthermore, all four of the *stu1^{sup}* alleles fail to complement one of the *tub1^{sup}* alleles for suppression of *tub2-406*. Unlinked noncomplementation, like synthetic lethality, is also observed between genes whose products interact. For example, the *tub1-1* allele was originally isolated as an unlinked noncomplementer of the *tub2-401* allele (Stearns and Botstein, 1988). Thus, three genetic criteria—allele specificity, synthetic lethality, and unlinked noncomplementation—suggest that Stu1p interacts with microtubules in vivo.

We have proposed a model in which *stu1^{sup}* alleles suppress *tub2-406* cold-sensitivity by restoring the interaction of Stu1p with mutant microtubules. Based on this model, one might predict that *stu1^{sup}* alleles should be dominant (*STU1/stu1^{sup} tub2-406/tub2-406* cells should grow at 14°C). However, the *stu1^{sup}* alleles are recessive to wild-type. If wild-type Stu1p cannot bind to *tub2-406* microtubules, why should it interfere with the function of the suppressor Stu1p? First, it is possible that a single *stu1^{sup}* allele may not provide enough protein to allow growth of a *tub2-406* homozygous diploid. This explanation is unlikely, however, because homozygous *TUB2^{+}* diploid cells containing a deletion of one copy of *STU1* grow at wild-type rates. A more plausible explanation is that Stu1p also binds to another protein, and formation of this complex is essential for Stu1p to interact with microtubules. In *STU1/stu1^{sup}* diploids, the wild-type Stu1p will compete with suppressor Stu1p for binding to this protein. If this protein is limiting, the amount of suppressor Stu1p complex will be reduced by half which may not be sufficient for growth.

Stu1 Is an Essential Component of the Mitotic Spindle

Cells lacking Stu1p fail to assemble a normal short spindle and are unable to segregate chromosomes. However, they contain extensive cytoplasmic microtubules that are capable of moving the nucleus to the bud neck. Thus, a deletion of *STU1* produces a defect in spindle assembly that is indistinguishable at the level of immunofluorescence from that produced by the *tub2-406* mutation. This observation suggests that the *STU1* deletion and the *tub2-406* mutation block the same step in spindle formation. We propose that interaction of Stu1p with β-tubulin is required for this step, and the *tub2-406* mutation, like the *STU1* deletion, prevents this interaction.

Epitope-tagged Stu1p colocalized with microtubules in both short and long spindles. Staining was brighter along short spindles, suggesting that the same amount of Stu1p on short spindles was distributed along the length of elongated spindles. We also observed diffuse staining in the nucleus surrounding these spindles. Staining of the nucleoplasm could be an artifact of the experimental conditions. The *STU1::HA$_3$* gene was carried on a plasmid and may have been expressed at levels that are higher than normal. Excess Stu1p may remain in the nucleoplasm once all Stu1p binding sites on the mitotic spindle are occupied. Nucleoplasmic staining may also be due to the epitope tag itself. The epitope-tagged Stu1p may not bind to spindles as well as wild-type Stu1p, producing a larger pool of soluble protein that remains in the nucleoplasm. However, it is unlikely that the HA$_3$ tag dramatically affects Stu1p localization because *STU1::HA$_3$* fully complements the *stu1-Δ1::HIS3* deletion. We did not observe Stu1p staining along microtubules or in the nucleoplasm of cells without spindles, suggesting that Stu1p is synthesized and/or transported into the nucleus just before spindle formation. Stu1p did not colocalize with cytoplasmic microtubules at any point of the cell cycle, consistent with the observation that a *STU1* deletion did not block cytoplasmic microtubule assembly or function. The pattern of Stu1p localization is similar to that of the kinesin-like proteins, Cin8p and Kip1p (Hoyt et al., 1992; Roof et al., 1992). These proteins are proposed to interdigitate polar microtubules from each half spindle. Likewise, Stu1p may also cross-link polar microtubules, a hypothesis that is consistent with the phenotype of the *STU1* deletion.

In summary, the intracellular location of Stu1p and the phenotype of cells lacking Stu1p demonstrate that Stu1p is an essential component of the yeast mitotic spindle. However, the precise role of Stu1p in spindle assembly has yet to be elucidated; conditional lethal alleles of the *STU1* gene may provide further insight into Stu1p function. In addition to our cytological observations, genetic evidence is also consistent with the model that Stu1p directly interacts with microtubules. Biochemical experiments that show binding of Stu1p to microtubules in vitro will be necessary to prove this model.

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