The Essential Yeast *Tcp1* Protein Affects Actin and Microtubules

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Previously, we showed that the yeast *Saccharomyces cerevisiae* cold-sensitive mutation *tcp1-1* confers growth arrest concomitant with cytoskeletal disorganization and disruption of microtubule-mediated processes. We have identified two new recessive mutations, *tcp1-2* and *tcp1-3*, that confer heat- and cold-sensitive growth. Cells carrying *tcp1* alleles were analyzed after exposure to the appropriate restrictive temperatures by cell viability tests, differential contrast microscopy, fluorescent, and immunofluorescent microscopy of DNA, tubulin, and actin and by determining the DNA content per cell. All three mutations conferred unique phenotypes indicative of cytoskeletal dysfunction. A causal relationship between loss of Tcp1p function and the development of cytoskeletal abnormalities was established by double mutant analyses. Novel phenotypes indicative of allele-specific genetic interactions were observed when *tcp1-1* was combined in the same strain with *tub1-1*, *tub2-402*, *act1-1*, and *act1-4*, but not with other tubulin or actin mutations or with mutations in other genes affecting the cytoskeleton. Also, overproduction of wild-type Tcp1p partially suppressed growth defects conferred by *act1-1* and *act1-4*. Furthermore, Tcp1p was localized to the cytoplasm and the cell cortex. Based on our results, we propose that Tcp1p is required for normal development and function of actin and microtubules either through direct or indirect interaction with the major cytoskeletal components.

INTRODUCTION

Mouse *TCP1*, which codes for Tcp1p (tailless complex polypeptide 1), is abundantly expressed during spermiogenesis. The *TCP1* gene is located on chromosome 17 in a region called the *t* complex. This region is associated with unusual genetic properties and has been under study for more than 60 years. Recessive *t*-alleles were originally discovered by their interaction with a dominant *T* locus mutation to produce a tailless phenotype in double mutant animals (Dobrovalskaia-Zawadskaia, 1927; Chesley, 1932; Gluecksohn-Waelsch, 1989).

One of the effects conferred by the *t*-chromosomes is transmission ratio distortion (TRD), which results in male-specific chromosome transmission in vast excess of Mendelian expectations. Mouse Tcp1p has been implicated to play a role in TRD (Silver and Remis, 1987).

Tcp1 homologues, proteins of ~60 kDa, have been identified in organisms ranging from archaeobacteria to humans (Silver *et al.*, 1979; Silver, 1981; Willison *et al.*,

1986, 1987; Ursic and Ganetzky, 1988; Ahmad and Gupta, 1990; Morita *et al.*, 1991; Trent *et al.*, 1991; Ursic and Culbertson, 1991; Mori *et al.*, 1992). The yeast *Saccharomyces cerevisiae* *TCP1* gene is essential for cell viability. Tcp1p in yeast, *Drosophila melanogaster*, and mouse share between 61 and 72% amino acid sequence identity (Ursic and Ganetzky, 1988; Ursic and Culbertson, 1991), suggesting a primordial function for the protein.

The intracellular localization of Tcp1p has not been clearly established. The mouse homologue was suggested to be an extracellular matrix protein (Silver and White, 1982). It was also reported to localize to the trans-Golgi membranes (Willison *et al.*, 1989), but recent experiments using monospecific antibodies suggested that much of the protein is soluble in the cytosol and with a minor amount in the nucleus (Horwich and Willison, 1993).

Several lines of evidence suggest that Tcp1p may function in a manner resembling the GroE subclass of

molecular chaperonins. This class of proteins was proposed to mediate the folding of monomeric polypeptides as well as the subunits of oligomeric proteins (Craig *et al.*, 1993). Tcp1p shares ~16.5–18% amino acid sequence identity with *Escherichia coli* GroEp, most of which centers around a putative cyclic AMP-dependent ATP binding domain (Hanks *et al.*, 1988; Ellis, 1990; Gupta, 1990; Lewis *et al.*, 1992). Archaeobacterial and mammalian Tcp1p form a homo- or heteromeric complex, respectively, resembling the ring structure of chaperonins (Trent *et al.*, 1991; Frydman *et al.*, 1992; Gao *et al.*, 1992; Lewis *et al.*, 1992; Craig *et al.*, 1993). However, similar structures have also been described for an ATPase, proteasome, glutamine synthetase, and outer membrane channels, indicating that ring structures are not a unique property of chaperonins (Almassy *et al.*, 1986; Jap *et al.*, 1991; Cowan *et al.*, 1992; Goldberg and Rock, 1992). Mammalian Tcp1p complexes have been shown to bind in vitro to denatured α - and β -tubulin and actin monomers, which are released upon ATP hydrolysis in altered conformation consistent with chaperonin function (Frydman *et al.*, 1992; Gao *et al.*, 1992, 1993; Yaffe *et al.*, 1992). The Tcp1p complex also binds to newly synthesized rabbit reticulocyte tubulin and actin monomers (Sternlicht *et al.*, 1993).

Despite the similarities between Tcp1p and the GroE type chaperonins, some notable differences call into question whether they perform similar functions. Unlike GroEp, Tcp1p is not heat inducible (Ursic and Culbertson, 1992). It does not require the presence of cochaperonin-10 (Frydman *et al.*, 1992; Gao *et al.*, 1992, 1993; Yaffe *et al.*, 1992). Cochaperonins mediate the dissociation of the GroEp-substrate binary complex (Craig *et al.*, 1993). Also, the chaperonins form amphipathic helices that may form coiled coil domains, whereas this type of structure is not apparent in the Tcp1 proteins. These domains were proposed to be involved in the binding to nascent polypeptides (Webb and Sherman, 1992). In contrast to GroEp, which can bind to ~50% of the *E. coli* cell protein (Viitanen *et al.*, 1992), Tcp1p appears to be much more selective in its ability to interact with other proteins. The only reported native in vitro substrates for Tcp1p are α - and β -tubulin and actin monomers (Frydman *et al.*, 1992; Gao *et al.*, 1992, 1993; Yaffe *et al.*, 1992). Further studies will be necessary to determine whether Tcp1p performs a chaperonin-like function in vivo.

To assess TCP1 function in the yeast *S. cerevisiae*, we constructed a cold-impaired recessive mutation called *tcp1-1* (Ursic and Culbertson, 1991). At the restrictive temperature mutant cells displayed abnormal nuclear distribution and contained both aploid and multinucleate cells. The microtubules were abnormally distributed and disorganized, and the cells were hypersensitive to an inhibitor of microtubule polymerization. These observations led us to propose that Tcp1p affects microtubule-mediated functions. In addition, we found that

the *tcp1-1* mutation confers aberrant budding patterns, suggesting that Tcp1p plays a role in the function of the actin cytoskeleton (Ursic and Culbertson, 1991).

In this paper we present a genetic approach to further explore the role of Tcp1p on the cytoskeleton. The effects of loss of TCP1 function on microtubules and actin were analyzed by examining the phenotypes of cells carrying three different *tcp1* alleles.

MATERIALS AND METHODS

Chemicals

Enzymes and chemicals were obtained from Promega (Madison, WI), Sigma (St. Louis, MO), New England Biolabs (Beverly, MA), Pharmacia (Piscataway, NJ), Boehringer Mannheim (Indianapolis, IN), and GIBCO BRL (Grand Island, NY). Propidium iodide was purchased from Aldrich (Milwaukee, WI). Radiolabeled nucleotides ([α - 32 P]CTP and [α - 35 S]ATP) were purchased from Amersham (Arlington Heights, IL) and New England Nuclear (Boston, MA). Deoxyoligonucleotides were synthesized by the University of Wisconsin Biotechnology Center (Madison, WI). Sequenase was purchased from United States Biochemical (Cleveland, OH). Rat monoclonal antibodies against yeast α -tubulin, YOL1/34, were purchased from SERA-LAB (Accurate Chemical and Scientific, Westbury, NY). Fluorescein isothiocyanate-labeled rabbit and rat antiserum to IgG was purchased from Boehringer Mannheim. Rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR). Benomyl was a gift from duPont (Wilmington, DE).

