HETERODIMER FORMATION AND TUBULIN FUNCTION IN *S. CEREVISIAE*

by

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ABSTRACT

Many effectors of microtubule assembly *in vitro* enhance the polymerization of subunits. However, several *S. cerevisiae* genes that affect cellular microtubule-dependent processes appear to act at other steps in assembly, and to affect polymerization only indirectly. Here we use a mutant α-tubulin to probe cellular regulation of microtubule assembly. *tub1-724* mutant cells arrest at low temperature with no assembled microtubules. The results reported here demonstrate that the heterodimer formed between Tub1-724p and β-tubulin is less stable than wild type heterodimer. The unstable heterodimer explains several conditional phenotypes conferred by the mutation.

We also describe genes that affect formation of the tubulin heterodimer. Our approach to identify such genes is based on the observation that excess Rbl2p, a β-tubulin binding protein, is lethal in *tub1-724* mutant strains. We show that excess Rbl2p is similarly lethal to cells bearing mutations in *CIN1* and *PAC2*. Genetic and biochemical analysis demonstrates roles for each in heterodimer formation *in vivo*. Both haploid and heterozygous *tub1-724* cells are inviable when, *PAC2*, is over-expressed. These effects are explained by the ability of Pac2p to bind α-tubulin, a complex we demonstrate directly. And, excess Cin1p rescues the phenotypes of *tub1-724* cells, strongly supporting a catalytic role for Cin1p in heterodimer formation. Pac2p is associated with Cin1p and with the tubulin polypeptides. The activities of these proteins *in vivo* are both similar to and distinct from the role of their homologues *in vitro* assays for tubulin folding. Our results uncover potential roles for these proteins in catalyzing tubulin heterodimerization *in vivo* and so in maintaining the balance between individual tubulin polypeptides.

Both excess β-tubulin and benomyl lead to loss of microtubules, large budded cell-cycle arrest and cell death. This parallel is supported by the consequences that changes in the level of either Rbl2p or α-tubulin have for both of these microtubule poisons. Overexpression of either gene rescues cells from the effects of β-tubulin overexpression and confers increased resistance to benomyl. We screened for galactose-induced cDNAs that confer resistance to benomyl in wild type cells. We describe the identification and initial characterization of two genes that allow cells to **L**ive on **B**enomyl when **O**verexpressed (LBO).

Thesis Supervisor: Frank Solomon

Title: Professor of Biology

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I dedicate this thesis to the memory of my grandfather, Antonio Alfredo Benito Vega (17 January 1911- 6 January 1999), who ever since I was a little girl called me "La Cientifica" (The Scientist).

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CHAPTER I :

Introduction

Microtubules are found in all eukaryotic cells and are composed of heterodimeric subunits of α- and β-tubulin. The filamentous arrays formed by microtubules are essential for life and microtubules are known to function in a variety of cellular processes including mitosis, meiosis, intracellular transport, morphogenesis, and cell motility (reviewed in (Dustin, 1984)). Some of the properties specifying microtubule assembly are intrinsic to the tubulin heterodimer. For example, the microtubule is a polar structure with a fast growing plus end and a slow growing minus end. And microtubules are dynamic, exhibiting periods of rapid growth and rapid shrinkage. *In vitro* , purified tubulin is sufficient to recreate microtubule polymers. The conditions for the assembly of microtubules from tubulin have been well defined. Among the factors that influence microtubule assembly are: concentration of tubulin; presence of GTP; concentration of cation; temperature and pH (reviewed in (Dustin, 1984)). However, other factors are necessary to specify the full range of structures, organelles and movements that microtubules specify in living cells. In addition, unlike microtubule assembly *in vitro*, which begins with purified tubulin and looks at the formation of polymer; cells must assemble heterodimer *de novo* from the individual α- and β- tubulin monomers. Recent work from a variety of different sources have identified proteins that may participate in tubulin heterodimer formation and suggest that formation of heterodimer is itself a regulated event.

Experiments in this thesis probe the activities of isolated tubulin polypeptides and tubulin binding proteins *in vivo*. Our thinking has been greatly influenced by the realization that free α- and β- tubulin are functionally distinct *in vivo* ; excess β-tubulin is a microtubule poison. We have used the activity of free β-tubulin to investigate various aspects of microtubule assembly *in vivo*. That analysis has allowed us to look at genes that impact the state and activities of tubulin chains in the cell and to uncover possible regulatory mechanisms.

Tubulin Structure

The tubulin heterodimer serves as the building block for the assembly of microtubules. The microtubules of most cells are arranged as 13 parallel protofilaments that close to form an intact hollow cylinder. Each protofilament is made up of linear arrays of tubulin heterodimers. Longitudinal bonds connect alternating α- and β-tubulin subunits in a protofilament. The subunits of adjacent protofilaments slightly staggered and are connected by lateral bonds that are primarily α-α and β-β interactions. However, since microtubules form a shallow helix with respect to the lateral bonds; lateral bonds also form α-β and β-α interactions when the subunits make a complete turn (reviewed in (Downing and Nogales, 1998; Dustin, 1984; Erickson and Stoffler, 1996).

The high degree of homology between the tubulins of very different organisms suggests that the molecular basis for conserved microtubule structure in part arises from the primary sequence of the α- and β-tubulins. Recently, the structure of the αβ tubulin dimer has been solved by electron crystallography (Nogales et al., 1998). This

work corroborates many of the insights into tubulin structure gained though more indirect biochemical analysis and genetic analysis.

The α- and β- tubulin monomers are about 55,000 daltons. Both monomers bind GTP and share about 40% amino acid identity. Nogales and coworkers found that α- and β- tubulin are also very similar at the structural level (Nogales et al., 1998). The GTP binding site on α-tubulin is buried with the tubulin dimer and is non-exchangeable (N). The β-tubulin GTP binding site is exchangeable (E). The state of the nucleotide at this E site is thought to contribute to microtubule dynamics (Nogales et al., 1998). According to their model, there are three possible states for the nucleotide bound at the E site, GTP, GDP + inorganic phosphate (Pi), and GDP. The crystal structure of the tubulin dimer predicts that both the inter-and intra- dimer interactions are very tight. Thus, the conformation of the polymer could be altered by the state of the nucleotide, by microtubule associated proteins, or by drugs (Nogales et al., 1998). The authors suggest that fine tuning of these interactions could regulate assembly and the dynamic instability of microtubules (Downing and Nogales, 1998; Nogales et al., 1998).

Interestingly, tubulin was found to be similar in structure to FtsZ, a GTPase that is essential for cell division in prokaryotes (Nogales et al., 1998); (Lowe and Amos, 1998; Nogales et al., 1998). FtsZ forms a filamentous ring-shaped septum at the division site in prokaryotes. In addition, FtsZ shares the "tubulin" signature sequence with α, β, and γ which is involved in GTP binding. Like tubulin, FtsZ can polymerize into protofilaments and tubules in a GTP dependent manner (Bramhill and Thompson,

1994; Erickson et al., 1996; Mukherjee and Lutkenhaus, 1994). Because of this, FtsZ has been described as the prokaryotic precursor of tubulin (Erickson, 1998; Erickson, 1995; Erickson and Stoffler, 1996). That tubulin and FtsZ share structural similarity suggests that studies of FtsZ a might provide insight into tubulin biology and *vice versa.*

Microtubule associated proteins

Potential regulators of microtubule assembly and organization have been identified by virtue of their ability to cycle with brain tubulin though several rounds of assembly and disassembly (Borisy et al., 1975; Cleveland et al., 1977). These proteins present in substochiometric amounts may serve to regulate both quantitative and qualitative aspects of microtubule assembly. Some microtubule associated proteins or MAPs (Weingarten et al., 1975) include MAP1A (Bloom et al., 1984), MAP1B (Noble et al., 1989), MAP2 (Shiomura and Hirokawa, 1987), MAP4 (Parysek et al., 1984), XMAP230 (Andersen et al., 1994), and Tau (Drubin et al., 1984). Fractionation schemes using microtubules (Magendantz and Solomon, 1985; Pillus and Solomon, 1986) and microtubule affinity chromatography (Barnes et al., 1992) have also been used with varying degrees of success to isolate microtubule binding proteins. Many of the proteins identified by all these methods co-localize with microtubules or microtubule organizing centers *in vivo* and enhance microtubule stability both *in vivo* and *in vitro* (Cleveland et al., 1977; Knops et al., 1991; Leclerc et al., 1996; Sandoval and Vandekerckhove, 1981).

In addition, proteins that inhibit microtubule polymerization and so have the opposite effect of MAPs have also been identified. XKCM1 is a kinesin related protein

from Xenopus ovary that increases the catastrophe rate of microtubules (Walczak et al., 1996). Oncoprotein 18/stathmin binds to tubulin dimer and affects tubulin assembly by increasing the catastrophe rate and decreasing the growth rate of microtubules (Belmont and Mitchison, 1996).

There are limitations in using biochemical approaches for isolating proteins that affect microtubule assembly. For example, proteins that are important for microtubule assembly but that only transiently interact with microtubules might not be identified by these methods. Moreover, that a protein can bind to microtubules does not mean that it functions with microtubules or that the binding is biologically significant.

Phage assembly as a model for microtubule assembly

Parallels between microtubule assembly *in vivo* and other assembly pathways allow us to exploit observations made in these systems and apply them to studies of microtubule assembly. In particular, the study of bacteriophage assembly provides several useful paradigms for studying the interactions required for assembly of a multi-subunit structure (reviewed in (Weinstein and Solomon, 1992)). By using a combination of biochemical and genetic methods, a detailed and ordered pathway of the protein-protein interactions required to assembly a bacteriophage particle was generated.

One approach that was successfully used in the phage system to identify interacting components was pseudo-revertant analysis. In the phage P22 system, Jarvik and Botstein (1975) showed that revertants of missense mutations were often conditional lethal mutations in gene products that physically interact with the protein product of the original mutant gene (Jarvik and Botstein, 1975). A second lesson of

bacteriophage assembly is that assembly pathways are sensitive to stoichiometry; a deficit in one component relative to another may result in qualitative rather than quantitative defects in assembly (Floor, 1970; Sternberg, 1976). These studies suggest that one can identify interacting proteins genetically by screening for stoichiometric suppressors of the original phenotypes. Both of these approaches have been successfully applied to the problem of microtubule assembly in yeast and are described below (Weinstein and Solomon, 1992).

Microtubule assembly in yeast

Yeast offers the opportunity to study microtubule function in a genetically tractable organism (Huffaker et al., 1988). The genes encoding the primary components of microtubule structure, α- and β-tubulin, have been cloned and characterized (Neff et al., 1983; Schatz et al., 1986). *Saccharomyces cerevisiae* has two genes encoding α-tubulin, *TUB1* and *TUB3,* that are 90% identical at the amino acid level (Schatz et al., 1986). *TUB1* is essential for mitotic growth while *TUB3* is non-essential (Schatz et al., 1986). The differences between the two can be accounted for by differences in their level of expression (Schatz et al., 1986). β-tubulin function is encoded by a single, essential gene, *TUB2* (Neff et al., 1983).

Microtubule arrays in yeast cells are known to be important for specific functions: mitosis, meiosis and nuclear fusion during mating (Delgado and Conde, 1984; Huffaker et al., 1988; Jacobs et al., 1988). Collections of mutations in tubulin genes that affect both quantitative and qualitative aspects of microtubule function are

available (Huffaker et al., 1988; Reijo et al., 1994; Schatz et al., 1988). The analysis of tubulin mutants can provide insight into those steps. For example, the phenotypes of cells containing *tub2-401*, a mutant β-tubulin that affects only the cytoplasmic microtubules, suggest that cytoplasmic microtubules are needed to position the spindle in the bud neck but are not required for spindle elongation at anaphase B (Sullivan and Huffaker, 1992). Yet, the defects of mutant tubulins are largely understood in terms of the arrest phenotype rather than their execution point.

For another important cytoskeletal polymer; actin, analysis of the requirements for actin assembly was facilitated by the availability of both structural information about actin and collections of actin mutants. Consequently, studies in yeast show that many actin alleles have properties that are readily interpreted in light of the actin structure (Wertman et al., 1992). The available three dimensional structure of the α β heterodimer will make a similar analysis of tubulin possible (Nogales et al., 1998).

Pseudo-revertant analysis of tubulin mutations has yielded both tubulin and non-tubulin suppressors. For example, *in S. cerevisiae* suppressers of conditional α-tubulin alleles have been isolated in β-tubulin (Schatz et al., 1988). In *Aspergillus nidulans*, Morris and co workers isolated α-tubulin revertants of conditional alleles in β-tubulin (Morris et al., 1979). Non-tubulin suppressors have also been identified in this manner. The most striking example comes from the work of Oakley and Oakley (1989) in *Aspergillus nidulans* . By screening for suppressors of a mutation in β-tubulin they identified *mipA* , the gene encoding γ-tubulin (Oakley and Oakley, 1989).

γ-tubulin is an evolutionarily conserved tubulin family member involved in microtubule nucleation (Oakley et al., 1990; Steams et al., 1991; Zheng et al., 1991). More recently in *S. cerevisiae* screens for suppressors of β-tubulin mutations have identified mutations in the genes encoding the microtubule associated proteins *STU1* (Pasqualone and Huffaker, 1994) and *STU2* (Wang and Huffaker, 1997).

Genetic approaches that focus directly on processes known to involve microtubules in *S. cerevisiae* have also yielded a number of microtubule associated proteins. For example, *BIK1* which localizes to the nuclear spindle was identified in a screen for karyogamy defects (Berlin et al., *1990*). Karyogamy is the process during mating by which two haploid yeast nuclei fuse to produce a single diploid nucleus. , This process requires nuclear congression, a microtubule dependent process, and nuclear envelope fusion (reviewed in (Rose, 1996)). Cells deleted for *BIK1* exhibit defects in chromosome segregation and nuclear migration. Similar functional screens in yeast have identified a variety of microtubule based motors (Eshel et al., 1993; Hoyt et al., 1992; Li et al., 1993; Lillie and Brown, 1992; Meluh and Rose, 1990; Roof et al., 1992) spindle pole body components (Nguyen et al., 1998; Page and Snyder, 1992; Schutz et al., 1997; Winey et al., 1991) and proteins involved in the spindle assembly checkpoint (Hoyt et al., 1991; Li and Murray, 1991).

Another approach to identify gene products involved in microtubule assembly is to screen for synthetic lethal interactions with deletions or defective alleles of known microtubule related proteins. For example, *ASE1* was isolated as a mutation that is synthetic lethal with *BIK1*. Ase1p localizes to the spindle midzone until the end of

mitosis (Pellman et al., 1995). Kem1/Sep1, a putative MAP, was isolated in a screen for mutations that enhanced the nuclear fusion defect of *kar1-1* (Kim et al., 1990).