Strains, Plasmids, and Media

Yeast strains used in this study are listed in Table 1. Genetic methods and media for growth of yeast and bacterial cells are as described previously (Ursic and Culbertson, 1991). Standard yeast techniques were used (Sherman *et al.*, 1979). For each genetic cross ≥ 23 tetrads were analyzed. Possible interactions between pairs of mutations were further pursued by performing additional genetic crosses and analyzing growth differences using drop tests. Growth rate differences were analyzed by drop tests performed as follows. Exponentially growing cells were serially diluted and placed on plates containing the appropriate growth media followed by incubation at 9, 15, 24, 30, 34, or 37°C. Benomyl (20 mg/ml in dimethyl sulfoxide) was added to YEP media. The percentage of viable cells after shift to the restrictive temperature was determined by plating appropriate dilutions of culture to YEP plates at 30°C.

Plasmid and DNA Manipulations

Plasmid DNA isolation and transformation of *E. coli* were described previously (Ursic *et al.*, 1983). Yeast strains were transformed by the method of Ito *et al.* (1983). Yeast DNA was prepared as described by Sherman *et al.* (1979).

Measurement of the DNA Content of Yeast Cells

DNA was stained with propidium iodide using the protocol of Hutter and Eipel (1978). For each sample the DNA content of 10 000 individual cells was determined using a Becton Dickinson Immunocytometry Systems (San Jose, CA) flow cytometer using their Lysis II version 1.1 software.

Immunofluorescence Staining of Yeast Cells

Indirect immunofluorescence microscopy and fluorescence microscopy was performed as described by Kilmartin and Adams (1984) with some modifications. Cells that were at an exponential phase of growth were fixed with 5% formaldehyde for 5 min at 37°C followed by a

2-h incubation at 24°C. Furthermore, spheroplasts were processed as previously described (Kilmartin and Adams, 1984) or treated with methanol (6 min at -20°C) and acetone (30 s at -20°C). Also, best results were obtained when the antibodies were allowed to bind overnight at 24°C. Cells were stained with 4,6-diamidino-2-phenylindole (DAPI) or rhodamine phalloidin to visualize DNA and actin, respectively (Adams and Pringle, 1991; Ursic and Culbertson, 1991). To visualize Tcp1p, we used antibodies designated 91a that recognize amino acids 315–501 of the mouse Tcp1 protein (Willison *et al.*, 1989). The preparations were examined using a Nikon epifluorescence microscope (Garden City, NY).

Replacement of the Wild-Type TCP1 Gene with *tcp1-1*

We replaced the wild-type TCP1 gene with a gene carrying the *tcp1-1* mutation using the two step gene replacement method (Scherer and Davis, 1979). The *tcp1-1* allele, contained on an *EcoRI* fragment in plasmid pDU25-4 (Ursic and Culbertson, 1991), was cloned into the *EcoRI* restriction site of vector YIp352, resulting in the plasmid pDU33. pDU33 was linearized by endonuclease digestion with *Mlu*I and used to transform the TCP1 strain M10 to a Ura⁺ phenotype (Table 1) (Ursic and Culbertson, 1991). Ura⁻ derivatives of one transformant were selected in the presence of 5-fluoroorotic acid (5-FOA) (Boeke *et al.*, 1984), resulting in the isolation of strain DUY48 (Table 1), which has the same cold-sensitive, benomyl-sensitive growth phenotype as strain DUY4a1 (*tcp1::LEU2 [tcp1-1]*) (Table 1). We verified that correct gene replacement had occurred by performing Southern blot analysis. The restriction endonuclease patterns of DUY48 and M10 were identical, consistent with correct gene replacement. We also crossed strain DUY48 to strain DUY2 that has *LEU2* integrated adjacent to the wild-type TCP1 gene as a scorable marker, see Table 1. Forty-eight tetrads from this cross all segregated 2 *leu*⁻ *cs*⁻ *ben*^S (leucine auxotrophic, cold-sensitive, benomyl-sensitive): 2 *leu*⁺ *cs*⁺ *ben*^R (leucine prototrophic, cold-insensitive, benomyl resistant), indicating replacement of TCP1 with *tcp1-1*.

Induction of New *tcp1* Alleles

We induced mutations in the TCP1 gene by in vitro hydroxylamine mutagenesis (Rose and Fink, 1987). Two alleles were identified using a plasmid shuffle (Ursic and Culbertson, 1991) in strain DUY4a (Table 1), which carries a chromosomal copy of the *tcp1::LEU2* null allele and is kept viable by the centromeric plasmid pDU22, which carries TCP1 *URA3*. DUY4a was transformed with the in vitro mutagenized centromeric plasmid pDU25-4, which carries *TRP1* and TCP1. Transformants were placed on 5-FOA plates to select for loss of plasmid pDU22. Approximately 5000 5-FOA resistant colonies were screened for conditional lethality at either 15 or 37°C or in the presence of 30 µg/ml benomyl. The heat-sensitive mutant DUY326 and the cold-sensitive mutant DUY327 were identified among the 5-FOA resistant colonies (Table 1). We confirmed that the mutations in DUY326 and DUY327, designated *tcp1-2* (*ts*) and *tcp1-3* (*cs*), respectively, were both within the TCP1 gene by complementation and plasmid rescue as described previously for the *tcp1-1* allele (Ursic and Culbertson, 1991). Plasmids carrying the *tcp1-2* and *tcp1-3* mutations, named pDU357 and pDU359, respectively, were recovered from the yeast strains DUY326 and DUY327 by transformation of *E. coli* as described in Ursic and Culbertson (1991). These plasmids were reintroduced into the diploid yeast strain DUY4 (Table 1). They restored the mutant phenotypes, as verified by meiotic segregation analysis in which only the Trp⁺ Leu⁺ prototrophic spores showed cosegregation with the heat- or cold-sensitive growth phenotype and with benomyl sensitivity. This shows that the wild-type gene can supply the function lacking in the mutant and the mutations are recessive.

DNA Sequence Analysis of the *tcp1* Alleles

pDU5-4, pDU357, and pDU359 were used to determine the DNA sequence of the *tcp1-1*, *tcp1-2*, and *tcp1-3* alleles. The entire TCP1

open reading frame was sequenced as described by Sanger *et al.* (1977) using oligonucleotide primers and compared to the wild-type TCP1 sequence (GeneBank, NCBI, Bethesda, MD, DNA sequence accession number M21160) (Ursic and Culbertson, 1991).

RESULTS

Gene- and Allele-specific Synthetic Interaction Between *Tcp1p* and the Tubulins

The interaction of specific proteins is often indicated by the observation that two partially defective alleles show negative synergism, resulting in so-called synthetic-lethality in the double mutant. Allele specificity is typically found for synthetic-lethal genetic interactions that are a consequence of altered protein-protein interactions (Hoyt *et al.*, 1992; Roof *et al.*, 1992). We looked for gene-specific and allele-specific interactions between strains carrying the conditional lethal mutation *tcp1-1* (Ursic and Culbertson, 1991) and a number of alleles in other genes whose product affects the cytoskeleton. Spore germination and mitotic growth of single and double mutants were compared to detect synthetic phenotypes that were unique to the double mutants. We observed synthetic-lethality and growth impairments when *tcp1-1* was placed in combination with particular alleles of genes coding for β -tubulin, α -tubulin, and actin, but not with mutations in *BIK1*, *CDC31*, and *ESP1*, all of which code for products that have cytoskeletal function (Table 2). We found no unique phenotypes for *tcp1-1* in combination with *cdc31-1*, *esp1-1*, or *bik1-518* (Tables 1 and 2). Esp1p and Cdc31p are required for spindle pole body duplication (Baum *et al.*, 1986a, b). Bik1p affects bilateral karyogamy and colocalizes with microtubules (Berlin *et al.*, 1990).

A synthetic-lethal phenotype was observed in strains carrying *tcp1-1* and *tub2-402* (β -tubulin). Thirty-three tetrads from two crosses (DUY246 × DBY2304 and DUY268 × DBY2304) (Table 1) were analyzed for spore germination at 30°C, which is a permissive temperature for both *tcp1-1* and *tub2-402*. Tetrads containing four, three, and two live segregants were found in a ratio of 4:24:5. This is statistically similar to the 1:4:1 ratio expected for two unlinked mutations that confer synthetic-lethality when they cosegregate. There were no double mutants among the surviving spores as indicated by their growth phenotypes. To show that the lethal spore germination pattern was due to synergism between *tcp1-1* and *tub2-402*, strain DBY2304, which carries *tub2-402*, was crossed to strain DUY332 (Table 1), which carries chromosomal copies of *tcp1-1* and *ura3-52* and a centromeric plasmid containing the wild-type TCP1 and *URA3* genes. Segregants from the cross were identified and tested for the ability to grow in the presence of 5-FOA (Figure 1, bottom). Segregants carrying *tcp1-1* and *tub2-402* were not viable in the absence of the extrachromosomal wild-type TCP1 gene. These results indicate that lethality is because of the combined presence of *tcp1-1* and *tub2-402*.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
DBY1197	MATa <i>act1-1 ura3-52</i>	D. Botstein
DBY1237	MATa <i>act1-2 ura3-52 CAN1</i>	D. Botstein
DBY1238	MATa <i>act1-3 ura3-52</i>	D. Botstein
DBY1384	MATa <i>ura3-52 his4-539 tub2-104</i>	T. Huffaker
DBY2023	MATa <i>ura3-52 lys2-801 his4-539 tub2-401</i>	T. Huffaker
DBY2304	MATa <i>ura3-52 lys2-801 his4-539 tub2-402</i>	T. Huffaker
DBY2305	MATa <i>ura3-52 lys2-801 his4-539 tub2-403</i>	T. Huffaker
DBY2308	MATa <i>ura3-52 lys2-801 his4-539 tub2-404</i>	T. Huffaker
DBY2309	MATa <i>ura3-52 lys2-801 his4-539 tub2-405</i>	T. Huffaker
DBY5230	MATa <i>act1-2 ura3-52</i>	D. Botstein
DBY5232	MATa <i>act1-4 his4-619</i>	D. Botstein
DUY1	MATa <i>leu2-3, -112 ura3-52 {URA3 YEp352}</i> ^a	D. Ursic
DUY2	MATa <i>his3-11 his3-15 CAN1 leu2-3, -112 TCP1/TCP1::LEU2::Ylp26</i> ^b	D. Ursic
DUY246	MATa <i>ura3-52 lys2-801 ade2-201 tcp1-1 leu2-3, -112</i>	This study
DUY268	MATa <i>ura3-52 leu2-3, -112 tcp1-1</i>	This study
DUY293	MATa <i>ura3-52 tcp1-1</i>	This study
DUY315	MATa <i>his4-539 tcp1-1 ura3-52 {TCP1 URA3 YCp50}</i>	This study
DUY316	MATa <i>lys2-801 leu2-3, -112 tub2-402 ura3-52 {TCP1 URA3 YCp50}</i>	This study
DUY317	MATa <i>tcp1-1 tub2-402 ura3-52 {TCP1 URA3 YCp50}</i>	This study
DUY318	MATa <i>lys2-801 his4-539 leu2-3, -112 ura3-52 {TCP1 URA3 YCp50}</i>	This study
DUY324	MATa <i>leu2-3, -112 ura3-52 trp1-7 tcp1::LEU2 {tcp1-2 TRP1 YCpMS38}</i>	This study
DUY326	MATa <i>leu2-3, -112 ura3-52 trp1-7 tcp1::LEU2 {tcp1-2 TRP1 YCpMS38}</i>	This study
DUY327	MATa <i>leu2-3, -112 ura3-52 trp1-7 tcp1::LEU2 {tcp1-3 TRP1 YCpMS38}</i>	This study
DUY329	MATa <i>leu2-3, -112 ura3-52 trp1-7 tcp1::LEU2 {tcp1-3 TRP1 YCpMS38}</i>	This study
DUY332	MATa <i>ura3-52 leu2-3, -112 tcp1-1 {TCP1 URA3 YCp50}</i>	This study
DUY4	MATa/ α <i>ura3-52/ura3-52 trp1-7/trp1-7 leu2-3, -112/leu2-3, -112 TCP1/tcp1::LEU2</i>	D. Ursic
DUY417	MATa <i>ura3-52 ade2-101 his3-Δ201 tub1-1</i>	This study
DUY418	MATa <i>ura3-52 leu2-3, -112 tub1-1 tcp1-1 {TCP1 URA3 YCp50}</i>	This study
DUY419	MATa <i>ura3-52 leu2-3, -112 tcp1-1 {TCP1 URA3 YCp50}</i>	This study
DUY420	MATa <i>ura3-52 ade2-101 his3-Δ201</i>	This study
DUY471	MATa <i>ura3-52 leu2-3, -112 his4-619 act1-4</i>	This study
DUY476	MATa <i>ura3-52 leu2-3, -112 tcp1-1 act1-1</i>	This study
DUY477	MATa <i>ura3-52 leu2-3, -112 tcp1-1 act1-2</i>	This study
DUY478	MATa <i>trp1-7 tcp1-1 act1-4</i>	This study
DUY48	MATa <i>leu2-3, -112 ura3-52 trp1-7 tcp1-1</i>	This study
DUY4a	MATa <i>ura3-52 trp1-7 leu2-3, -112 tcp1::LEU2 {TCP1 URA3 YCp50}</i>	D. Ursic
DUY4a1	MATa <i>ura3-52 trp1-7 leu2-3, -112 trp1::LEU2 {tcp1-1 TRP1 YCpMS38}</i>	D. Ursic
DUYT1	MATa <i>leu2-3, -112 ura3-52 {TCP1 URA3 YEp352}</i>	D. Ursic
F1073	MATa <i>tub1-1 ura3-52 ade2-101 his3-Δ201 leu2-3, -112</i>	D. Botstein (DBY2501)
L2751	MATa <i>bik1-518 ura3-52 leu2-3, -112</i>	J. Trueheart (JY148)
M10	MATa <i>leu2-3, -112 ura3-52 trp1-7</i>	W. Boorstein
M11	MATa <i>leu2-3, -112 ura3-52 trp1-7</i>	W. Boorstein
PB9-33B	MATa <i>ura3 leu2-3, -112 trp1 cdc31-1</i>	B. Byers
SN127-3B	MATa <i>ura3-52 ade2 lys2 leu2-3, -112 cyh2 esp1-1</i>	B. Byers

^a The braces enclose the name of an autonomously replicating plasmid and the relevant genes.

^b The symbol :: separates a locus and an integrated gene and/or plasmid.

The interaction between *tcp1-1* and *tub2-402* is allele-specific. Genetic crosses between strains containing *tcp1-1* and four other alleles, *tub2-104*, *tub2-403*, *tub2-404*, and *tub2-405* (Table 1), resulted in double mutants with no unique growth properties (Table 2). The allele specificity is not simply because of the differing severity of the *tub2* mutants examined. The *tub2-402* allele has one of the least severe phenotypes. Unlike the other *tub2* mutations analyzed, *tub2-402* retains some ability to form microtubules and exhibit nuclear migration (Huffaker *et al.*, 1988).