However, from these same types of functional analysis there is a growing list of gene products that affect microtubule-dependent processes but do not interact directly with the microtubule polymer. For example, several genes that affect chromosome instability (Hoyt et al., 1990) sensitivity to microtubule depolymerizing drugs (Stearns et al., 1990) excess β-tubulin (Archer et al., 1995) dependence upon a mitotic motor (Geiser et al., 1997), and γ-tubulin function (Geissler et al., 1998) encode proteins that act on microtubules at some step other than the polymerization reaction. That mutations in proteins not present on the assembled polymer affect microtubule stability *in vivo* suggests that there are other regulatory steps for crucial microtubule assembly. In addition, such regulatory functions are evident in the response of cells to tubulin imbalances described below.

The dynamic behavior of microtubules suggest that fluctuations of tubulin concentration in the cell may regulate quantitative and qualitative aspects of microtubule assembly (Mitchison and Kirschner, 1984). Indeed, in animal cells, manipulation of tubulin levels by drug induced depolymerization or by microinjection of tubulin was found to affect tubulin mRNA stability (Cleveland, 1988). And overexpression of α-tubulin transgene in CHO cells was found to downregulate endogenous α-tubulin protein levels, perhaps by translational repression (Gonzalez-Garay and Cabral, 1996). Thus, animal cells may contain regulatory mechanisms to maintain homeostasis with respect to tubulin levels.

In yeast it is possible to vary tubulin levels by varying the copy number and/or the expression levels of tubulin genes. Cells with increased copies of α- and β-tubulin genes, or of α- tubulin genes alone, downregulate tubulin levels (Katz et al., 1990). However, in *S. cerevisiae* the absolute cellular levels of tubulin can vary from 0.5X to 1.2X without very little effect in mitotic growth or microtubule organization (Katz et al., 1990). In contrast, experiments designed to alter the cellular ratio of α-tubulin relative to β-tubulin show that yeast cells can tolerate high levels of α-tubulin; in contrast, even a small excess of β-tubulin is lethal (Katz et al., 1990; Weinstein and Solomon, 1990).

Effects of β-tubulin overexpression

Acute overexpression of β-tubulin is lethal in budding yeast (Burke et al., 1989; Weinstein and Solomon, 1990), but overexpression of α-tubulin is not (Weinstein and Solomon, 1990). The lethality associated with excess β-tubulin is preceded by the rapid disassembly of microtubules in the cell (Weinstein and Solomon, 1990). Moreover, the β-tubulin that accumulates co-localizes with the spindle pole body (SPB) (M. Magendantz, personal communication). These effects depend on the ratio of β- to α- tubulin rather than the absolute level of either subunit since concomitant overexpression of α-tubulin rescues both the microtubule phenotype and the lethality of excess β-tubulin (Weinstein and Solomon, 1990). This situation is similar to some aspects of bacteriophage assembly (described above); a deficit in one component

relative to another results in qualitative defects rather than quantitative defects in assembly. Thus, *in vivo*, normal heterodimer assembly requires that the components be held in balance, while an imbalance permits illegitimate interactions that can be deleterious to the cell (reviewed in (Weinstein and Solomon, 1992) . We and others have identified proteins that may participate in this process. For example the yeast protein, Rbl2p, affects how cells survive alterations in the ratios of α- to β-tubulin (described below and (Archer et al., 1995). Levels of Pac10p and the *GIM* genes affect α- to β-tubulin ratios (Alvarez et al., 1998; Geissler et al., 1998). That excess β-tubulin appears to interfere with microtubule assembly might be explained if the excess β-tubulin forms inappropriate interactions with other components that might be limiting for assembly (Weinstein and Solomon, 1992).

Rbl2p, a β-tubulin monomer binding protein.

Our lab identified *RBL2* as a high copy suppressor of excess β-tubulin lethality (Archer et al., 1995). Rbl2p is able to rescue β-tubulin lethality to the same extent as α-tubulin. Overexpressed Rbl2p can suppress the effects of both too much β-tubulin or too little α-tubulin (Archer et al., 1995). Rbl2p shares other properties with α-tubulin: both confer increased resistance to microtubule depolymerizing drugs when overexpressed, and both bind to β-tubulin *in vivo* (Archer et al., 1995)

Rbl2p binds β-tubulin to form a complex that excludes α-tubulin and is less stable than the α-β heterodimer (Archer et al., 1998). *In vivo*, Rbl2p is competent to bind β-tubulin both before and after it has been in the α-β heterodimer suggesting that

the form of β-tubulin recognized by Rbl2p is in equilibrium with the α-β heterodimer (Archer et al., 1998). Thus, the Rbl2p-β-tubulin complex defines a second pool of β-tubulin in the cell. Our analysis of the Rbl2p-β-tubulin complex suggests that the ability of Rbl2p to rescue cells from excess β-tubulin lethality likely requires binding of β-tubulin *in vivo*.

Rbl2p can be deleterious in both wild type and mutant cells by competing with α-tubulin for β-tubulin binding. We have characterized a mutant α-tubulin allele that is synthetically lethal with both *RBL2* deletion and over-expression (Vega et al., 1998). Our analysis of this mutant, detailed in Chapter Two, suggests that tubulin heterodimers containing the mutant α-tubulin protein, *tub1-724* are less stable than the wild type heterodimers (Vega et al., 1998). These results provide both a structure-function analysis of tubulin as well as information about the function of non-tubulin genes important for microtubule assembly *in vivo* (see below).

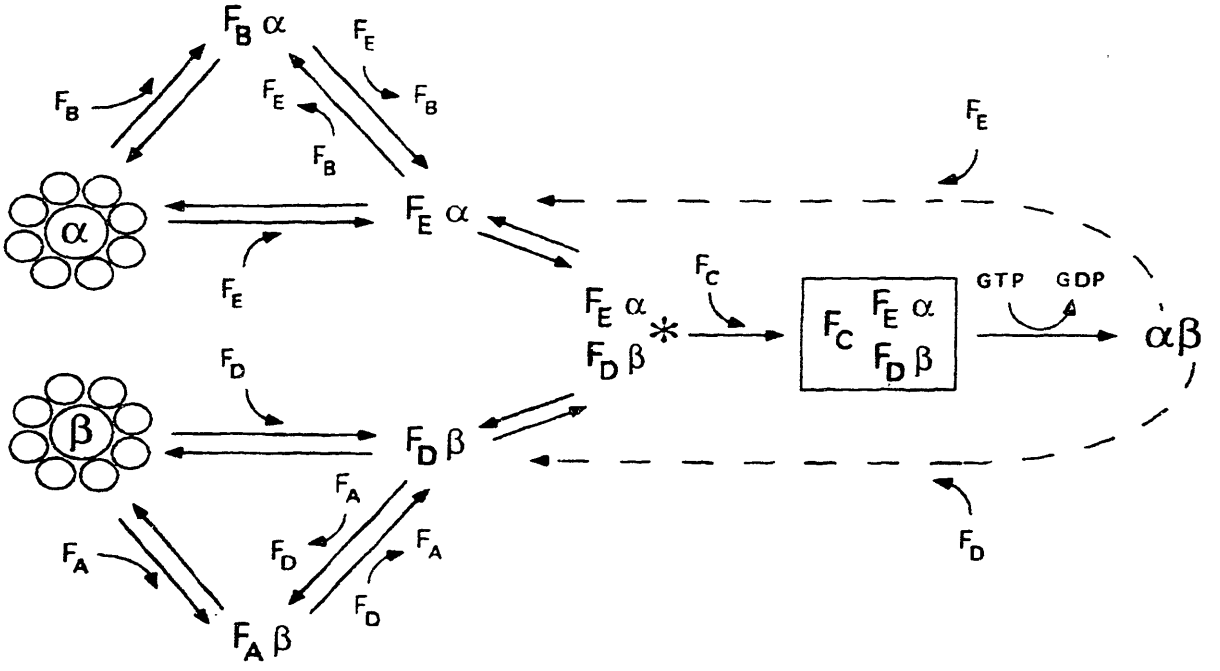
Tubulin Folding and heterodimer formation

Protein folding is the process by which the linear information contained in the primary amino-acid sequence of a polypeptide gives rise to the native, three dimensional structure of the functional protein (Hartl, 1996). Some proteins, such as ribonuclease A, can spontaneously acquire their native conformations *in vitro* (Anfinsen, 1973). However, the folding of other polypeptides is facilitated by the action of molecular chaperones that bind non-native states of proteins and assist them to reach a native conformation (Bukau and Horwich, 1998)).

The evolutionarily conserved heat shock family of molecular chaperones were first identified as proteins that are rapidly induced by stress. Although they are stress proteins, they play essential roles in the quality control of proteins under non-stress conditions as well. These functions include: folding of nascent polypeptides, translocation of proteins across membranes, promoting assembly and disassembly of oligomeric proteins; and facilitating the degradation of misfolded proteins (reviewed in (Hartl, 1996). Tailless complex polypeptide 1 (TCP-1), a cytoplasmic homologue of hsp60 chaperonin, forms a hetero-oligomeric torodial complex that is responsible for folding a specific subset of cellular proteins, including tubulins and actin (Lewis et al., 1992; Sternlicht et al., 1993).

In vitro, incubation with TCP-1 and ATP is sufficient for both actin and γ-tubulin to achieve a native state (Gao et al., 1992). However, Cowan and colleagues found that in addition to TCP-1 and ATP, α- and β-tubulin require the presence of GTP and of additional protein cofactors to interact with the tubulin chains before they are competent to assemble into exogenously added heterodimers (Campo et al., 1994; Gao et al., 1992; Gao et al., 1993; Tian et al., 1996; Tian et al., 1997). In that assay, β-tubulin released from the chaperone is bound independently by cofactors A or D, and α-tubulin is bound by either cofactor B or E. The β-tubulin released from cofactor A and the α-tubulin released by cofactor B fails to exchange into exogenous heterodimer as assayed by native gel electrophoresis. Instead, cofactor A and cofactor B enhance the efficiency the exchange reaction by 90% (see Figure 1-1 and (Tian et al., 1996; Tian et al., 1997).

Figure 1-1. Model for the *in vitro* tubulin folding assay and *S. cerevisiae* cofactor homologues. β-tubulin or α-tubulin is released from the chaperone (eight subunit toroid) and is bound independently by cofactors A or cofactor D for β-tubulin; or cofactor B or cofactor E for α-tubulin . The β-tubulin released from cofactor A and the α-tubulin released from cofactor B are not competent to exchange into exogenous heterodimer. The formation of native heterodimer requires that the α- and β-tubulin monomers bind cofactors D and E, respectively. Cofactors D bound to β-tubulin and cofactor E bound to α-tubulin form a quaternary complex. Finally, cofactor C in a GTP dependent step mediates the release of the α-β tubulin heterodimer.



From: Tian et al., Jour. Cell Biol. 138: 821-832 (1997)

Mamalian Cofactor	***S. cerevisiae* homologue**
A*	Rbl2p@
B#	Alf1p#
C*	not identified
D*	Cin1p^
E*	Pac2p&

* Tian et al., 1996
@ Archer et al., 1995
\# Tian et al., 1997
^ Hoyt et al., 1990; Stearns et al., 1990
& Hoyt et al., 1997

In yeast, TCP1 and TCP1 related genes are essential and conditional mutations in the genes encoding TCP1 subunits, *TCP1*, *BIN2*, *BIN3* and *ANC2* exhibit cytoskeleton defects, including abnormal nuclear distribution, aberrant microtubule structure, sensitivity to microtubule depolymerizing drugs, (Campo et al., 1994; Miklos et al., 1994; Ursic and Culbertson, 1991; Ursic et al., 1994; Vinh and Drubin, 1994) and allele specific interactions with tubulin genes and with actin (Ursic et al., 1994). Thus, proper folding of tubulin *in vivo* likely requires the action of the cytosolic chaperonin. More recently, Oka et al., (1998) reported that loss function of hsp70 /Ssa1p in *S. cerevisiae* led to microtubule defects and showed synthetic lethality with mutations in the gene encoding γ-tubulin, *TUB4* suggesting that hsp70-hsp40 may also have a role in γ-tubulin biogenesis (Oka et al., 1998). Alice Rushforth has shown that SSA and SSB co-purify with both α- and β-tubulin *in vivo* (A. Rushforth., personal communication), and Rbl2p was found to have homology to the "J domain" of DNA (Llosa et al., 1996). It will be interesting to see if the hsp70 family of heat shock proteins have a role in α β heterodimer formation.

Homologues of many of the mammalian cofactors involved in the tubulin folding assay have also been identified in yeast by diverse screens for mutations that affect microtubule processes. Rbl2p is structurally and functionally homologous to cofactor A (Archer et al., 1995). Cin1p, the cofactor D homologue, was first identified in two independent screens for mutation that increase sensitivity to microtubule depolymerizing drugs (Hoyt et al., 1990) and fidelity of mitotic chromosome transmission (Stearns et al., 1990). Pac2p, the cofactor E homologue was found as a

genetic interactor with the mitotic motor *CIN8* (Hoyt et al., 1997). Only Alf1p was identified by virtue of its homology to the mammalian cofactor B rather than in a genetic screen (Tian et al., 1997). Thus far there is no known *S. cerevisiae* homologue of the mammalian cofactor C. However, *S. cerevisiae* has two additional proteins *CIN2* and *CIN4* that were not identified in the *in vitro* assay. *CIN2* and *CIN4*, were identified in the same screens that identified *CIN1* ; and appear to function with *CIN1 in vivo* (Hoyt et al., 1990; Hoyt et al., 1997; Stearns et al., 1990). Perhaps in S. cerevisiae, *CIN2* and *CIN4* might take the place of the mammalian cofactor C. *In vivo*, deletion in any one of these genes- *RBL2; CIN's 1,2,4; ALF1;* and *PAC2*; -confers increased sensitivity to the microtubule depolymerizing drug, benomyl (Archer et al., 1995; Hoyt et al., 1990; Stearns et al., 1990; Tian et al., 1997). However, the benomyl supersensitivity of the strains varies depending on the null.

In the *in vitro* assay cofactor D and cofactor E are essential (Tian et al., 1996; Tian et al., 1997). In fission yeast, the cofactor D,B,E homologues are required for viability and mutations in these genes affect cell polarity (Hirata et al., 1998). However, in budding yeast, none of the gene products that is required for the *in vitro* folding assay are essential [Hoyt, 1997 #1833; and unpublished results]. Only under conditions of stress, such as cold, or in combination with other mutations affecting microtubules are these gene products essential. There may be redundant functions in yeast specified by genes as yet undetected, or the *in vivo* tubulin folding could follow a different pathway. Thus, the extent to which the *in vitro* tubulin folding assay is relevant to the *in vivo* situation is not clear. Some of the work in this thesis, detailed in Chapter Two and Chapter Three addresses this question.