We also observed a synthetic phenotype between *tcp1-1* and *tub1-1* (α -tubulin) (Stearns and Botstein, 1988). Double mutant segregants from a cross between strains DUY268 and F1073 (Table 1) grew slowly at 24°C, a permissive temperature for either allele (Table 2). We verified that the slow growth was because of synergism between *tcp1-1* and *tub1-1*. Strain DUY293, which carries chromosomal copies of *tcp1-1* and *ura3-52* and the centromeric plasmid pDU22 [TCP1 URA3], was crossed to strain F1073 [*tub1-1*]. Segregants from the cross were identified and tested for the ability

Table 2. Genetic interaction of *tcp1-1* with cytoskeletal mutants

Allele (strain ^a)	Conditional lethal temperature ^b	Growth of double mutant ^c
<i>tcp1-1</i> (DUY48)	15°C	not applicable
<i>act1-1</i> (DBY1197)	37°C	growth impaired
<i>act1-2</i> (DBY1237)	37°C	viable
<i>act1-3</i> (DBY1238) ^d	37°C	growth impaired
<i>act1-4</i> (DBY5232)	34°C	growth impaired
<i>bik1-518</i> (L2751)	14°C	viable
<i>cdc31</i> (PB9-33B)	37°C	viable
<i>esp1-1</i> (SN127-3B)	37°C	viable
<i>tub1-1</i> (F1073)	14°C	growth impaired at 24°C
<i>tub2-104</i> (DBY1384)	11°C	viable
<i>tub2-402</i> (DBY2304)	11°C	lethal
<i>tub2-403</i> (DBY2305)	16°C	viable
<i>tub2-404</i> (DBY2308)	11°C	viable
<i>tub2-405</i> (DBY2309)	11°C	viable

^a Genotypes of strains are shown in Table 1.

^b The conditional lethal temperature for cells containing each single mutation is indicated.

^c Growth of each single mutant in combination with *tcp1-1* at the permissive temperature of 24 and 30°C is indicated. Double mutants that produce a synthetic interaction are shown in bold-face.

^d *act1-1* and *act1-3* have the same mutational changes (Shortle *et al.*, 1984).

to grow in the presence of 5-FOA (Figure 1, top). Segregants carrying *tcp1-1* and *tub1-1* were growing slowly at 24°C in the absence of the extrachromosomal wild-type *TCP1* gene. These results indicate that the growth impairment is because of both *tcp1-1* and *tub1-1* mutations. Overall, the results of double mutant analyses indicated that the synthetic phenotypes displayed by *tcp1-1* are both gene and allele specific.

Genetic Evidence for Interaction Between *Tcp1p* and Actin

To find out if *Tcp1p* genetically interacts with actin, two experiments were performed. We asked if cells expressing *tcp1-1* show a novel phenotype in combination with mutations in the *ACT1* gene, which codes for actin (Novick and Botstein, 1985). Also, we determined if overproduction of wild-type *Tcp1p* could suppress the heat-sensitive growth phenotype conferred by *act1* mutations. To analyze double mutants, strains DUY268 and DUY48 (Table 1), which carry *tcp1-1*, were crossed with strains DBY1197, DBY5230, DBY1238, and DBY5232 (Table 1), which carry *act1-1*, *act1-2*, *act1-3*, and *act1-4*, respectively. Because *act1-1* and *act1-3* have the same mutational changes (Shortle *et al.*, 1984), further studies were done only with strains containing *act1-1*.

We found no new phenotype among *tcp1-1 act1-2* double mutant segregants at 24 or 30°C, which are permissive temperatures for all of the mutations (Table 2 and Figure 2A). In addition to a normal growth rate,

tcp1-1 act1-2 double mutant cells appeared morphologically normal (Figure 3, compare panels A3 and B3). However, the growth of *tcp1-1 act1-1* and *tcp1-1 act1-4* double mutant segregants was impaired at 24 and 30°C (Table 2 and Figure 2A). Double mutant cells carrying *tcp1-1* and *act1-1* had the most severe growth-defective

Figure 1. Synthetic interactions between *tcp1-1* and *tub1-1* (α -tubulin) and *tub2-402* (β -tubulin). (Top) Double mutants *tcp1-1 tub1-1* show a synthetic phenotype when grown at 24°C in absence of the wild-type *TCP1* gene that is present on an extrachromosomal plasmid containing *URA3*. Strains were grown in YEP or media lacking uracil (-Ura) at 24°C and then replica plated onto YEP, -Ura, and minimal media containing 5-FOA. Failure to grow in the presence of 5-FOA indicates a requirement for the wild-type *TCP1* gene carried on the *URA3* plasmid pDU22. The relevant genotypes are indicated as follows (top to bottom): *tcp1-1 TUB1 {TCP1}* (DUY332), *TCP1 tub1-1* (F1073), *TCP1 TUB1* (DUY420), *tcp1-1 TUB1 {TCP1}* (DUY419), *tcp1-1 tub1-1 {TCP1}* (DUY418), and *TCP1 tub1-1* (DY417). The slow growth of strain DUY418 in the presence of 5-FOA indicates that *TCP1* is necessary for growth. (Bottom) Growth impairment of *tcp1-1 tub2-402* double mutants was shown to result from synthetic-lethal interaction by demonstrating that growth of the double mutant requires the presence of the wild-type *TCP1* gene on a centromeric *URA3*-containing plasmid. The experimental procedure was the same as described for the top panel, except that the cells were incubated at 30°C. The relevant genotypes are indicated as follows (top to bottom): *tcp1-1 TUB2* (DUY246), *TCP1 tub2-402* (DBY2304), *TCP1 TUB2 {TCP1}* (DUY318), *tcp1-1 tub2-402 {TCP1}* (DUY317), *TCP1 tub2-402 {TCP1}* (DUY316), and *tcp1-1 TUB2 {TCP1}* (DUY315) (Table 1). The failure of strain DUY317 to grow in the presence of 5-FOA indicates that *TCP1* is required for growth.

Figure 2. *TCP1* genetically interacts with *ACT1*. (A) Synthetic interactions between *tcp1-1* and alleles of *ACT1* (actin). Strains grown at 30°C that were in exponential growth phase and of same optical density were plated onto YEP medium and incubated for 3–6 d at 30°C, a temperature permissive for all alleles. The following strains were tested: DUY48 [*tcp1-1*], DBY1197 [*act1-1*], DBY5230 [*act1-2*], DBY5232 [*act1-4*], DUY476 [*tcp1-1 act1-1*], DUY477 [*tcp1-1 act1-2*], and DUY478 [*tcp1-1 act1-4*] (Table 1). Growth of the double mutants *tcp1-1 act1-1* and *tcp1-1 act1-4* was impaired. (B) Overproduction of Tcp1p from a multicopy plasmid partially suppresses the conditional-lethal growth phenotype of cells containing actin

alleles. Growth was assayed using strains containing the three actin alleles and either a multicopy plasmid {YEp352} (control) or the wild-type *TCP1* gene on the multicopy plasmid {*TCP1* YEp352}. Cells grown at 30°C that were in exponential growth phase and of same cell density were plated onto media and incubated at 30, 34, and 37°C for 2–4 d. The restrictive temperature for *act1-1* and *act1-2* alleles is 37°C. The restrictive temperature for *act1-4* is 34°C. The results show that *act1-1* and *act1-4* are partially suppressed by overproduction of *TCP1* despite a modest inhibition of growth at 37°C resulting from Tcp1p overproduction in a strain carrying wild-type *ACT1*.

phenotype, but neither double mutant was completely growth-arrested, because both were eventually able to form microcolonies. The slow growth of the *tcp1-1 act1-1* double mutant results from a high frequency of cell lysis (Figure 3, compare panels A2 and B2). Gaps develop in the cell wall and granular material accumulates outside the cells. Cells carrying *tcp1-1 act1-4* do not lyse as frequently but are much larger and appear to lack organized intracellular structures (Figure 3, compare panels A4 and B4). We also found that the slow growth of the *tcp1-1 act1-1* and *tcp1-1 act1-4* double mutants was not suppressed in the presence of 1 M sorbitol, an osmotic stabilizer. This suggests that lysis of the *tcp1-1 act1-1* double mutant cells is not a simple consequence of osmotic sensitivity.