Based on the observation that an excess of Rbl2p is lethal in cells containing a less stable heterodimer; experiments in chapter three of this thesis, describe how we have applied *RBL2* overexpression to identify non-tubulin genes that influence heterodimer stability. From that analysis we found that excess Rbl2p is lethal in cells bearing mutations in *CIN1*, the cofactor D homologue, and *PAC2*. the cofactor E homologue. Our genetic and biochemical analysis demonstrates roles for each in heterodimer formation.

Tubulin and Microtubule drugs

Tubulin polymerization is affected by various antimitotic drugs that either inhibit or enhance microtubule polymerization and inhibit cell proliferation. At high concentrations, drugs such as colchicine, the vinca alkaloids and nocodazole inhibit polymerization *in vivo* and *in vitro* (reviewed in (Jordan and Wilson, 1998)). Other drugs such as taxol act by stabilizing microtubules against depolymerization (Schiff et al., 1979; Schiff and Horwitz, 1980).

Only a subset of microtubule drugs affect *S. cerevisiae*. For example nocodazole, benomyl and methyl benzimidazole-2yl carbamate (MCB) are known to cause the depolymerization of yeast microtubules *in vivo* (Jacobs and Huitorel, 1979; Kilmartin, 1981; Quinlan et al., 1980) but colchicine does not (Baum et al., 1978). In addition, yeast microtubules are not sensitive to the microtubule stabilizing drug taxol (Barnes et al., 1992). Benzimidazole treatment in yeast results in the failure of microtubule mediated processes such as nuclear division, nuclear migration and nuclear fusion (Delgado and Conde, 1984; Jacobs et al., 1988).

Altered sensitivity to benomyl is a phenotype associated with impaired microtubule function in *S. cerevisiae* and is often used to suggest that a gene product is involved in a microtubule related process. Mutations in the α-tubulin genes, *TUB1* and *TUB3* , as well as the β-tubulin gene, *TUB2*, can confer increased benomyl supersensitivity (Reijo et al., 1994; Schatz et al., 1988; Stearns and Botstein, 1988). In addition the tubulin genes, mutations in a number of genes involved in microtubule morphogenesis are known to be supersensitive to benomyl. Some examples include mutations in genes encoding: microtubule motors (Cottingham and Hoyt, 1997), mitotic checkpoints (Hoyt et al., 1991; Li and Murray, 1991), spindle pole body function (Brachat et al., 1998), centromere function, (Baker and Masison, 1990; Foreman and Davis, 1993) microtubule associated proteins (Kim et al., 1990; Schwartz et al., 1997), and tubulin folding (Chen, et al., 1994 ; Hoyt et al., 1990; Hoyt et al., 1997; Stearns et al., 1990). Mutations in genes that confer increased resistance to microtubule depolymerizing drugs are less common. For the most part, mutations that confer increased resistance to benomyl map to the β-tubulin encoding gene, *TUB2* (Reijo et al., 1994; Thomas et al., 1985). However, mutations in genes other than *TUB2* have also been isolated that confer Ben^R. These include: nulls in *CDP1,* which requires centromere binding factor I (Cbf1p) for viability (Foreman and Davis, 1996), and alleles of SAC3, isolated as a suppressor of *act1-1* (Bauer and Kolling, 1996).

Increased resistance to microtubule depolymerizing drugs can also be acquired by overexpression of various gene products. For example, the *Candida albicans* multidrug resistance gene, BENr, is an efflux pump that confers increased resistance to benomyl in *S. cerevisiae.* (Ben-Yaacov et al., 1994). Our laboratory showed that

overexpression of the β-tubulin binding proteins, α-tubulin or Rbl2p confers increased resistance to benomyl in wild-type cells (Archer et al., 1995; Schatz et al., 1986). We designed a screen to identify genes in *S. cerevisiae* that confer resistance to benomyl when overexpressed. In particular, were interested in genes that might be involved in microtubule assembly. We identified two genes that like Rbl2p and -αtubulin confer a Ben^R phenotype. These results are described in chapter Four of this thesis.

Finally, in Chapter Five of this thesis I have described some of the ongoing questions in our laboratory and I have proposed a few experiments that may address some of these questions.

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CHAPTER 2:

An α-tubulin mutant destabilizes the heterodimer: phenotypic consequences and interactions with tubulin binding proteins.

INTRODUCTION

Microtubules participate in a variety of specific functions crucial for morphological differentiation, cell growth, and cell movement. The diversity of these functions requires that microtubules assemble into quite different structures even within the same cell. Many of those structures are dynamic, allowing them to disassemble rapidly and thus provide the components necessary to form another microtubule organelle. Possible mechanisms for regulation of these processes can be envisioned at several levels: primary sequences of tubulin genes (Hoyle and Raff, 1990; Joshi and Cleveland, 1989), message stability (Pachter et al., 1987), folding and dimerization of the protein subunits (Chen et al., 1994; Ursic and Culbertson, 1991), properties of the polymer (Mitchison and Kirschner, 1984; Saxton et al., 1984), and the interaction of the polymer with non-tubulin proteins (Caceres and Kosik, 1990; Dinsmore and Solomon, 1991).

Recently, several diverse experimental approaches have identified proteins that may participate in tubulin heterodimer formation. *In vitro* assays for proper folding of denatured α- and β-tubulins require several protein co-factors that interact transiently with the individual polypeptide chains (Melki et al., 1996; Tian et al., 1996; Tian et al., 1997). These complexes of tubulin polypeptides with co-factors may be intermediates that form in the interim between release of tubulin polypeptide from the TCP1-containing ring complex (TRiC) and its incorporation into pre-existing heterodimers by exchange. In at least some cases, those polypeptides form binary or higher order

complexes with the tubulins that are stable enough to be isolated but are still reactive (Tian et al., 1997).

Homologs of these cofactors (except cofactor C) are identified by diverse screens for mutations that affect microtubule processes in budding yeast. The processes affected include sensitivity to microtubule depolymerizing drugs (Stearns et al., 1990), fidelity of mitotic chromosome transmission (Hoyt et al., 1990), response to over-expression of β-tubulin (Archer et al., 1995), and interactions with mitotic motors (Geiser et al., 1997). Although most of these co-factors are essential for the *in vitro* assay, none of their *S. cerevisiae* homologs are essential for viability. Therefore, they may participate in the folding and heterodimerization of tubulin polypeptides, but there must be pathways that do not depend upon them.

The genetic data alluded to above suggest that there may be multiple steps in tubulin assembly subject to cellular control. Analysis of tubulin mutants can provide access to those steps. A panel of α-tubulin mutants cold-sensitive for growth arrest at their restrictive temperature with diverse microtubule phenotypes (Schatz et al., 1988). Some of the mutants arrest with no microtubules (class 1), some with a normal complement of microtubules (class 2), and the rest with an apparent excess of microtubules (class 3). This variability suggests that the conditional defects in these mutant α-tubulin proteins can affect different aspects of microtubule assembly and function. Certain of these mutations are suppressed by specific mutations in β-tubulin (Schatz et al., 1988), and others by extra copies of the mitotic check point *BUB* genes (Guénette et al., 1995) or by yeast homologues of the mammalian checkpoint gene

RCC1 (Kirkpatrick and Solomon, 1994). However, there is insufficient structure-function information for tubulin to permit an understanding of the phenotype in terms of the tubulin mutation itself.

Another distinction among the *tub1* mutants is uncovered when they are assayed in the presence of varying Rbl2p levels. Rbl2p binds β-tubulin to form a 1:1 complex (Archer et al., 1998; Melki et al., 1996). Rbl2p binding to β-tubulin excludes α-tubulin binding to β-tubulin. Four class 1 α-tubulin mutants are synthetically lethal with deletion of *rbl2*. Two of those are also synthetically lethal with over-expression of *RBL2*, but several other class 1, 2 or 3 mutants show no such interactions (see Table 2-1 and Archer et al., 1995).

The present study analyzes and exploits the properties of one of those two mutants. The *tub1-724* mutation fails to support growth at 18°C, and only partially supports growth at 25°C, but grow as well as wild type cells at 30°C (Schatz et al., 1988). The lethality and loss of microtubules at the non-permissive temperature is not a consequence of degradation of α-tubulin; the steady state α-tubulin levels in these cells is the same as that in an isogenic wild type control (our unpublished results). Upon induction of *GAL-RBL2*, *tub1-724* cells at permissive temperature rapidly lose assembled microtubule structures, and within 20 hours fewer than 0.1% of the cells are viable (Figure 2-1 and Archer et al., 1995). After 5 hours in galactose, *tub1-724* cells overexpressing Rbl2p lose most of their assembled microtubules (see Figure 2-2 (B) and Archer et al., 1995).

Table 2-1. Synthetic Lethality of *RBL2* Overexpression and Null Strains

Allele	*RBL2* Overexpression	Δ*RBL2*
tub1-724,-728	-	-
tub1-738,-759	+	-
tub1-704,-714, -744,-750	+	+
tub1-727,-730,-733, -741,-746,-758	+	nd

Ability of mutants to grow at permissive temperatures upon induction of *pGAL-RBL2* or in *RBL2* nulls. nd = not determined

Figure 2-1. Synthetic Interaction of *RBL2* Overexpression with *tub1-724*. Haploid cells contain two plasmid each: either *TUB1* or *tub1-724* on a *CEN* plasmid as their only source of α tubulin, and either *GAL-RBL2* or a *YCpGAL* (control) plasmid. These strains were grown overnight in selective raffinose media at 30°C. At T=0 hours, galactose was added to 2%. Cell viability equals the number of colonies arising on glucose plates divided by cell number counted in a light microscope.

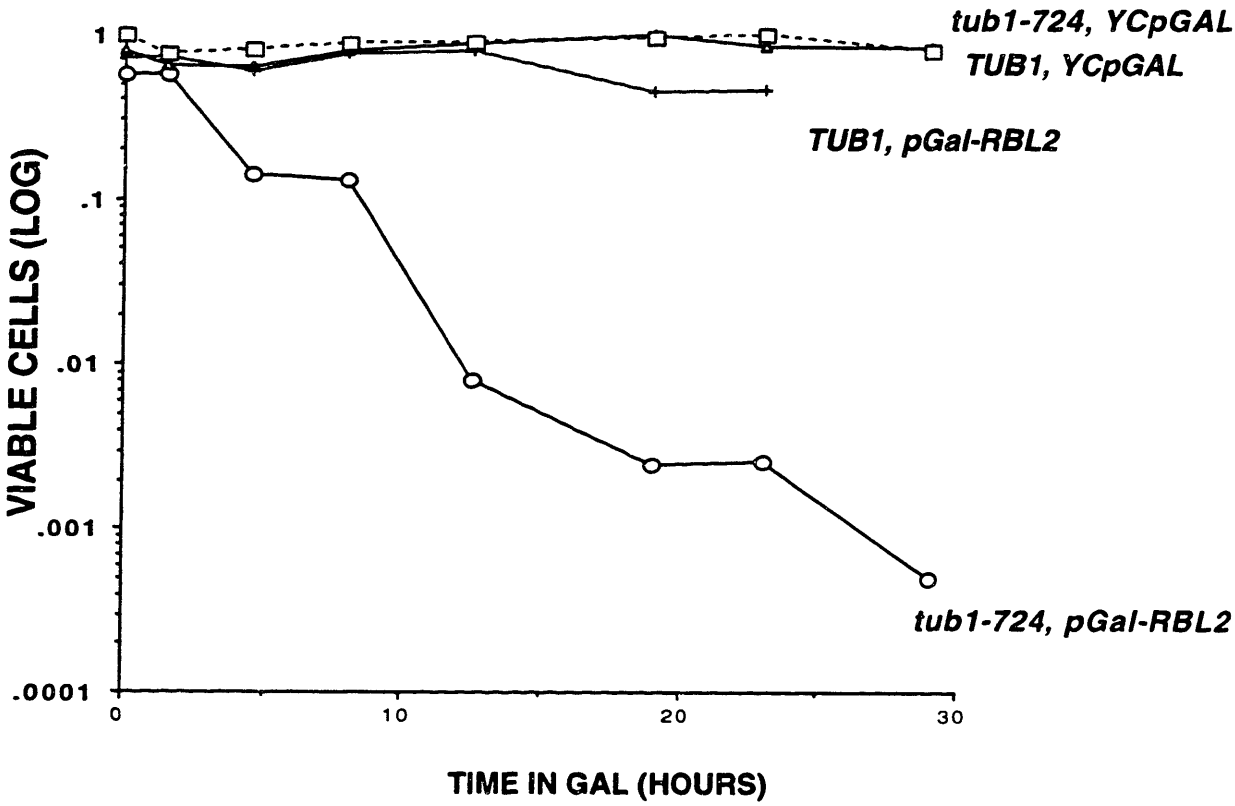
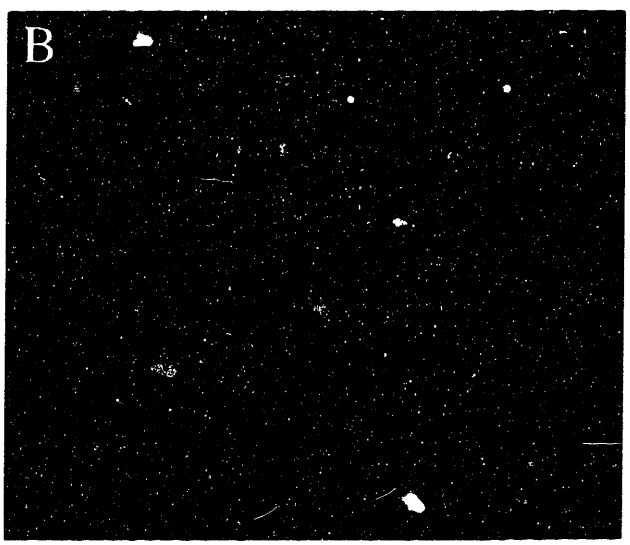

tub1-724, YCpGAL
TUB1, YCpGAL
TUB1, pGal-RBL2
tub1-724, pGal-RBL2
VIABLE CELLS (LOG)
1
.1
.01
.001
.0001
0
10
20
30
TIME IN GAL (HOURS)

Figure 2-2. Microtubule disassembly in *tub1-724* cells over-expressing *RBL2*. *tub1-724* cells growing at 30 °C and containing either control (A) or *pGAL-RBL2* (B) plasmids were fixed and processed for anti-tubulin immunofluorescence after 5 hours in galactose. In control cells, there are a variety of tubulin staining patterns. In cells overexpressing Rbl2p, large budded cells contain little or no localized staining.


A


B

The data presented here demonstrate that tubulin heterodimer containing this mutant α-tubulin protein is less stable than the wild type heterodimer. We use this property to analyze interactions between *tub1-724* and altered levels of two of the cofactor homologues mentioned above. The results provide a structure-function correlation for tubulin as well as insight into the cellular activities of the β-tubulin binding protein Rbl2p and the putative α-tubulin binding protein Pac2p.