In the second set of experiments, we determined if overexpression of wild-type *TCP1* suppressed the heat-sensitive growth phenotypes associated with *act1* mutations. Suppression was assayed by comparing the growth rates of *act1-1* (DBY1197), *act1-2* (DBY5230), and *act1-4* (DUY471) mutant cells containing *TCP1* on a multicopy plasmid to otherwise isogenic cells containing only the vector plasmid and to wild-type cells having single or multiple copies of *TCP1* (Figure 2B). We showed previously by Western blotting that increasing the copy number of the *TCP1* gene results in overproduction and increased accumulation of protein product (Ursic and Culbertson, 1991).

To assess the extent of multicopy suppression of *act1-1* and *act1-4*, growth was compared to *ACT1* strains that carry single or multiple copies of *TCP1* (Figure 2B, bottom two strains). For each set of transformants, five independent colonies were tested for growth, one set of which is presented in Figure 2B. We found that overproduction of Tcp1p partially suppressed the heat-sensitive growth phenotype of the *act1-1* and *act1-4* mutants. The growth because of *TCP1* multi-copy

suppression was reduced compared to the growth rate supported by a wild-type *ACT1* gene. However, restoration of actin function may be more complete than it appears, because overproduction of Tcp1p by itself causes a modest inhibition of growth rates at 37°C. We corroborated that the suppression resulted from overproduction of Tcp1p and not from a suppressing mutation on a chromosome by testing the cells for their ability to grow on 5-FOA at 30, 34, and 37°C. We observed that in the absence of the wild-type *TCP1* gene the heat-sensitive growth phenotypes of *act1-1* and *act1-4* were restored, implying that partial suppression was because of plasmid-born *TCP1*.

Mutations in *tcp1* Block Normal Nuclear Distribution and Completion of the Cell Cycle

Because previous conclusions regarding Tcp1p function were based on the phenotype of a single mutation, we isolated additional mutations in this gene (Ursic and Culbertson, 1991) (see MATERIALS AND METHODS). A heat-sensitive and a cold-sensitive mutation, *tcp1-2* and *tcp1-3*, respectively, were isolated. Their growth phenotypes are a direct consequence of mutations in *TCP1* based on two criteria. We performed recombination and complementation tests that showed that the mutations are genetically and functionally linked to *TCP1*.

The alleles were cloned, and the nucleotide changes of all three alleles were determined by DNA sequence analysis. The results show that each mutant allele corresponds to a single base pair change (see Table 3). Each mutation is recessive to wild-type and is located in a unique span of 10 to 20 amino acids that has shared identity among all Tcp1p homologues isolated to date (Willison *et al.*, 1986, 1987; Ursic and Ganetzky, 1988; Ahmad and Gupta, 1990; Morita *et*

Figure 3. *tcp1-1* affects the morphology of cells containing *act1-1* and *act1-4*. Cells in exponential phase of growth were analyzed by DIC as follows: (A1) strain M10 [TCP1 ACT1], (A2) strain DBY1197 [TCP1 *act1-1*], (A3) strain DBY5230 [TCP1 *act1-2*], (A4) strain DBY5232 [TCP1 *act1-4*], (B1) strain DUY48 [*tcp1-1* ACT1], (B2) strain DUY476 [*tcp1-1 act1-1*], (B3) DUY477 [*tcp1-1 act1-2*], and (B4) strain DUY478 [*tcp1-1 act1-4*] (Table 1). Incubation was at 30°C. *tcp1-1 act1-1* double mutant cells were abnormally large and misshapen with indications of cell lysis. *tcp1-1 act1-4* double mutant cells were abnormally large. Bar, 2.5 μ m.

al., 1991; Trent *et al.*, 1991; Ursic and Culbertson, 1991; Mori *et al.*, 1992).

Previously, we showed that the *tcp1-1* strain DUY4a1 (Table 1) exhibits a linear rather than exponential increase in cell number for ~ 100 h after shift to the restrictive temperature of 15°C. The number of viable cells, as assayed by the ability to form colonies at the permissive temperature of 30°C, remains constant. Both multinucleate and anucleate cells accumulate with time, suggesting that the linear growth kinetics may be explained by the generation of anucleate buds incapable of further cell division (Ursic and Culbertson, 1991).

In this study we found that the *tcp1-2* strain DUY324 (Table 1) undergoes an increase in the number of viable

cells for ~ 1.0 – 1.5 h after shift to the restrictive temperature of 37°C. By 2 h ~ 85 – 90% of the cells are still viable, but cell viability drops to $\sim 1\%$ by 17 h. The loss of viability appears to be caused by cell lysis as evidenced by the appearance of gaps in the cell wall and hypersensitivity to mechanical stress such as sonication and centrifugal forces. The *tcp1-3* strain DUY329 (Table 1) exhibits an increase in the number of viable cells for ~ 4 h after shift to the restrictive temperature of 15°C followed by a decrease in cell viability to $\sim 40\%$ after 24 h.

We compared cells carrying TCP1, *tcp1-1*, *tcp1-2*, and *tcp1-3* to assess how each mutation effects cellular morphology and nuclear distribution. Cells from asynchro-

Table 3. *tcp1* alleles

Allele	Base change	Amino acid change	Possible functional* domain
<i>tcp1-1</i>	GAC ₂₈₈ Δ GAA	Asp ₉₆ Δ Glu ₉₆	cAMP dependent protein kinase ATP binding
<i>tcp1-2</i>	GG ₁₂₆₈ T Δ GAT	Gly ₄₂₃ Δ Asp ₄₂₃	tau/MAP2 microtubule binding
<i>tcp1-3</i>	G ₁₃₃ GC Δ AGC	Gly ₄₅ Δ Ser ₄₅	not yet established

Indices correspond to the positions in the complete wild-type nucleotide and amino acid sequences.

* This assignment is based on sequence similarity (see DISCUSSION).

nous cultures of strains M10 [*TCP1*], DUY48 [*tcp1-1*], DUY324 [*tcp1-2*], and DUY329 [*tcp1-3*] (Table 1) were stained with DAPI and analyzed by differential contrast microscopy (DIC) and by fluorescence microscopy (DNA) (Figure 4). Exponentially growing cells were shifted from the permissive temperature of 30°C to the restrictive temperatures of 15°C for *tcp1-1* and *tcp1-3*,

and 37°C for *tcp1-2*. Cells carrying *TCP1* were analyzed at both 15 and 37°C (Figure 4 shows the result at 15°C). *TCP1* and *tcp1-1* cells were grown for 16 h, and *tcp1-2* and *tcp1-3* cells for 2 h at the restrictive temperatures. Using these incubation conditions, most (100, 85–90, and 100% for *tcp1-1*, *tcp1-2*, and *tcp1-3*, respectively) of the mutant cells were viable at the time points of

Figure 4. Mutations in *TCP1* affect nuclear distribution and cell cycle progression. Cells were analyzed by DIC and fluorescence microscopy using DAPI staining to visualize DNA and by flow cytometric DNA analysis of cells stained with propidium iodide to measure DNA content/cell. The following strains were examined: M10 [*TCP1*], DUY48 [*tcp1-1*], DUY324 [*tcp1-2*], and DUY329 [*tcp1-3*] (Table 1). For the DIC and DAPI analyses, M10 and DUY48 were photographed 16 h after shift to 15°C, DUY329 cells at 2 h after shift to 15°C, and DUY324 cells at 2 h after shift to 37°C. The incubation temperatures for the flow cytometric analysis were the same as for DIC and DAPI. The samples were taken at various times (h) after temperature shift as indicated in the figure. The vertical axis shows the relative cell numbers, and the horizontal axis shows the relative DNA content. The fluorescence signal of Aploid cells and cells with a 1N, 2N and >2N DNA content are indicated. Bar, 5 μ m.

analysis. For the remainder of the experiments, we used the same strains and incubation conditions and we will refer to them by the *tcp1* alleles.

We observed that the cell morphology and the relative distribution of cells at different stages of the yeast cell cycle were unique for each mutant (Figure 4, DIC). About 30% of the wild-type *TCP1* cell population consists of large budded cells (as reported previously, Ursic and Culbertson, 1991). DAPI staining revealed that 77–82% of these cells had divided and segregated their chromosomes into the mother and daughter cells. In all of the remaining large-budded cells the chromosomal DNA was localized in the mother-bud neck. We did not observe any wild-type anucleate or multinucleate cells (Figure 4, DNA). The *tcp1-1* mutant strain accumulates 15% multinucleate and 6% aploid cells (Figure 4) (as shown previously, Ursic and Culbertson, 1991). In the *tcp1-2* mutant strain, 44% of the large budded cells were mononucleate, and 12% contained two nuclei in the mother cell and none in the bud. These cells can undergo cytokinesis, because 5% of the unbudded cells were anucleate (Figure 4). In the *tcp1-3* mutant strain, 73% of the cells were large budded and elongated, 63% of which contained one nucleus near the mother-bud neck (Figure 4). Thus, the nuclei were abnormally distributed in strains carrying any of the three *tcp1* mutations.

To distinguish between defects in DNA replication and nuclear migration, we used flow cytometry analysis of asynchronous haploid cultures. About 30% of wild-type cells had 1N DNA content and ~55–60% had 2N DNA content. The remainder of the cells were intermediate in DNA content and were therefore in the process of DNA replication (Figure 4). The *tcp1-1* and *tcp1-2* cells resembled wild-type cells when grown at the permissive temperature. In contrast, cells carrying *tcp1-1* accumulate ~11% aploid cells and 67% cells with 2N and greater DNA content within 40.5 h after shift to the restrictive temperature (Figure 4). Cells carrying *tcp1-2* accumulate 21% aploid cells and 62% cells with 2N and greater DNA content within 5 h after shift to the restrictive temperature (Figure 4). At the permissive temperature, ~65% of cells carrying *tcp1-3* had 2N DNA content compared with 55–60% for wild-type (Figure 4). After 22.5 h of incubation at the restrictive temperature, 5% of the *tcp1-3* carrying cells were aploid and 83% had 2N and greater DNA content. Thus, these results suggest that the *tcp1* mutations perturb nuclear division and migration and block the completion of the cell cycle, whereas DNA synthesis appears to proceed normally.

Mutations in *TCP1* Alter the Organization of Microtubules

To examine the organization of microtubules in strains carrying *TCP1*, *tcp1-1*, *tcp1-2*, and *tcp1-3*, we used direct

fluorescence and indirect immunofluorescence microscopy. Each pair of panels in Figure 5 shows cells carrying *TCP1* alleles, labeled with anti- α -tubulin antibodies to visualize the microtubules and with DAPI to stain DNA (MATERIALS AND METHODS). There was no significant difference in the distribution of DNA or microtubules in wild-type *TCP1* cells grown at 30°C and then shifted to 15°C for 16 h. Similar results were obtained when the same cells were shifted to 37°C, indicating that temperature changes alone do not significantly alter the distribution of DNA or microtubules. Cells carrying *tcp1-1*, *tcp1-2*, or *tcp1-3* each developed microtubules that were abnormal in appearance after a shift to the appropriate restrictive temperature. The abnormalities were different for each mutant.

Similar to previous findings (Ursic and Culbertson, 1991), the microtubules in cells carrying *tcp1-1* became abnormally positioned, frayed, and fanned after incubation at 15°C. Cells carrying *tcp1-2* developed abnormal microtubules after incubation at 37°C. The spindles stained much more intensely than in wild-type cells. The appearance of the spindles suggests either hyperpolymerization or increased accessibility of α -tubulin to the antibodies. Cells carrying *tcp1-3* also developed abnormal microtubules after incubation at 15°C. The spindles stained slightly more intensely compared with wild-type cells. The astral microtubules formed fine, often fanned structures that extended across the entire cell. In cells carrying any of the three *tcp1* alleles the microtubules appear to be positioned at random (Figure 5, arrows).

We also found that the growth of strains carrying *tcp1-1*, *tcp1-2*, and *tcp1-3* were hypersensitive to benomyl, a drug that specifically prevents microtubule polymerization (Davidse, 1986). The mutants were unable to form colonies at 24°C when grown on YEP plates containing 12.5 μ g/ml benomyl, a concentration that permits the growth of wild-type cells. Cells carrying *tcp1-3* were somewhat more sensitive to benomyl than cells carrying *tcp1-1* and *tcp1-2*, which have similar sensitivity to this drug (Ursic and Culbertson, 1991). The benomyl sensitivity is recessive relative to wild-type in each mutation (see MATERIALS AND METHODS).

Mutations in *TCP1* Alter the Distribution of Actin Filaments

We assessed the distribution of actin filaments in cells carrying *TCP1*, *tcp1-1*, *tcp1-2*, and *tcp1-3* using phalloidin (Figure 6), a toxin that stains filamentous F actin rather than the unpolymerized G form of actin (Adams and Pringle, 1991). Wild-type *TCP1* cells incubated at 15°C (or 37°C) had typical asymmetric actin structures with buds that contained intensely stained patches and actin cables localized along the long axis of the mother cell (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Novick and Botstein, 1985). Some cells had an

Figure 5. *Tcp1p* is required for the proper organization and distribution of microtubules. Each pair of photographs shows fluorescent microscopy and indirect immunofluorescent microscopy analysis of cells labeled with DAPI to visualize the DNA and anti- α -tubulin antibodies to visualize the microtubules. We used the strains, incubation conditions, and time points after shift to the restrictive temperature as described in Figure 4. Cells carrying *tcp1-1* have frayed and fanned microtubules. Cells carrying *tcp1-2* have abnormally wide anti- α -tubulin-stained spindles and broader astral microtubules than found in wild-type cells. Cells carrying *tcp1-3* have pronounced, fanned astral microtubules, and more intensely stained spindles. Each *tcp1* allele appears to cause random positioning of the microtubules (marked by arrows) when compared to wild-type cells, which usually contain microtubules extending from mother cell to bud. Bar, 2.5 μ m.

intense phalloidin-stained ring at the bud neck (Figure 6, *TCP1*). Cells carrying *tcp1-1*, *tcp1-2*, or *tcp1-3*, respectively, each exhibited a unique change in actin distribution when shifted to the appropriate restrictive temperature.

Cells carrying *tcp1-1* had a symmetric actin filament distribution after shift to 15°C for 16 h. Disorganized fine cables and surface patches were observed. The actin distribution in cells carrying *tcp1-2* appeared nearly normal after shift to 37°C for 2 h. Actin structures were typically asymmetric, but the mother cells had disorganized fine actin cables similar to those present in cells carrying *tcp1-1*. Also, *tcp1-2* cells that were undergoing cell lysis developed a gap in the cell wall and had in-

tensely stained actin patches localized near the cell surface (Figure 6, arrow). Cells carrying *tcp1-3* had intensely stained actin cables instead of the normal patches and short disorganized actin filaments after incubation at 15°C for 2 h. These results suggest that mutational alterations in *Tcp1p* lead to perturbations in the normal distribution of actin filaments.