MATERIALS AND METHODS

Strains, Plasmids, and Media

All yeast strains are derivatives of FSY185 (Weinstein and Solomon, 1990) with the exception of the *tub1* mutants (Schatz et al., 1988). We used standard methods for yeast manipulations (Sherman et al., 1986; Solomon et al., 1992). All the relevant strains are listed in table2-2.

To construct pLV27- an Aat II-Sph I fragement of pRB624 containing *tub1-724* was ligated into Aat II-Sph I digested YCp50. The plasmids pLV30, 32,36,37,38 were constructed by*in vivo* recombintation between the Pvu II digested backbone of pLV27 and the Aat II-Sph I fragment containing the mutant *tub1* allele. pGP2L was constructed by isolating the Sal I-Not I fragment from pPA45 and inserted into the Sal I-Not I site of the pGLR vector. pLV56 was constucted by P.C.R. of *PAC2* from genomic DNA the 5' primer was 5' GCAAAACGAATTTCAGAGATAGCATG and the 3' primer inserts 6 histidine tag and a Not I site with the sequence 5'CTACCGCGGAGCTCTTAGTGATGGTGATGGTGATGGCGGCCGCCCGATGGGCTG TTAACCTTCTGAATGCTCTTGTTATTTACTGG. The resulting PCR. product was cloned into pRS316-GAL1 (Liu et al) then a 111 bp NotI fragment containing the triple HA epitope from B2385 (Fink Lab) was cloned into the Not I site of pLV56. For pLV62, the Sal I -Sac II fragment from pLV56 was ligated into Sal I -Sac II digested pGLR.

Synthetic Lethality

RBL2 Overexpression - FSY182 (wild type)or haploid strains containing *tub1* alleles were transformed with pA5. Transformants on were grown SC-ura-leu, viablity

assessed by plating strains to glucose and to galactose plates. The cell number and size of the colonies were assesed by visual inspection in most cases.

Δrbl2 - JAY422 (*Δrbl2* haploid) was crossed to haploid *tub1* mutants. The diploids were sporulated, and dissected. Synthetic interactions were judged by two criteria: % dead spores and marker analysis (inability to recover *tub1* allele + *Δrbl2* products).

Viability measurements and Immunofluoresence

LTY6, LTY1, LTY374, LTY8, LTY376 and LTY11 were grown overnight in SC -ura -leu raffinose media. Log phase cells were then induced with 2% galactose and at various time points aliquots of cells were taken and counted using a haemocytometer. Known numbers of cells were then plated to SC -ura glucose plates. Cell viability was measured as the percent of counted cells able to form colonies on the SC -ura glucose plates. At various time points cells were fixed for immunofluorescence in 3.7% formaldehyde. Anti β-tubulin staining was done with #206 (Bond et al., 1986) at 1/2000 in phosphate buffered saline containing 0.1% bovine serum albumin.

Phenotypes of *TUB1* or *tub1-724* heterozygous diploids

Δtub1, *Δtub3* strains containing *tub1-724* or *TUB1* gene on *LEU2:CEN* plasmids were crossed to FSY183(wild type) containing YCpGAL, pPA45 or pA5. The diploid strains were grown to saturation overnight in SC -ura -leu -his glucose liquid media. The saturated cultures were serially diluted in 96 well dishes, and spotted onto SC -ura glucose and SC -ura galactose plates.

α-tubulin rescue of *tub1-724* haploids double overexpressors

FSY157 was transformed with various combinations of pGP2L, pJA3, pRS317, YCpGAL, and pPA45. Transformants were selected on SC-ura-lys plates. The haploid cells were grown to saturation overnight in SC-ura-lys glucose liquid media. The saturated cultures were serially diluted in 96 well dishes, and spotted onto SC -ura-lys glucose and SC-ura-lys galactose plates at 30°C.

Rescue of JAY47

JAY47 (Archer et al., 1995) was transformed with genomic *CEN: URA3* plasmids containing *TUB1*, *tub1* alleles or with *CEN:URA3:RBL2*. Cells were plated to SC -leu -ura glucose plates at 30°C and to SC -leu-ura galactose plates 30°C, 18°C and 15°C. The number of colonies on galactose relative to glucose was measured.

DNA Sequencing

DNA sequencing was performed using modified T7 DNA polymerase Sequenase with the dideoxy chain termination method (U.S. Biochemical Corporation).

Immune techniques

Immunoblots: Standard procedures were used (Solomon et al., 1992). After gel electrophoresis and transfer to nitrocellulose membranes, we blocked blots with TNT (0.025M Tris, 0.17M NaCl, 0.05% Tween-20, pH 7.5) for 30-120 minutes. Primary antibodies were incubated for >12 hours at 1/3500 (#206 or #345; Weinstein and Solomon, 1990) or at 1/100 (#250 (Archer et al., 1995) and then washed 5 times (5 min. each) in TNT. Bound antibody was detected by ^{125}I Protein A (NEN) or (for 12CA5) ^{125}I sheep anti-mouse IgG (NEN). Commerical preparations of anti-HA were used (Boehringer).

Immunoprecipitations: The procedure described previously (Archer et al. 1995) was used with slight modifications. The monoclonal antibodies A1BG7 (anti -α) and B1BE2 (anti-β), raised against the carboxy terminal 12 amino acids of Tub1p and Tub2p, respectively, were affixed to Affigel-10 beads (BioRad). Yeast strains FSY157 and FSY182 were grown up at 30°C. Total protein was harvested by glass bead lysis in PME (0.1M Pipes, 2mM EGTA, 1 mM magnesium chloride, pH 6.9) plus protease inhibitors and was added to antibody beads for one hour incubation with rotation at 4°C. We washed the beads eight times with PME + protease inhibitors. Bound proteins were eluted by boiling in SDS sample buffer and resolved by SDS-PAGE analysis.

Purification of His_6 tagged proteins

The Ni-NTA nickel slurry and column materials were from Qiagen. We used protocols from the Qiagen handbook and modifications of this protocol that have been previously described (Magendantz et al., 1995).

In vivo His_6-Rbl2p-β-tubulin association experiments

Yeast strains LTY291 and LTY292 are FSY157 and FSY182 transformants with a CEN p*GAL-RBL2-HIS*$_6$ (pGHR). We grew LTY 291 and LTY292 overnight at 30°C in selective media containing raffinose to about $2X10^9$ cells per experiment. 2% galactose was added to induce His_6-RBL2 expression. After 0,1, and 2 hours protein was harvested by glass bead lysis in 1ml PME buffer plus protease inhibitors. We applied 0.85 mls of protein extract to 130ul Ni-NTA beads. We washed and eluted the bound proteins as previously described (Magendantz et al., 1995). Eluted proteins

were subjected to SDS-PAGE analysis and probed for α-tubulin, β-tubulin and Rbl2p and quantitated by densitometry.

In vivo HIS_6-(HA)-Pac2p-α-tubulin association experiments

We grew yeast strains LTY539, LTY541, LTY439 and LTY 440 overnight in selective raffinose media at 30°C. 2% galactose was added to induce Pac2p-(HA)-His_6 and α-tubulin or β-tubulin expression. $6.0X10^9$ cells were harvested by glass bead lysis per experiment in 1.1ml PME buffer plus protease inhibitors. We applied 1ml of protein extract to 25ul Ni-NTA beads. We washed and eluted the bound proteins as previously described (Magendantz et al., 1995). Eluted proteins were subjected to SDS-PAGE analysis and probed for α-tubulin, β-tubulin and HA(12CA5). For Pac2p, the bead eluants represent 120x the load of whole cell extract. For α- and β-tubulin, the bead eluants represent 500x the load of whole cell extract.

TABLE 2-2 Strains and Plasmids

Strains	Genotype	Reference
FSY185	*a/α; ura3-52/ura3-52, leu2-3,112/leu2-3,112, his3Δ200/his3Δ200, lys2-801/lys2-801, ade2/ADE2*	(Weinstein and Solomon, 1990)
FSY183	*a; ura3-52; leu2-3,112; his3Δ200; lys2-801*	(Weinstein and Solomon, 1990)
FSY157	*α; ura3-52; leu2-3,112; his3Δ200; lys2-801; Δtub1::HIS3, Δtub3::TRP1* [pRB624]	(Schatz et al., 1988)
FSY182	*α; ura3-52; leu2-3,112; his3Δ200; lys2-801; Δtub1::HIS3, Δtub3::TRP1* [pRB539]	(Schatz et al., 1988)
LTY8	FSY157 plus YCpGAL	(Archer et al., 1995)
LTY11	FSY182 plus YCpGAL	This study
LTY291	FSY157 plus pGHR	This study
LTY292	FSY182 plus pGHR	This study
LTY374	FSY157 plus pPA45	This study
LTY376	FSY182 plus pPA45	This study
JAY47	*a/α, ura3-52/ura3-52, leu2-3,112/leu2-3,112, his3Δ200/his3Δ200, lys2-801/lys2-801, ade2/ADE2, TUB2/TUB2-LEU2-GAL-TUB2*	(Archer et al., 1995)
LTY319	JAY47 plus YCp50	This study
LTY321	JAY47 plus A21A	This study
LTY323	JAY47 plus pLV32	This study
LTY325	JAY47 plus pA1A5106	This study
LTY338	JAY47 plus pLV30	This study
LTY340	JAY47 plus pLV38	This study
LTY343	JAY47 plus pLV36	This study
LTY345	JAY47 plus pLV37	This study
LTY392	*a/α; ura3-52/ura3-52; leu2-3,112/leu2-3,112; his3Δ200/his3Δ200; lys2-801/lys2-801; TUB1/Δtub1::HIS3, TUB3/Δtub3::TRP1;* [pRB539, pA5]	This study
LTY393	like LTY392 but with pPA45 rather than pA5	This study
LTY395	like LTY392 but with YCpGAL rather than pA5	This study
LTY396	*a/α; ura3-52/ura3-52; leu2-3,112/leu2-3,112; his3Δ200/his3Δ200; lys2-801/lys2-801;TUB1/Δtub1::HIS3,TUB3/Δtub3::TRP1,* [pRB624, pPA45]	This study
LTY397	like LTY396 but with pA5 rather than pA45	This study
LTY440	JAY47 plus YCpGAL	This study
LTY439	JAY47 plus pLV56	This study
LTY399	like LTY396 but with YCpGAL rather than pA45	This study
LTY539	FSY183 plus pJA3 and pLV62	This study
LTY541	FSY183 plus pJA3 and pRS317	This study
LTY540	FSY183 plus YCpGAL and pLV62	This study

TABLE 2-2

Plasmid	Genotype	Reference
YCp50	*CEN-URA3*	(Kirkpatrick and F., 1994)
pA1A5106	*TUB1-CEN-URA3*	(Kirkpatrick and F., 1994)
pA21A	*RBL2-CEN-URA3*	(Archer et al., 1995)
pA5	*GAL 1-10-RBL2-URA3*	(Archer et al., 1995)
pGHR	*GAL 1-10-HIS$_6$-RBL2-URA3*	(Archer et al., 1998)
pRB624	*tub1-724-CEN-LEU2*	(Schatz et al., 1988)
pRB539	*TUB1-CEN-LEU2*	(Schatz et al., 1988)
pPA45	*GAL 1-10-PAC2-CEN-URA3*	(Alvarez et al., 1998)
YCpGAL	*GAL1-10-CEN-URA3*	(Archer et al. 1995)
pLV30	*tub1-704* in YCp50	This study
pLV32	*tub1-724* in YCp50	This study
pLV36	*tub1-737* in YCp50	This study
pLV37	*tub1-747* in YCp50	This study
pLV38	*tub1-714* in YCp50	This study
pLV56	*GAL 1-10-PAC2-HA-HIS$_6$-CEN-URA3*	This study
pLV62	*GAL1-10-PAC2-HA-HIS$_6$-CEN-LYS2*	This study
pRS317	*CEN-LYS2*	(Sikorski and Hieter, 1989)

Results

A model for the interactions between tub1-724 and altered levles of Rbl2p

We reasoned that the conditional loss of assembled microtubules in class 1 α-tubulin mutants, incuding *tub1-724*, could arise from cold sensitivity of any of several steps in microtubule morphogenesis. However, the synthetic lethality of Tub1-724p with both Rbl2p deletion and over-expression suggest that the mutant defect arises from a weaker heterodimer. This model is depicted in Figure 2-3. If the heterodimer formed by the Tub1-724p dissociates more readily than does wild type heterodimer, the increase in free, undimerized β-tubulin could be toxic in the absence of the β-tubulin binding capacity provided by Rbl2p. Conversely, an excess of Rbl2p, which has only minor phenotypes in a wild type cell, could compete effectively with the mutant α-tubulin protein for β-tubulin and so diminish the level of tubulin subunits to cause loss of microtubules and cell death. The experiments in this chapter present tests of this model.

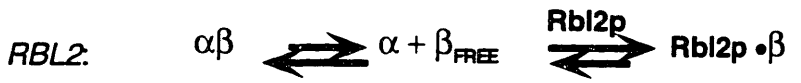
Co-immunoprecipitation of α- and β-tubulin from wild type and *tub1-724* mutant cells

We assessed the stability of the wild type and mutant α-β heterodimers by co-immunoprecipitation. Extracts from *tub1-724* mutant cells and wild type cells grown at 30°C were incubated with antibodies to either α-tubulin or β-tubulin coupled to Affigel beads. The beads were washed extensively to remove adventitiously adhering proteins, and specifically bound proteins were released by SDS. The tubulin chains in

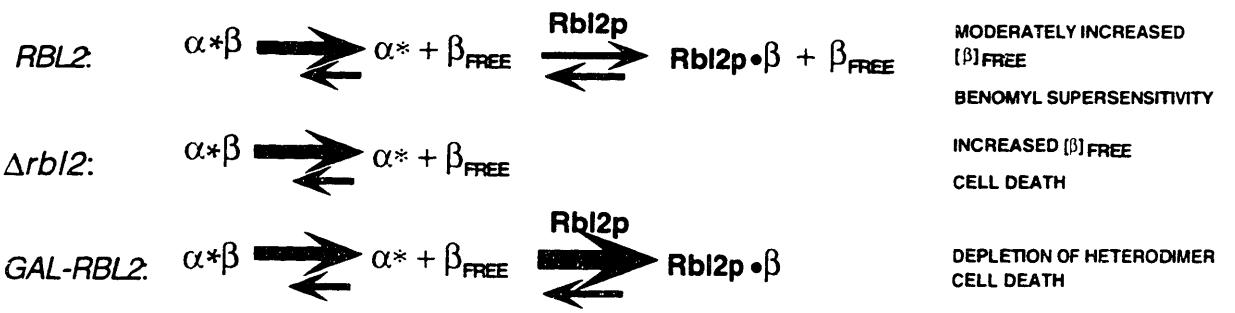
Figure 2-3. Synthetic lethal interactions between *tub1-724* and altered levels of Rbl2p: a model. Cells expressing *tub1-724* as their sole source of α-tubulin die either when Rbl2p is absent or over-expressed. Those relationships are explicable if the heterodimer formed by the Tub1-724p (α*β) dissociates more readily than that formed by the wild type Tub1p (αβ). In the presence of a normal complement of *RBL2*, the mutant cells would have a high concentration of free β-tubulin (β_{FREE}), which may be responsible for the conditional phenotypes of the mutant (*e.g.* benomyl supersensitivity). In the absence of Rbl2p, the activity of β_{FREE} would increase to toxic levels. In contrast, an excess of Rbl2p could bind to β-tubulin and so enhance dissociation of the mutant heterodimer, promoting dissociation to levels below those necessary for viability.