***Tcp1p* Localizes to the Cytoplasm and to Patches at the Cell Cortex**

The subcellular localization of *Tcp1p* was examined by indirect immunofluorescence microscopy using anti-mouse *Tcp1p* antibodies designated 91a by Willison *et*

Figure 6. *TCP1* is required for normal actin organization. Photographs show fluorescence microscopy of cells stained with rhodamine phalloidin. We used the strains, incubation conditions, and time points after shift to the restrictive temperature as described in Figure 4. Cells carrying *TCP1* have actin patches in the bud and fine cables along the long axis in the mother cell. Some large budded cells have an intense actin-stained ring at the mother bud neck. Cells carrying *tcp1-1* do not have the asymmetric actin distribution typical of wild-type, but have fine, disrupted actin filaments. Cells carrying *tcp1-2* have a near normal actin distribution, except that some cells develop a break at the cell cortex corresponding to intensely stained actin patches (marked by an arrow). Cells carrying *tcp1-3* have intense actin cables rather than patches in buds. Bar, 5 μ m.

al. (1989). Previously, we showed by Western blotting that 91a antibodies specifically recognize yeast Tcp1p when overproduced in strains carrying *TCP1* on a multicopy plasmid. 91a sera did not detectably cross-react with any other yeast proteins (Ursic and Culbertson, 1991). Recognition of yeast Tcp1p by the anti-mouse Tcp1p antibodies was too weak to produce a band on Western blots from strains carrying a single *TCP1* gene copy. Despite the lack of detection on Western blots, we were able to detect yeast Tcp1p by immunofluorescence analysis of strain DUY1, which carries a single chromosomal copy of the *TCP1* gene.

Tcp1p localizes predominantly at the cell surface in DUY1 cells (Figure 7A). The Tcp1p distribution was similar in DUYT1 cells (Table 1) that overproduce Tcp1p, indicating that overexpression of Tcp1p does not cause any obvious mislocalization (Figure 7, B–D). Because cells overproducing Tcp1p provide a much clearer and more intense staining pattern, we chose these cells to assess its distribution. The similar distribution combined with the differential intensity of labeling of Tcp1p in single and multicopy strains indicates that the observed staining is specific for Tcp1p. Figure 7 shows a cell when viewed from two distinct focal planes, through the center of the cell (B) and at the cell surface (C). D shows a field of cells to illustrate the extent of variability in the staining pattern from cell to cell. The staining has a granular appearance at the cell surface and a ring that becomes apparent by comparing different focal planes. The results show that Tcp1p localizes to the cell cortex, but some of the protein appears

to be distributed throughout the cytoplasm. Tcp1p cannot be detected in the nucleus.

DISCUSSION

Functional Relationship Between Tcp1p and the Microtubules

TCP1 interacts in a gene- and allele-specific manner with mutations in both *TUB1* and *TUB2*, genes encoding α - and β -tubulins, respectively. We observed a synthetic-lethal phenotype in a *tcp1-1 tub2-402* double mutant and a slow growth phenotype in a *tcp1-1 tub1-1* double mutant. The *tcp1-1* mutant had no obvious phenotypic effect when placed in combination with mutations in genes coding for other cytoskeletal components, including Bik1p, Cdc31p, and Esp1p. This kind of genetic behavior in double mutants is indicative of a close functional relationship between proteins and in some cases has been found to be the result of direct protein-protein interaction (Hoyt *et al.*, 1992; Roof *et al.*, 1992).

By examining the distribution of nuclei and microtubules in *tcp1-1*, *tcp1-2*, and *tcp1-3* mutant cells using fluorescence and indirect immunofluorescent microscopy, we found that each mutation was associated with allele-specific abnormalities in the microtubules. At the restrictive temperature cells carrying *tcp1-1* displayed frayed and fanned microtubules. Cells carrying *tcp1-2* developed extensively stained spindles. Cells carrying *tcp1-3* showed expansive astral microtubules. In each case, the majority of the mutant cells were viable at the

Figure 7. Tcp1p localizes to the cytoplasm and the cell cortex. Wild-type cells (DUY1) and cells overproducing Tcp1p (DUYT1) were labeled with monoclonal antibodies to mouse Tcp1p and visualized by immunofluorescence microscopy. Wild-type cells have a patchy staining pattern at the vicinity of the cell cortex (A). Cells overproducing Tcp1p have a similar, but more intense staining pattern (B–D). B and C show a cell photographed at two focal planes, through the center of the cell (B) and the top of the cell (C). These images show that Tcp1p localizes to the cytoplasm and to patches at the cell cortex. D shows a field of cells. Depending on the focal plane, some cells show a staining pattern that extends almost to the center of the image. All cells have an unstained nuclear region. Bar, 1 μ m.

time these phenotypes were initially observed. Thus, the kinetics of appearance of these abnormalities suggests that they are a direct consequence of altered Tcp1p function and not a delayed, indirect consequence of cell death. We did not observe any deformed microtubule structures in wild-type cells.

The aberrant microtubule structures in the *tcp1* mutants do not precisely match those observed for mutations in a number of other genes affecting microtubules. However, the microtubules in the *tcp1-2* mutant cells do resemble those found in strains carrying the heat-sensitive *cdc20* mutation and in strains that overproduce both α - and β -tubulins (Burke *et al.*, 1989; Sethi *et al.*, 1991; Weinstein and Solomon, 1990). Cdc20p was proposed to modulate microtubule structures either by promoting microtubule disassembly or by altering the surface of the microtubules (Sethi *et al.*, 1991).

Even though the *tcp1* mutations map at different locations in the Tcp1p protein, all three confer similar phenotypes with regard to microtubules. At the restrictive temperature they develop frayed and fanned microtubule bundles, and in some cells these structures are positioned randomly in budded cells rather than oriented toward the incipient bud. This type of abnor-

mality is suggestive of defects in both the microtubules and in the actin filaments (Palmer *et al.*, 1992). In addition, the *tcp1* alleles cause cells to be hypersensitive to benomyl (Ursic and Culbertson, 1991; this paper), an anti-mitotic compound shown to specifically prevent microtubule polymerization (Davidse, 1986). Furthermore, all three alleles cause abnormal nuclear distribution and perturb cell-cycle progression. They do not appear to affect DNA replication. Most of the cells are large budded (52–73%) after shift to the nonpermissive temperature. It was shown previously that mutants affecting the microtubules often arrest as large budded cells at the restrictive temperature (Huffaker *et al.*, 1987). Taken together, our findings imply a role for Tcp1p in the function of microtubules. We are now in a position to test whether the genetic interactions between α - and β -tubulin are the consequence of direct physical interactions.

Functional Relationship Between Tcp1p and Actin Filaments

We detected two distinct allele-specific interactions between the *tcp1-1* mutation and mutations in *ACT1*, the

sole structural gene for actin (Novick and Botstein, 1985). *tcp1-1 act1-1* and *tcp1-1 act1-4* double mutants have much slower growth rates than either single mutant. In addition, overproduction of wild-type Tcp1p partially suppresses the heat-sensitive growth phenotypes of *act1-1* and *act1-4*. There were no novel phenotypes found in the *tcp1-1 act1-2* double mutant nor did overproduction of Tcp1p suppress the growth defects caused by *act1-2*.

The *act1-1* and *act1-4* mutants have similar phenotypes at the restrictive temperature: absence of actin cables and the persistence of actin patches. In contrast, the *act1-2* mutant cells have heavy bars of actin-stained material at the permissive temperature and disorganized fine cables and surface patches at the restrictive temperature (Novick and Botstein, 1985; Dunn and Shortle, 1990). Although the *act1-1* and *act1-4* mutants show similar phenotypes at the restrictive temperature, the mutations map to different positions in the *ACT1* gene (Novick and Botstein, 1985; Dunn and Shortle, 1990). Based on a three-dimensional atomic model proposed for actin, the mutations are positioned in two different lobes of the actin protein (Wertman *et al.*, 1992; Schroeder *et al.*, 1993).

Depletion of wild-type *SAC7*, a gene identified as an extragenic suppressor of the heat-sensitive *act1-4* mutation, affects actin mutant phenotypes in a manner opposite of the interaction observed for *tcp1-1* (Dunn and Shortle, 1990). Depletion of *SAC7* is lethal in strains carrying *act1-2* but results in suppression of the heat-sensitive growth of *act1-1* and *act1-4*. The *tcp1-1* mutation is near lethal in combination with *act1-1* and *act1-4* but has no effect with *act1-2*. Thus, the *act1-1* and *act1-4* mutants share similar phenotypes and functions at the restrictive temperature that are different from cells containing *act1-2*.

Mammalian Tcp1p was shown to bind in vitro to actin (Gao *et al.*, 1992). Based on analyses of budding patterns of cells containing the *tcp1* null allele (strain DUY4) (Table 1) and secretory vesicles in *tcp1-245* mutant cells, Horwich and Willison (1993) and Miklos *et al.* (1994) saw no evidence for an in vivo role of Tcp1p on actin. However, genetic analyses and actin staining of cells containing mutations in *TCP1* substantiate the observed in vitro interaction between actin and Tcp1p.

Possible Functional Domains in Tcp1p

We scanned the SWISS-PROT (Geneva, Switzerland) protein sequence data bank (release 28) for possible functional domains related to the observed phenotypes of our *tcp1* mutants. We found three regions within Tcp1p that have sequence similarity with known functional domains.

The first region, amino acids 95–99 (gly-asp-gly-thr), comprises part of the putative ATP-binding domain, which resembles the ATP-binding domain of the

cyclic AMP-dependent protein kinase (Hanks *et al.*, 1988). Lewis *et al.* (1992) first noticed the sequence similarity of this Tcp1p site and the ATP-binding domain. It is known that Tcp1p homologues bind to and hydrolyze ATP in vitro (Trent *et al.*, 1991; Frydman *et al.*, 1992; Gao *et al.*, 1992; Yaffe *et al.*, 1992). Therefore, it is likely that the *tcp1-1* mutation, which localizes within this domain (Table 3), alters the binding affinity or rate of hydrolysis of ATP.

The second region, amino acids 406–423, shows amino acid sequence similarity to the MAP2/tau microtubule-binding domain (Table 4) (Lewis *et al.*, 1988; Paschal *et al.*, 1989). MAP2 and tau are mammalian tubulin-binding proteins that share incomplete repeats of an 18 amino acid motif. This motif is relatively common among the 36 000 proteins of the SWISS-PROT data bank, as a scan revealed that ~0.5% of its entries have a region with some similarity to it. However, the mammalian MAP2/tau sequence is sufficiently rare and conserved among eukaryotes to be successfully used to clone a Tcp1-like protein from *Tetrahymena* (Soares *et al.*, 1994). The *tcp1-2* mutation localizes within this region. It is therefore possible that the mutation alters the affinity of Tcp1p to the tubulins, being the cause of the observed allele-specific lethal interaction with the *tub2-402* mutation.

The third region, amino acids 66–80, shows similarity to a domain that has been associated with actin binding (amino acids 516–530 of Myo4p) (Table 4) (Haarer and Brown, 1992; Schroeder *et al.*, 1993). This amino acid motif is rare. A scan of the SWISS-PROT data bank revealed that besides the Tcp1p homologues, seven other proteins contain it. They are the myosin-2 and myosin-4 isoforms of *S. cerevisiae*, important for vesicle transport and bud formation (Haarer and Brown, 1992; Johnston *et al.*, 1991), and the myosin-like protein ABMA of *Dictyostelium*, involved in a range of motile

Table 4. Sequence similarity between Tcp1p and putative functional domains

Protein	Sequence similarity
Tcp1-2p mutation ₄₂₃	D
Tcp 1p ₄₀₆	LSVVK RTLESGNVVPGGG : : :::
mouse MAP2 ₁₇₃₇	AQA KVGSLDNAHHVPGGG
consensus MAP2/tau	K GS N P GG
Tcp 1p ₆₆	GATILSLLDVQ HPAG
<i>S. cerevisiae</i> Myo4p ₅₁₆	G ILSLLDEESRLPFG

Protein names indices correspond to the position of the first amino acid subsequence in the complete wild-type protein. Identical amino acids are indicated with | and conserved amino acid similarity by :. The groups of similar amino acids are as follows: (A, G), (D, E), (F, Y), (I, M, V, L), (N, S, T, Q), and (R, K).

processes (Titus *et al.*, 1989). Myosin binds laterally to filamentous actin (Vandekerckhove and Vancompernelle, 1992; Schroeder *et al.*, 1993). The remaining proteins that contain this amino acid motif are the maize *Zea mays* protein, associated with a DNA binding complex (De Vetten *et al.*, 1992); Prp8, an *S. cerevisiae* protein involved in pre-mRNA splicing (Jackson *et al.*, 1988); the seryl-tRNA synthetase (Fujinaga *et al.*, 1993); and the turnip yellow mosaic virus RNA replicase polyprotein (Keese *et al.*, 1989). Hence, among all the proteins in the data bank, the Tcp1p homologues, three myosin isoforms, and four other proteins, which affect nucleic acids, share this motif.

Tcp1 Localizes to the Cell Cortex and the Cytoplasm

We found that Tcp1p localizes in discrete granular structures at the cell cortex extending into the cytoplasm. The observed pattern of Tcp1p distribution resembles that of the mammalian protein vinculin (Samuels *et al.*, 1993). In higher eukaryotes vinculin plays a role in specialized structures in the plasma membrane. At these sites actin filaments make lateral connections to the membranes. In view of the observed relationship of Tcp1p with actin and the analogy between the subcellular distribution of Tcp1p and of vinculin, it is plausible to speculate that Tcp1p could perform functions related to vinculin.

Cell fractionation experiments by Silver and White (1982) have shown that mouse Tcp1p has biochemical properties resembling cell surface-associated components. The authors also noted a difference in Tcp1p sublocalization and protein levels when comparing F9 teratocarcinoma cells and mouse testicular cells using biochemical fractionations. These findings were interpreted by the authors to mean that the level of Tcp1p expression and the intra- and extramolecular Tcp1p interactions are under tissue-specific control. This explanation is consistent with our observations that Tcp1p interacts with the cytoskeleton, which is a dynamic network of both structural and motor proteins that maintain the integrity of the cell and generate cellular movement. It also may explain some of the apparent differences in Tcp1p subcellular localization observed by various researchers (Willison *et al.*, 1989; Lewis *et al.*, 1992; Horwich and Willison, 1993).

Microtubules and Actin Filaments Perform Comparable Functions that Are Affected by Tcp1p

All available data show that defects in Tcp1p lead to abnormal cellular functions for which microtubules and actin filaments are required. Microtubules and actin filaments perform comparable functions by transferring mechanical stress across distances many orders of magnitude larger than would be possible with typical macromolecular interactions. These processes must require

the cooperative interactions of large numbers of cell components.

Mutations in *TCP1* have been shown to result in cells defective in microtubules and actin structures. Hence, this is the first known protein that affects both of these fundamental cytoskeletal structures. Tcp1p should provide an appealing focus for elucidating the microtubules-actin connection.

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