A MODEL FOR INTERACTIONS BETWEEN *tub1-724* AND ALTERED LEVELS OF RBL2P

In wild type cells:



In *tub1-724* cells:

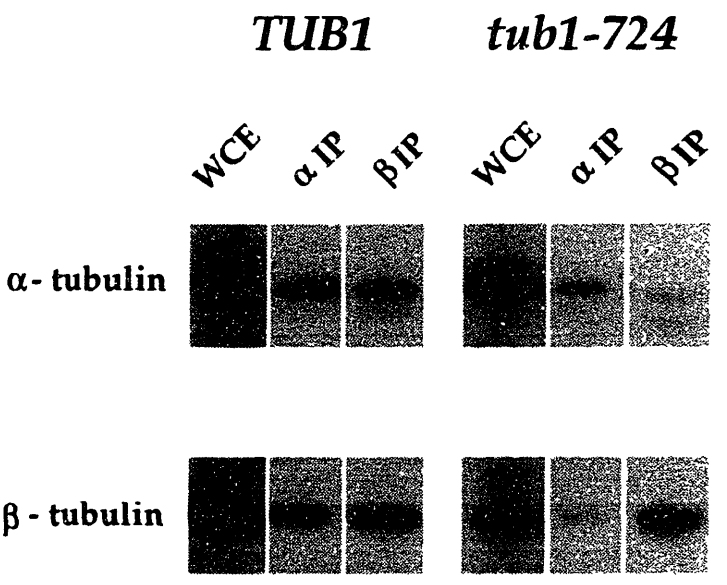


the immunoprecipitates were analyzed by immunoblotting with antibodies to α and β tubulin. An example of this is shown in figure 2-4. From extracts of wild type cells, antibodies against each of the tubulin polypeptides co-precipitates the other chain with high efficiency; the ratio of the tubulins in the co-precipitates is comparable to the original extracts. This result suggests that under the conditions of tubulin immunoprecipitation, normal heterodimer largely remains intact. From extracts of *tub1-724* cells, however, the anti-tubulin antibodies complex efficiently with the specific tubulin chain against which they are directed, but precipitate the other tubulin chain only poorly.

Because we recover only a small fraction of Tub1-724p heterodimer by immunoprecipitation, we can not directly compare the stability of the mutant and wild type heterodimers. We previously established that at least 98% of the β-tubulin in wild type cells is in the form of α-β heterodimer (Archer et al., 1998). Since *tub1-724* cells grow normally at 30°C, presumably most of the tubulin in those cells is in heterodimer *in vivo*. Thus, the dissociation of the heterodimer likely occurs in the course of the immunoprecipitation itself, which exposes the heterodimer to large dilutions at low temperature (4°C). Under similar conditions, we showed that the the wild type heterodimer has a half life of about 10 hours (Archer et al., 1998).

The only difference between the primary sequences of *TUB1* and *tub1-724* genes predicts substitution of threonine for arginine at codon 106 (AGA becomes ACA) (data not show). Arginine-106 is a highly conserved residue among α-tubulins. The single mutation in Tub1-724p predicted from DNA sequence is loss of a positive

Figure 2-4. α- and β- tubulin co-immunoprecipitate with low efficiency from *tub1-724* cells. Immunoblots with anti-α-tubulin (top row) and anti-β-tubulin (bottom row) of whole cell extracts (WCE) and the precipitates with the two antibodies (αIP, βIP) from wild type *TUB1* or mutant *tub1-724* cells.


TUB1
tub1-724
WCE
α IP
β IP
WCE
α IP
β IP
α - tubulin
β - tubulin

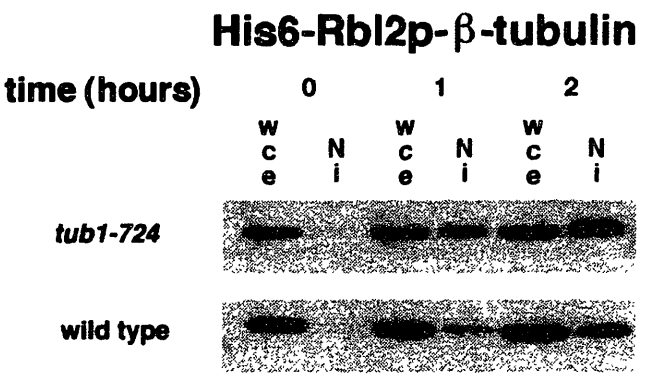
charge at position 106. Based upon the structure of tubulins reported by Nogales and her colleagues (Nogales et al., 1998), this residue occurs in the region between the B3 and H3 loops that contact the phosphates of the non-exchangeable GTP. That site is at the postulated interface between α- and β-tubulin in the heterodimer. The wild type arginine at this position probably contributes to phosphate binding, and so may indirectly participate in α-β interactions.

Formation of Rbl2p-β-tubulin complex in wild type and *tub1-724* mutant cells

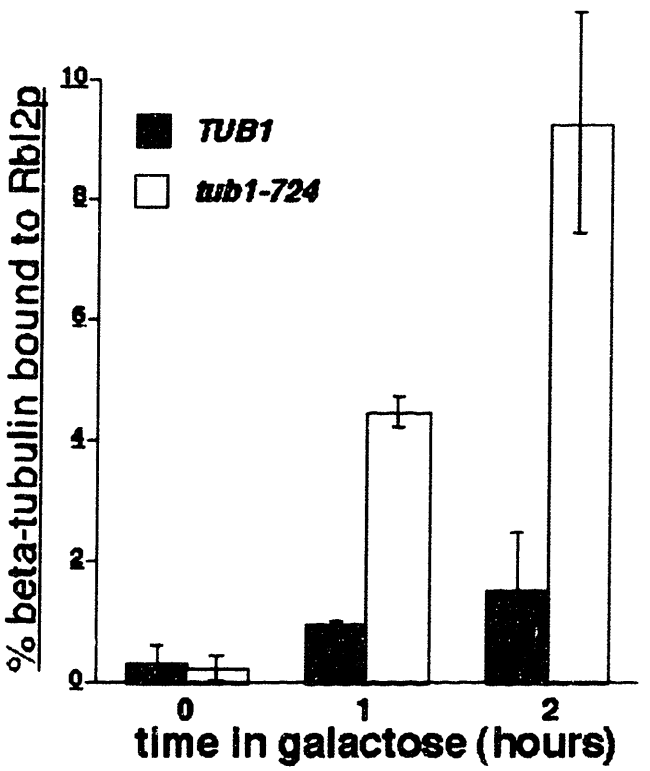
Rbl2p is complexed with β-tubulin *in vivo*, and the level of that complex increases as the cellular level of Rbl2p increases (Archer et al., 1998; Archer et al., 1995). The model presented in figure 2-3 predicts that over-expressed Rbl2p will form a complex with β-tubulin more readily in *tub1-724* cells than in wild type cells. To test that possibility, we introduced a plasmid encoding His_6-Rbl2p under the control of the galactose promoter into wild-type *TUB1* cells or *tub1-724* mutant cells. The transformants were grown at the permissive temperature for the mutant in non-inducing medium, and then were shifted to inducing medium containing galactose for 1 or 2 hours. We used nickel-agarose beads to purify the His_6-Rbl2p -β-tubulin complex. The bound proteins were eluted and analyzed by immunoblotting with antibodies against α-tubulin, β-tubulin or Rbl2p. As expected, the levels of His_6-Rbl2p-β-tubulin complex increase upon induction in both control and mutant cells, but as shown in figure 2-5 (A,B); 3-5 fold more complex forms in *tub1-724* cells relative to wild type. In these experiments, we detect only a trace of α-tubulin bound to the nickel

Figure 2-5. The Rbl2p-β-tubulin complex *in vivo* is enhanced in *tub1-724* cells. (A) Cells growing in raffinose ("0 hours") were induced with galactose to express His_6-Rbl2p for 1 or 2 hours. His_6-Rbl2p was isolated by affinity chromatography of the whole cell extracts, the levels of β-tubulin in the original extract and bound to Rbl2p assayed by immunoblotting. (B) The results are the averages of two independent experiments as in (A) for each strain and time point with the ranges indicated by error bars. In both of these experiments, the wild type strain produced slightly more His_6-Rbl2p upon induction (our unpublished results). black bars, *TUB1* cells; grey-bars, *tub1-724* cells.

A.


His6-Rbl2p-β-tubulin
time (hours)
0
1
2
wce
Ni
wce
Ni
wce
Ni
tub1-724
wild type

B.


% beta-tubulin bound to Rbl2p
10
8
6
4
2
0
TUB1
tub1-724
0
1
2
time in galactose (hours)

columns, and its level does not increase with time in galactose (Archer et al., 1998 and our unpublished results). This result suggests either that Rbl2p competes more efficiently with Tub1-724p than with wild type α-tubulin for binding to β-tubulin *in vivo,* or that there is a greater pool of free β-tubulin available for binding to Rbl2p in the *tub1-724* mutant (see Discussion). Either possibility is consistent with Tub1-724p forming a less stable heterodimer with β-tubulin than wild type α-tubulin.

Rescue of β-tubulin lethality by wild type and mutant α-tubulins

An excess of either α-tubulin or Rbl2p rescues cells from β-tubulin lethality (Archer et al., 1995; Alvarez et al., 1998); the rescue likely depends upon the ability of these two proteins to bind β-tubulin. Even a modest excess of α-tubulin, expressed under the control of its own promoter from a low-copy plasmid, increases the survival of cells over-producing β-tubulin by 2-3 orders of magnitude. If Tub1-724p binds β-tubulin with low affinity, we would expect it to rescue β-tubulin lethality poorly. To test this hypothesis, wild type or mutant alleles of α-tubulin were introduced into JAY47, a diploid strain with a normal complement of tubulin genes plus a third, integrated copy of the β-tubulin gene *TUB2* under the control of the galactose promoter. We measured the percent survivors on galactose relative to glucose at both the permissive (30°C) and the non-permissive temperature (18°C). As shown in Table 2-3 rescue of β-tubulin lethality by *tub1-724* is substantially less efficient (0.84%) than by wild type *TUB1* (15.4%). The efficiency of rescue is further diminished at the non-permissive temperature for the mutant: at 18°C, *tub1-724* rescues β-tubulin lethality (0.06%) to essentially the same extent as the negative control (0.03%). In contrast, four other mutant α-tubulins rescue at levels comparable to that of the wild type, and their

Table 2-3. Rescue of excess β-tubulin lethality by α-tubulin alleles

Plasmid	30°C	18°C
YCpGAL	.04	.03
RBL2	6.8	7.2
TUB1	12.0	15.4
tub1-724	.84	.06
tub1-704	10.9	18.9
-714	14.0	20.0
-737	4.1	8.5
-747	11.8	21.0

JAY47 cells, which contain an integrated *GAL-TUB2* gene, carrying the indicated α-tubulin alleles on plasmids are plated to media containing either galactose or glucose and incubated at either 30°C or 18°C. Rescue is reported as the percent of cells the form colonies on galactose plates compared to glucose plates.

Cold sensitivity of *TUB1/tub1-724* heterozygotes and their suppression by excess Rbl2p.

The *tub1-724* phenotype is not completely suppressed in a heterozygote with *TUB1*. A diploid strain containing only single chromosomal copies of *TUB1* and *TUB3* plus a low copy plasmid expressing *tub1-724* is cold sensitive for growth at 18°C. In contrast, heterozygotes containing *TUB1* and other *tub1* mutants show the same temperature sensitivity as do wild type cells (our unpublished results). The conditional growth of *TUB1/tub1-724* heterozygotes must reflect a property of the mutant heterodimer, rather than a deficiency in tubulin levels, since diploid cells with only 50% of their wild type complement of tubulin are wild type for growth at low temperatures (Katz et al., 1990).

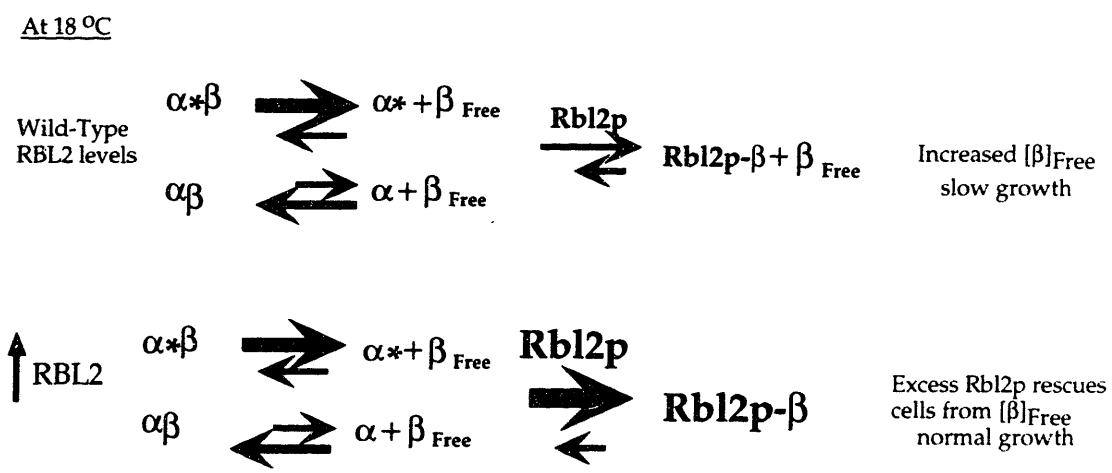
We hypothesized that the cold sensitivity of these *TUB1/tub1-724* heterozygous cells is due to the free β-tubulin produced by dissociation of the mutant heterodimer (see model in Figure 2-6 (A). Consistent with that explanation, the cold sensitivity of the heterozygotes is substantially suppressed by overexpression of *RBL2* from the galactose promoter as shown in figure 2-6 (B). The presence of excess Rbl2p can bind the free β-tubulin and so protect the cell from its deleterious consequences. This result is in striking contrast to the lethal effect of *GAL-RBL2* in cells expressing *tub1-724* as their sole source of α-tubulin (see Figure 2-1).

Figure 2-6 Rbl2p overexpression in *TUB1/tub1-724* heterozygous cells.

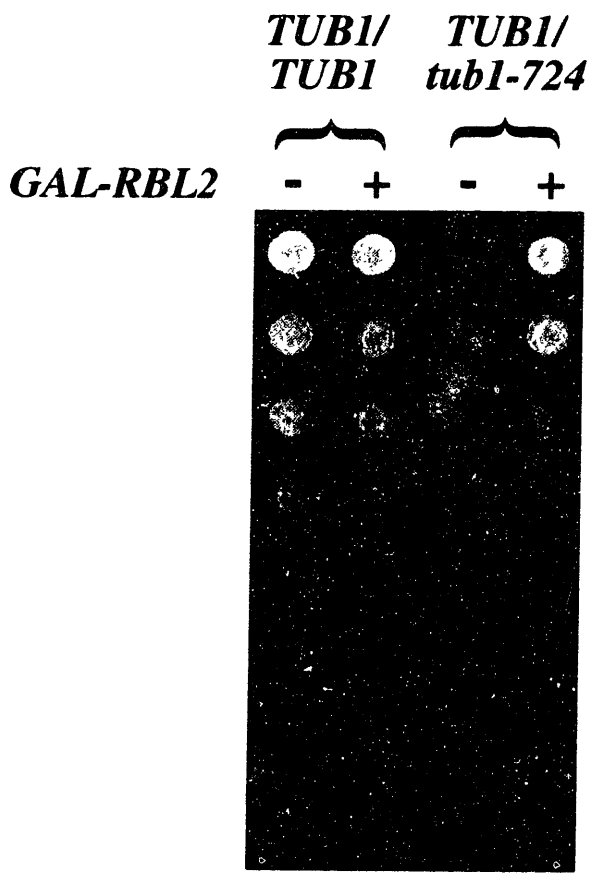
(A) Model: *TUB1/tub1-724* heterozygous cells contain both , wild-type (α/β) and mutant (α*β) heterodimers . At 18°C, a restictive temperature for the mutant α-tubulin allele, the mutant (α*β) heterodimers will tend to dissociate resulting in excess free β tubulin (β_{Free}) and sickness in these cells. Overexpression of Rbl2p can bind to the (β_{Free}) and rescue these cells from β-tubulin toxicity. (B) Over-expression of *RBL2* suppresses *TUB1/tub1-724* heterozygous cells. Serial (four-fold) dilutions of saturated cultures were plated to galactose containing media, and allowed to grow at 18°C. The cells were either wild type diploids or *TUB1/tub1-724* cells; carrying either YCpGAL or *CEN-GAL-RBL2*.

A.

Model for *tub1-724/TUB1* interactions with increased levels of Rbl2p



B.



GAL 18°C

Over-expression of *PAC2* in *tub1-724* cells

Pac2p is a candidate for an α-tubulin binding protein in yeast. It is the homolog of cofactor E in the *in vitro* system described above. Cofactor E plays an essential role in this assay: it is believed to bind to α-tubulin after its release from the TriC complex (Tian et al. 1997). This binary complex is then thought to form a quaternary complex with cofactor D and β-tubulin. The cofactor E-α-tubulin complex is rather unstable, and is detectable on native gels only after it is stabilized by glutaraldehyde fixation.

The *S. pombe* homolog of cofactor E is essential *in vivo* (Hirata et al., 1998). In budding yeast *PAC2* is not essential but mutations in *pac2* affect microtubule functions. *pac2* mutations are super-sensitive to benomyl (Hoyt et al. 1997). It is required in cells deleted for *cin8*, which encodes a kinesin-related protein that participates in anaphase (Geiser et al., 1997), or deleted for *pac10* (Alvarez et al., 1998.), which affects ratios of α-tubulin to β-tubulin (Alvarez et al., 1998.; Geissler et al., 1998).

If Pac2p is an α-tubulin binding protein, we would predict that at elevated levels it would be deleterious to cells containing the unstable *tub1-724* heterodimer. As shown in figure 2-7, induction of *GAL-PAC2* in haploid *tub1-724* cells grown at permissive temperature (30°C) causes rapid loss of viability, down 10-fold in approximately 3 hours. In contrast, *GAL-PAC2* has only a modest effect on the viability of wild type cells (figure 2-7). In that time, the induction of *GAL-PAC2* causes microtubule disassembly in the mutant (figure 2-8 [B]), but not in wild type cells (figure 2-8 [C]); representative micrographs are shown in figure 2-8 . From such fields, we

Figure 2-7. Over-expressing *PAC2* is lethal in *tub1-724* cells.

tub1-724 (triangles) and wild type (squares) containing either control plasmid (open symbols) or *GAL-PAC2* (filled symbols) cells growing at 30°C were shifted to galactose containing media at 0 time, and aliquots taken at intervals and scored for total cells and viable cells.

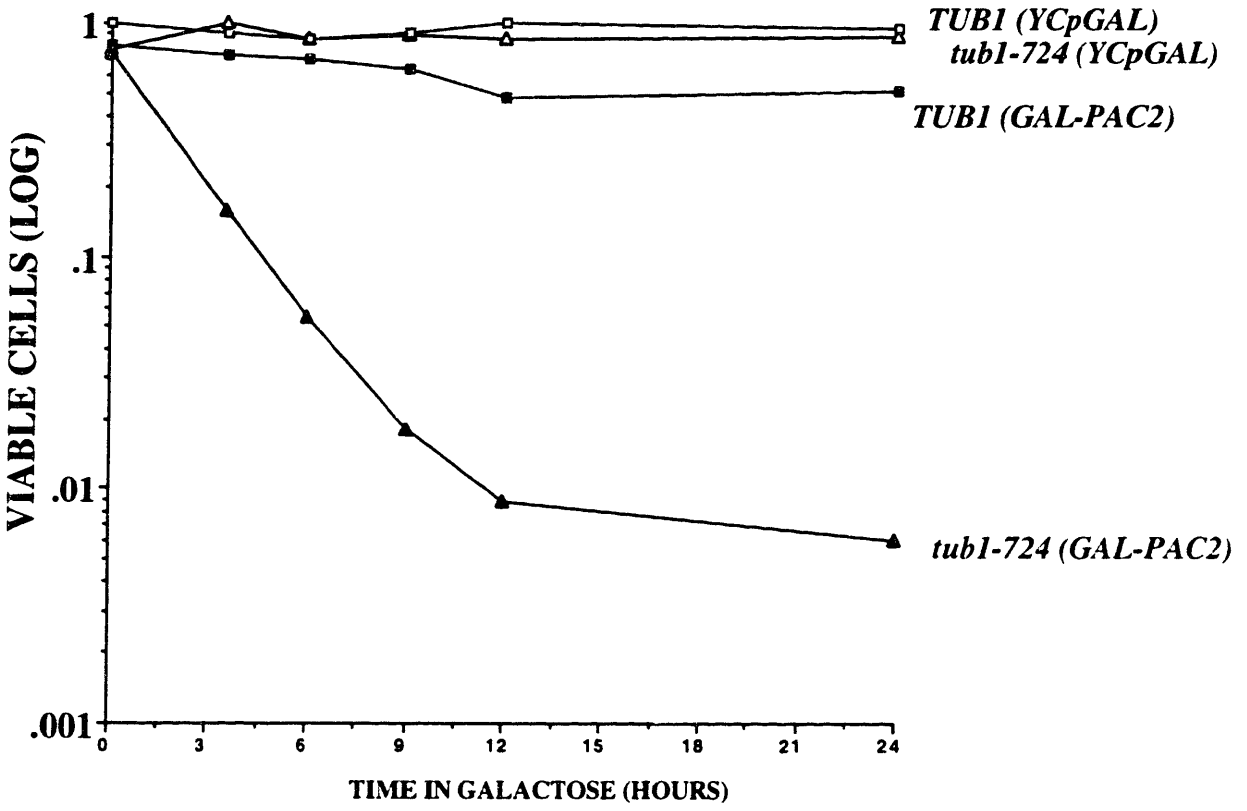
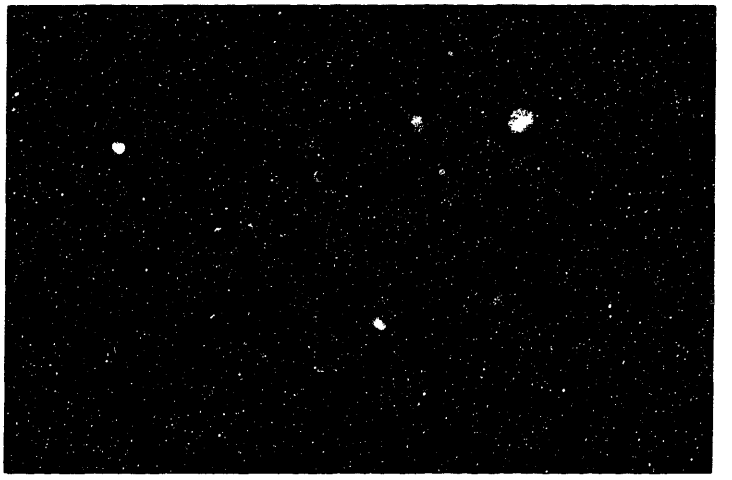

1
.1
.01
.001
VIABLE CELLS (LOG)
0
3
6
9
12
15
18
21
24
TIME IN GALACTOSE (HOURS)
TUB1 (YCpGAL)
tub1-724 (YCpGAL)
TUB1 (GAL-PAC2)
tub1-724 (GAL-PAC2)

Figure 2-8. Microtubule disassembly in *tub1-724* cells over-expressing *PAC2*. Anti-tubulin immunofluorescence of *tub1-724* cells containing the control plasmid YCpGAL (A), or a *CEN-GAL-PAC2* (B); and wild type cells containing a *CEN-GAL-PAC2* plasmid (C). Cultures were grown in galactose for 3.5 hours before fixation for immunofluorescence.

A.

B.



C.

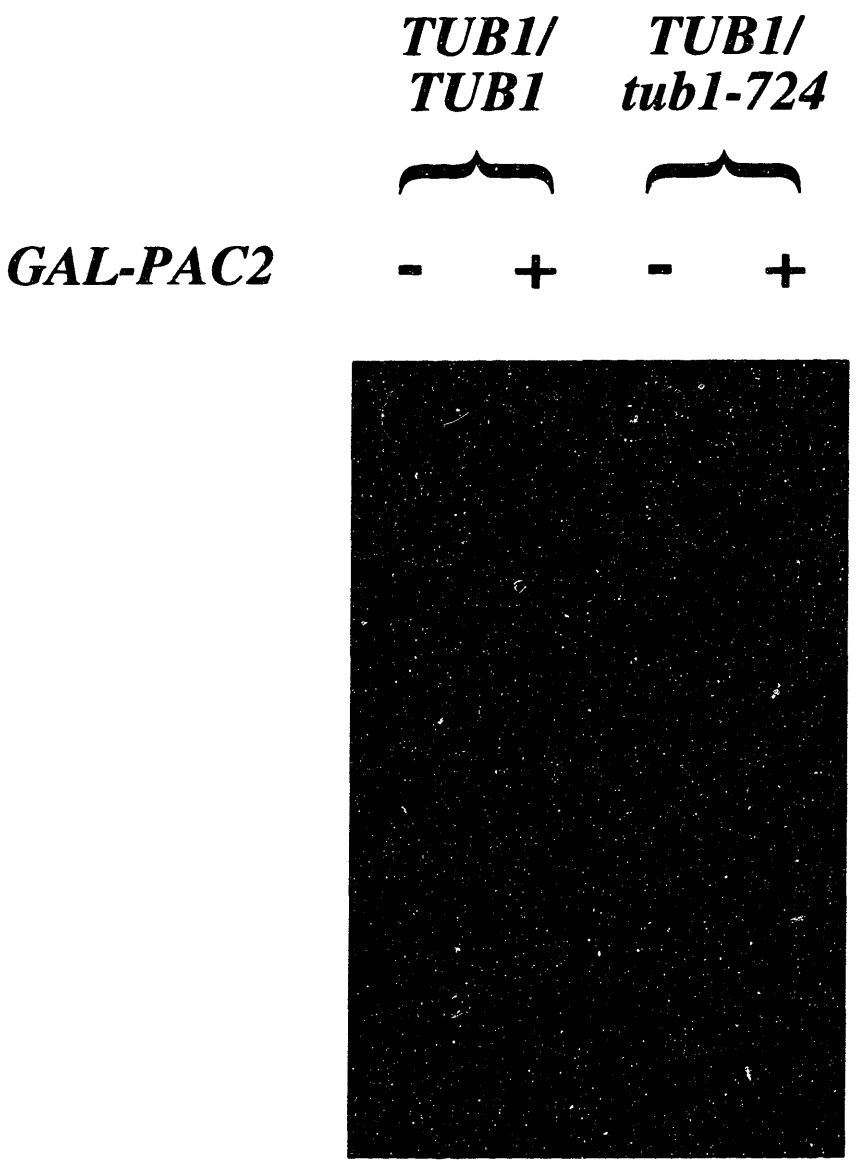
find that overexpression of *PAC2* increases the proportion of *tub1-724* cells that have no microtubules by 10-fold (53.2% versus 5.4%) but has no effect on wild type cells (10.1% for both strains).

Effect of elevated Pac2p levels in *tub1-724* heterozygotes

Both phenotypes of elevated Pac2p levels on *tub1-724* haploid cells are the same as produced by elevated levels of Rbl2p (compare 2-1; 2-7 and 2-2 ; 2-8 and Archer et al., 1995). Therefore, these results could represent Pac2p binding to either β-tubulin or α-tubulin. However, the effect of *GAL-PAC2* expression in *TUB1/tub1-724* heterozygotes does distinguish between these two possibilities. As shown in figure 2-9 , over-expression of *PAC2* in the heterozygotes causes a significant loss of cell viability at the permissive temperature. This result contrasts with that shown in figure 2-6 above, showing that over-expression of *RBL2* actually suppresses the phenotype of the *TUB1/tub1-724* heterozygotes.

These results are explicable if the Tub1-724p-β-tubulin heterodimer is relatively unstable (see figure 2-3). The increased levels of an α-tubulin binding protein might be expected to increase free β-tubulin to toxic levels in both *tub1-724* haploids and *TUB1/tub1-724* heterozygotes. This outcome is in contrast to the effect noted for excess Rbl2p in the heterozygotes, where the increased capacity to bind β-tubulin would be expected to reduce its levels and so suppress the *TUB1/tub1-724* phenotypes. Taken together, these results suggest that Pac2p can bind to α-tubulin *in vivo*, and so are consistent with the conclusion of the *in vitro* experiments (Tian et al., 1997).

Figure 2-9. Over-expression of *PAC2* is lethal in *TUB1/tub1-724* heterozygous cells. Serial (four-fold) dilutions of saturated cultures were plated to galactose containing media, and allowed to grow at 30°C. Strains were wild type diploids or *TUB1/tub1-724* cells containing either YCpGAL or *CEN-GAL-PAC2.*


TUB1/
TUB1
TUB1/
tub1-724
GAL-PAC2
-
+
-
+
GAL 30°C

Isolation of a Pac2p-α-tubulin complex

To demonstrate directly a Pac2p-α-tubulin complex, we used a form of Pac2p that contains the HA tag followed by 6 histidines at its carboxy terminus. This modified allele is functionally indistinguishable from wild type Pac2p in both *Δpac2* and *tub1-724* cells (our unpublished results). We can isolate a complex containing α-tubulin and Pac2p-(HA)-His_6 from extracts of cells over-expressing both proteins (figure 2-10, lane c); no β-tubulin is detected in this complex. We can not detect this complex unless both Pac2p and α-tubulin are overexpressed. In contrast, over-expression of both Pac2p and β-tubulin does not produce a complex between those two proteins (figure 2-10, lane g). These results support the conclusion that Pac2p can bind α-tubulin *in vivo*. Over-expression of Pac2p-(HA)-His_6 alone in *tub1-724* cells does not produce measurable levels of the Pac2p-α-tubulin complex (our unpublished results).

Co-overexpression of α-tubulin suppresses the synthetic effects of Pac2p overexpression in tub1-724.

We tested the ability of α-tubulin to rescue the lethal effects of Pac2p overxpression in *tub1-724* cells. As expected, we found that co-overexpression of *GAL-TUB1* is able rescue *tub1-724* haploid (see Figure 2-11) and *TUB1/tub1-724* heterozygous cells (data not shown) from the lethal effects of overproduction of Pac2p.

Figure 2-10. Binding of α-tubulin to Pac2p-(HA)-His_6 in double over-expressing cells. Whole cell extracts (lanes a, b, e, f) and eluants from nickel agarose beads (lanes c, d, g, h) were analyzed by SDS-PAGE and immunoblotting for HA-tagged Pac2p, α-tubulin and β-tubulin. The fractions were from cells overexpressing Pac2p-(HA)-His_6 and α-tubulin (a, c), Pac2p-(HA)-His_6 and β-tubulin (e, g); α-tubulin alone (b, d); or β-tubulin alone (d, h). For Pac2p, the bead eluants represent 120x the load of whole cell extract. For α- and β-tubulin, the bead eluants represent 500x the load of whole cell extract.

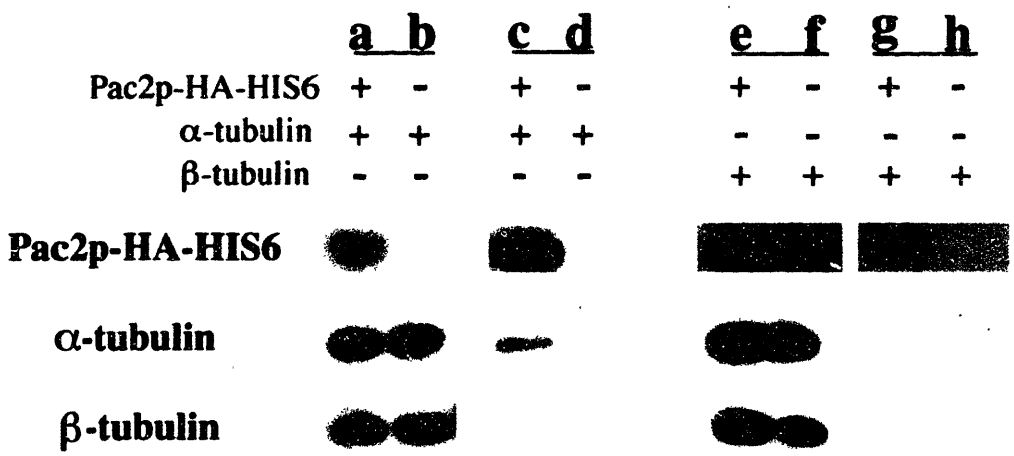
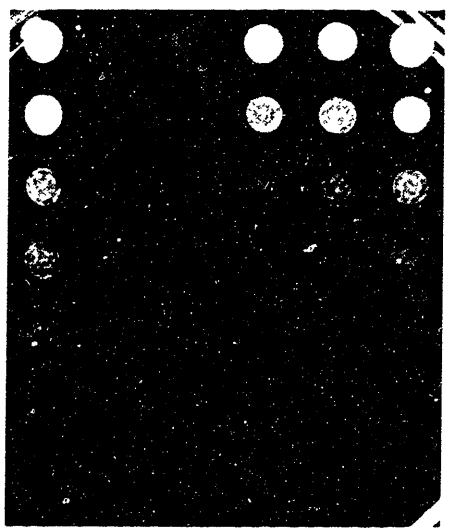

a b c d e f g h
Pac2p-HA-HIS6 + - + - + - + -
α-tubulin + + + + - - - -
β-tubulin - - - - + + + +
Pac2p-HA-HIS6
α-tubulin
β-tubulin

Figure 2-11. Co-overexpression of α-tubulin rescues Pac2p lethality in *tub-724* cells. Serial (five-fold) dilutions of saturated cultures were plated to galactose containing media, and allowed to grow at 30°C. From left to right: *tub1-724* cell containing YCpGAL and pRS317 (control) ; *CEN-GAL-PAC2-URA3* and pRS317; *CEN-GAL-PAC2-LYS2* and YCpGAL; *CEN-GAL-PAC2-LYS2* and *CEN-GAL-TUB1 -URA3*; *CEN-GAL-PAC2-LYS2* and *CEN-GAL-TUB1-URA3*; *CEN-GAL-TUB1 -URA3* and pRS317.

GAL-PAC2	-	+	+	+	+	-
GAL-TUB1	-	-	-	+	+	+



SC Galactose 30 °C

DISCUSSION

A tubulin mutation that affects heterodimer stability

tub1-724 is one of a set of α-tubulin mutants generated by chemical mutagenesis and selected on the basis of their conditional growth at low temperature. Because of the familiar cold lability of microtubules evident both *in vivo* and *in vitro*, a reasonable prediction might have been that mutants so selected would arrest because their microtubules were especially cold labile at temperatures permissive for wild type cells. Instead, only a subset of the mutants arrest with no microtubules; the others have at least normal complements of assembled tubulin.

Here we have characterized the properties of the protein encoded by one of the mutants that arrest with no microtubules, *tub1-724*. We previously showed that cells expressing only this α-tubulin allele are dead when Rbl2p is either over-expressed or absent. Since Rbl2p is a β-tubulin binding protein, we hypothesized that these lethal interactions could reflect an unstable heterodimer formed by Tub1-724p (Figure2-3). Several of the experiments presented above demonstrate that the mutant heterodimer does act as if it were unstable relative to wild type. The mutant heterodimer does not remain intact *in vitro* during immunoprecipitation. Similarly, *in vivo* the mutant heterodimer reacts more readily with excess Rbl2p to produce Rbl2p-β-tubulin. An alternative measure of Tub1-724p binding to β-tubulin is manifest in its inability to rescue cells from β-tubulin overexpression even at permissive temperature for the mutant (Table 2-3); success in that assay most likely depends upon the ability of the α-

tubulin protein to bind β-tubulin. These results indicate that Tub1-724p has a reduced affinity for β-tubulin. However, the normal growth of the mutant cells requires that most of its tubulin be in heterodimers, rather than as free α- and β-tubulin. We previously showed that the microtubules in 50% of cells overproducing β-tubulin are completely depolymerized when β-tubulin levels are 1.4-fold greater than wild type (Weinstein and Solomon, 1990).

A weaker heterodimer could readily explain the arrest phenotype of *tub1-724* cells. At the restrictive temperature, increased dissociation of the mutant heterodimer could be lethal either by decreasing the level of heterodimer below that necessary to maintain microtubules or by increasing the level of undimerized β-tubulin, which in turn causes microtubule disassembly and cell death even at modest excess (Katz et al., 1990; Weinstein and Solomon, 1990).

The single mutation in Tub1-724p predicted from DNA sequence is loss of a positive charge at position 106. Based upon the structure of tubulins reported by Nogales and her colleagues (Nogales et al., 1998), this residue occurs in the region between the B3 and H3 loops that contact the phosphates of the non-exchangeable GTP. That site is at the postulated interface between α- and β-tubulin in the heterodimer. The wild type arginine at this position probably contributes to phosphate binding, and so may indirectly participate in α-β interactions. Further analysis to understand the physical properties of mutations in this region are underway.

This analysis of Tub1-724p provides insight into the primary molecular defect that explains the mutant phenotypes. In general, the defects of mutant tubulins are largely understood in terms of the arrest phenotype rather than their execution point. For example, mutations in yeast β-tubulin can selectively affect a subset of microtubules (Sullivan and Huffaker, 1992), or cause cells to become benomyl dependent (Huffaker et al., 1988). Similarly selective tubulin mutations have been identified in other organisms as well (Oakley and Morris, 1980). However, the precise molecular basis for the defective arrest phenotype is not yet understood. A possible exception is the disruption produced by substitution of lysine for the highly conserved glutamate at position 288 in the *Drosophila* β2 protein; this mutation causes an apparent packing defect, so that the protofilaments do not close to form a tubule (Fuller et al., 1987). However, the same substitution in yeast β-tubulin has no apparent effect (Praitis et al., 1991). The generalizability of the mutation found in Tub1-724p also requires further testing.

Genetic interactions between *tub1-724* and *PAC2*

Instability of the Tub1-724-β-tubulin heterodimer predicts that over-expression of an α-tubulin binding protein should be deleterious to *tub1-724* cells, perhaps by producing more toxic free β-tubulin in the mutant cells. The work of Tian et al. (Tian et al., 1997) suggests the vertebrate homolog of the yeast protein Pac2p binds α-tubulin. As predicted, over-expression of *PAC2*, is lethal in *tub1-724* cells, and causes loss of all assembled microtubules. Consistent with this result, we can recover a complex containing Pac2p and α-tubulin from double over-expressing cells. These results

demonstrate for the first time that Pac2p can bind α-tubulin *in vivo.* This result does not distinguish among many possible functions for *PAC2.* It may act as does cofactor E in the *in vitro* assay, facilitating the incorporation of α-tubulin into heterodimers (Tian et al., 1997), but it is not essential for that reaction since *PAC2* is not an essential gene *in vivo* (Hoyt et al. 1997). *Δpac2* is synthetically lethal with other microtubule mutants - *Δcin8* (Geiser et al., 1997), *Δpac10* (Alvarez et al., 1998.), and *tub1-724* (unpublished results).

Regulating microtubule function

The first analyses of microtubules at a molecular level focused on protein factors that could be responsible for assembly in an *in vitro* reaction. It is striking that so many of the genes which appear to affect microtubules *in vivo* almost certainly do not participate in the polymerization reaction itself. In this sense, the *CIN* genes (Hoyt et al., 1990; Stearns et al., 1990), the *PAC* genes (Geiser et al., 1997), the *GIM* genes (Geissler et al., 1998) and the *RBL* genes (Archer et al., 1995), although identified - in some cases more than once - by a wide variety of approaches, have fundamental properties in common. They are not essential for cell viability in budding yeast, and their deletion does not confer a quantitative defect in microtubule assembly. Conversely, their over-expression does not increase the level of assembly, as could be expected for a modulator of microtubule assembly. For only one of these proteins - alp 1, a *CIN1* homolog in fission yeast - is there evidence suggesting that it binds along the length of the microtubule (Hirata et al., 1998).

A role for these proteins arises from the *in vitro* system for incorporating separated tubulin chains into heterodimer. Alone among proteins that have been analyzed in such assays, the tubulin polypeptides appear to require factors that act after release from the chaperonin. Without those factors, there is no exchange of newly folded polypeptide with the exogenously added heterodimer. Some of the protein factors are homologous to gene products in *S. cerevisiae* and *S. pombe* that affect microtubule functions, and in *S. pombe* some of them are essential (Hirata et al., 1998). That they are not essential in *S. cerevisiae*, however, suggests that there must be other mechanisms for folding tubulin and forming heterodimer in those cells.

These proteins may also have alternative functions. Rbl2p levels affect how cells survive alterations in the ratios of α- to β-tubulin (Archer et al., 1995). Levels of Pac10p and the *GIM* genes affect those ratios (Alvarez et al., 1998.; Geissler et al., 1998). It is clear that yeast cells are sensitive to those ratios. These proteins may participate in maintaining proper balance of the tubulin components, which may become an important step especially under times of stress. Such a role could help explain why expression of *RBL2* mRNA increases when cells are incubated with a microtubule depolymerizing drug (Velculescu et al., 1997), although there is no evidence that the tubulin chains themselves are expressed in greater amounts. The results from these several approaches suggest that the early steps of microtubule morphogenesis may be crucial for cell function.

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CHAPTER 3:

Tubulin Heterodimerization *in vivo*

INTRODUCTION

The spatial and temporal control of microtubule assembly is an essential aspect of many cellular functions, including division, motility, and organization of the cytoplasm. The development of a robust *in vitro* assembly reaction of microtubule polymers from heterodimeric subunits of α- and β-tubulin has had a major impact on the field. That assay led to identification of factors - structures, proteins, and small molecules - which influence the extent and organization of microtubule assembly. Reverse genetic techniques have enabled evaluation of the relevance of some of those factors to *in vivo* conditions. Surprisingly, such experiments have revealed that proteins that are required for the *in vitro* assembly reaction are not essential *in vivo*.

In principle, conventional genetic approaches should identify genes that are relevant to cellular microtubule function. Indeed, such screens have identified functions that directly modulate the assembly of subunits into microtubules (Pasqualone and Huffaker, 1994). What is notable, however, is the growing list of gene products that affect microtubule-dependent processes but do not interact directly with the polymer. For example, several genes that affect chromosome instability (Hoyt et al., 1990), sensitivity to microtubule depolymerizing drugs or excess β-tubulin (Archer et al., 1995; Stearns et al., 1990), dependence upon a mitotic motor (Geiser et al., 1997), cell polarity (Hirata et al., 1998), γ-tubulin function (Geissler et al., 1998) or phenotypes of tubulin mutants (Vega et al., 1998) encode proteins that must act on microtubule control at some step other than the polymerization reaction.

It is also striking that the screens enumerated above have, despite their diverse designs, frequently identified the same genes. For example, certain *CIN* (*C*hromosome *IN*stability) genes affect not only chromosome instability and sensitivity to benomyl - the contexts in which they originally were identified - but also yeast cells' ability to function without the kinesin Cin8p (Geiser et al., 1997). Similarly, mutations in the *PAC* genes perish in the absence of Cin8p, but some also participate in cellular responses to excess β-tubulin and to γ-tubulin function (Alvarez et al., 1998; Geissler et al., 1998).

Mammalian homologues of some of these proteins also have been identified as essential factors in an *in vitro* reaction that mediates formation of αβ-tubulin heterodimers from the unfolded individual polypeptides (Gao et al., 1994; Gao et al., 1992; Gao et al., 1993; Melki et al., 1996; Tian et al., 1996; Tian et al., 1997). Under the conditions of this reaction, the tubulin polypeptides released from the chaperonin complex are not competent to exchange efficiently into pre-existing heterodimers (Tian et al., 1997). Instead, a series of five factors are necessary to interact with monomeric α- and β-tubulin, and then to bring them together in a large complex that is the direct precursor of heterodimer (Tian et al., 1997). Four of those factors have homologues in budding yeast, three identified by independent genetic investigations - *CIN1* (Hoyt et al., 1990; Stearns et al., 1990), *RBL2* (Archer et al., 1995) and *PAC2* (Geiser et al., 1997) - and a fourth, *ALF1*, identified by its homology to the vertebrate protein (Tian et al., 1997). That none of these proteins is essential in *S. cerevisiae* suggests that the *in vitro* assay can not fully represent the early part of the *in vivo* pathway of microtubule

assembly. Even with that caveat, the *in vitro* assay strongly suggests *in vivo* activities for these proteins.

The *RBL2* (*R*escue excess β-tubulin *L*ethality) gene has properties that make it particularly valuable for exploring the processing of tubulin polypeptides *in vivo*. Over-expression of Rbl2p effectively rescues cells from the microtubule disassembly and cell death phenotypes of excess β-tubulin (Archer et al., 1995). Like its mammalian homolog cofactor A *in vitro* (Melki et al., 1996) Rbl2p binds β-tubulin both *in vivo* and *in vitro* to form a heterodimer that excludes α-tubulin (Archer et al., 1995; Archer et al., 1998). Unlike cofactor A, which binds only to a form of β-tubulin that is not competent to bind α-tubulin, Rbl2p can bind to β-tubulin molecules both before and after they have been incorporated into heterodimer (Archer et al., 1998). *RBL2* is not essential for mitotic growth but is essential for normal meiosis and normal resistance to microtubule depolymerizing drugs. In addition, its synthesis may be up-regulated at the G2/M stage of the cell cycle, when β-tubulin expression is apparently unchanged (Velculescu et al., 1997).

These properties suggest that Rbl2p functions may affect processes other than folding of β-tubulin. Analysis of genetic interactions of Rbl2p has helped to identify those processes. For example, *rbl2* deletion makes *PAC10* essential; *PAC10* regulates the ratio of α- to β-tubulin (Alvarez et al., 1998). Conversely, overexpression of *RBL2* is lethal in cells expressing a mutant α-tubulin that makes a weaker αβ-tubulin heterodimer (Chapter Two and Vega et al., 1998) .

In this study, we have applied *RBL2* overexpression to identify non-tubulin genes that influence heterodimer stability. We show here that null mutations in both *CIN1* and *PAC2* are synthetically lethal with excess Rbl2p. Both mutations enhance the formation of the Rbl2p-β-tubulin complex, which may deplete the pool of heterodimer and so cause cell death. The data presented here indicate that the effects of Cin1p and Pac2p are a consequence of their ability to promote heterodimer formation.

MATERIALS AND METHODS

Strains, Plasmids, and Media

All yeast strains are derivatives of FSY185 (Weinstein and Solomon, 1990) with the exception of the *tub1* mutants (Schatz et al., 1988). We used standard methods for yeast manipulations (Schatz et al., 1986; Solomon et al., 1992). All the relevant strains are listed in Table 3-1.

Screen for *erl* mutants

Wild-type cells containing p*GAL-RBL2:URA3:CEN* (pA5) were mutagenized with ethyl methanesulfonate (EMS) to 25% survival. The mutagenized strains were grown in YPD media for four hours. The cells were frozen at -70°C in 25% glycerol. Cells were plated from frozen stocks to SC -ura glucose plates (~200/plate) and after ~40 hours growth were replica plated to SC -ura galactose plates. Replica plated colonies that were unable to grow on galactose were retested by streaking to SC -ura galactose and SC -ura raffinose plates. Cells unable to grow on galactose were streaked to SC 5-fluoroorotic acid (5-FOA) plates to select for loss of the plasmid. Positive *erl* (*e*nhancer of *R*bl2p *l*ethality) mutants were able to grow on galactose after loss of the pA5 plasmid, but were unable to grow on galactose after retransformation with the same pA5 plasmid.

Construction of *CIN1* and *PAC2* knockout

The primers, 5'-GCACGACGTCGATAATATTTTTGGAAAGAACGCC and 5'-GCAGAGATCTGTTGATCGCGGCAATCGTCTGTTGGTGC were used to amplify DNA in the 5' UTR of *CIN1*. The primers 5'-GACCGTCGACGAGATAAAGAAATGCGGAATGAAGC and 5'-

GACCGCATGCGAGATAAAGAAATGCGGAATGAAGC were used to amplify DNA in the 3' UTR of *CIN1*. The PCR products from these primers were ligated into pNKY51 (Alani et al., 1987) on opposite ends of the *his*G-*URA*3-*hisG* sequence. The plasmid was digested with AatII and EagI and the *CIN1* 5' UTR-*hisG-URA3-hisG* -*CIN1* 3' UTR DNA fragment was isolated (Quaiex II from Quiagen) after electrophoresis on a 1% agarose gel. This DNA was then transformed into FSY185 to create a disruption of the entire *CIN1* open reading frame. The disruption was confirmed by Southern blot analysis of the diploids and of their haploid segregants, and by the phenotypic analysis of the haploid segregants. To disrupt *PAC2* pPA14 containing 1050 bp of 5' *PAC2* UTR and 800 bp of 3' *PAC2* UTR (Alvarez et al., 1998) joined together at a BamHI site in pGEM (Promega, Madison, WI) was digested. The BamHI-BglII fragment containing *hisG-URA3-hisG* from PNK51 was cloned into the BamHI site of digested pPA14. The resulting plasmid pLV59 was digested with BglII and NotI to release a 5.7kb disruption fragment to transform FSY183. The disruption was confirmed by PCR analysis.

Viability measurements

JFY203 (*Δcin1* containing pA5), JFY3 (wild-type containing pA5) and LTY500 (*Δpac2* containing pA5) were grown overnight in SC -ura raffinose media. Log phase cells were then induced with 2% galactose and at various time points aliquots of cells were taken and counted using a haemocytometer. Known numbers of cells were then plated to SC -ura glucose plates. Cell viability was measured as the percent of cells able to form colonies on the SC -ura glucose plates.

In vivo His_6-Rbl2p-β-tubulin association experiments

Yeast strains LTY503(*Δpac2*), JFY253(*Δcin1*) and LTY573 (wild-type) containing a *GAL-RBL2-HIS*$_6$ plasmid (pGHR) were grown overnight at 30°C in selective media containing raffinose to about $2X10^9$ cells per experiment. 2% galactose was added to induce *His*$_6$*-RBL2* expression. After 3 hours protein was harvested by glass bead lysis in 1ml PME buffer plus protease inhibitors. We applied 0.85 ml of protein extract to 50ul Ni-NTA beads (Qiagen). We washed and eluted the bound proteins as previously described (Magendantz et al., 1995). Eluted proteins were subjected to SDS-PAGE analysis and probed by immunoblotting for α-tubulin, β-tubulin and Rbl2p.

Immune Techniques

Immunoblots: Modifications of standard procedures (Solomon et al., 1992) were used to assay for Rbl2p-His_6-β-tubulin association. After gel electrophoresis and transfer to nitrocellulose membranes, we blocked with TNT (0.025M Tris pH 7.5, 0.17M NaCl, 0.05% Tween-20) for 30-120 minutes. Primary antibodies were incubated for >12 hours at 1/3500 (#206 or #345; (Weinstein and Solomon, 1990)) or at 1/100 (#250 (Archer et al., 1995) and then washed 5 times (5 min. each) in TNT. Bound antibody was detected by ^{125}I Protein A (NEN).

In other experiments, after gel electrophoresis (as above), we blocked with milk (5% Carnation) TBST (0.05M Tris pH8.0, 0.15M NaCl, 0.1% Tween-20) overnight. Primary antibodies were incubated for 1-2 hours 1/3500 fo :206 and #345 and at 1/5000 for 12CA5 (Boehringer) in milk TBST. The blots were washed 6 times (two 20

sec, one 15 min, three 5 min) in TBST alone. Blots were incubated with 1/3000 dilutions of horseradish peroxidase conjugated goat anti-rabbit (Jackson ImmunoResearch) for #206 and #345 and horseradish peroxidase conjugated rabbit anti-mouse (Jackson ImmunoResearch) for 12CA5, in milk TBST, washes were done in TBST as above, and detected by chemilumenescence (Renaissance NEN).

Immunofluorescence: We used standard techniques (Solomon et al., 1992). Primary antibody was #206 (anti-β-tubulin) and secondary antibody was fluorescein conjugated goat anti-rabbit IgG (Cappel). DAPI (Boehringer Mannheim) was used to visualize DNA.

Sensitivity to β-tubulin overexpression

We crossed *Δcin1* or *Δpac2* strains with a haploid derivative of JAY47 containing *TUB2-GAL-TUB2-LEU2* allele. The resulting diploids were sporulated and haploids that were *Δcin1* or *Δpac2* and contained the *TUB2-GAL-TUB2-LEU2* allele were obtained. The *Δpac2* ,*TUB2-GAL-TUB2-LEU2* were covered with pLV63 (LTY558) or with the control plasmid pRS313 (LTY559). The *Δcin1*, *TUB2-GAL-TUB2-LEU2* cells were covered with p18C (JFY238) or with the control plasmid pCT3 (JFY236). To test the sensitivity of these strains to β-tubulin overexpression, we grew up LTY558 and LTY559 in liquid SC -his-leu raffinose, and JFY236 and JFY238 in liquid SC -ura-leu raffinose or. β-tubulin overexpression was induced by adding galactose to 2%. At various time points aliquots of cells were taken and counted using

a haemocytometer. Known numbers of cells were then plated to SC-his-leu or SC-ura-leu glucose plates. Cell viability was measured as above.

Analysis of α-tubulin mutations synthetic lethal with *Δcin1 and Δpac2*

Δcin1 Δtub1 Δtub3 (JFY474) or *Δpac2 Δtub1 Δtub3* (LTY479) strains containing a plasmid with a genomic copy of *TUB1* on a *URA3 CEN* vector or *TUB3* on a *URA3 2um* vector, respectively were transformed with *LEU2 CEN* plasmids containing the various α-tubulin mutations. The strains containing both the wild type and a mutant form of *TUB1* were grown on 5-FOA plates to select for cells that have lost the wild type α-tubulin plasmid, since 5-FOA kills *URA3*$^+$ but not *ura3*$^-$ cells. *Δpac2* or *Δcin1* strains that are synthetic lethal with the α-tubulin mutations will be unable to lose the wild-type α-tubulin plasmid and cannot survive on 5-FOA. However, strains that are viable without the wild-type α-tubulin allele are able lose this plasmid along with the *URA3* gene and form colonies.

Construction of *GAL-CIN1*

The *CIN1* ORF and additional 5' and 3' UTR was amplified by P.C. R. The 5' primer (5'-GACACGCGTCATGAACAATATTCGGGCCTTGC) contained a MluI site and the 3' primer, (5'-CAGCCGCGGATTATATGTAAAATTTGCCGTTTAC) contained a SacII site. The PCR product was ligated into the pT7-Blue plasmid from Novagen. This DNA was then digested with MluI and SacII and ligated into the pRS316-Gal plasmid (Liu et al., 1992). The construct (pJF10) suppressed the benomyl supersensitive phenotype of cells deleted for *CIN1*.

Interactions of α-tubulin mutant alleles with overproduced *CIN1*

Δtub1; *Δtub3* strains containing mutant alleles of the *TUB1* gene on *LEU2:CEN* plasmids (listed in Figure 2) were transformed with pJF10 and YCpGAL. These strains were grown to saturation overnight in SC -ura glucose liquid media. The cultures were serial diluted in 96 well dishes, and spotted onto SC -ura galactose plates containing 10ug/ml benomyl and to SC -ura galactose plates incubated at 25°C (a semipermissive temperature for the growth of *tub1-724* mutant strains) as well as to galactose and glucose plates at 30° as a growth control.

Effect of *CIN1* overproduction on excess Pac2p *tub1-724* lethality

Δtub1:*Δtub3* strains containing the *tub1-724* mutant α-tubulin allele on a *LEU2 CEN* plasmid were transformed with the following plasmids: p*GAL-CIN1 CEN URA3* and p*GAL-PAC2 CEN LYS2* (JFY475), p*GAL-CIN1 CEN URA3* and p*CEN LYS2* (JFY476), or p*CEN URA3* and p*GAL-PAC2 CEN LYS2* (JFY477). The strains were grown overnight in SC - lys -ura glucose liquid media. The cultures were serial diluted in 96 well dishes, and spotted onto SC -lys -ura galactose plates. Cells were also spotted onto glucose plates as a growth control.

Construction of *GAL-CIN1-HA* and *GAL-CIN1-HA-His*$_6$

The 3' third of the *CIN1* open reading frame was amplified using PCR. The 5' primer (5'-GATGTAGGACGTCTGGTAAGAATACAGGC) contained an AatII site. Two 3' primers were used. To make the GAL-CIN1-HA construct we used the 3' primer (5' CTCACCGCGGCTAGCGGCCGCCTAAAGTGATATCAGACTCTAATATATTCGC) containing a NotI site followed by two stop codons and a SacII site. To make GAL-

CIN1-HA-His_6 we used the 3' primer (5' CTCACCGCGGCTAGTGATGGTGATGGTGATGGCGGCCGCCTAAAGTGATATCAGAC TCTAATATATTCGC) containing 6 in frame histidine residues, a NotI site, two stop codons, and a SacII site. The PCR products were ligated into pT7-Blue plasmid (Novagen). The AatII SacII fragments were then ligated into pJF10 to create pJF11 and pJF12 respectively. A 111 bp NotI fragment containing the triple HA epitope from B2385 (provided by G. Fink, M.I.T.) was cloned into the Not I site of pJF11 and pJF12 to create pJF14 and pJF15 respectfully. pJF14 and pJF15 suppressed the benomyl supersensitive phenotype of cells deleted for *CIN1* to a similar extent as did pJF10. pJF14 and pJF15 also suppressed the conditional phenotypes of the *tub1-724* mutant as well as the pJF10 construct (as described in results).

In vivo Cin1p-HA-His6 and Pac2p-HA-His6 association experiments

We grew yeast strains overnight in selective raffinose media at 30°C. Galactose (2%) was added to induce the tagged constructs for ~4 hours. $6.0X10^9$ cells were harvested by glass bead lysis per experiment in 1.1ml PME buffer plus protease inhibitors. We applied 1ml of protein extract to 25ul Ni-NTA beads. We washed and eluted the bound proteins as previously described (Magendantz et al., 1995). Eluted proteins were subjected to SDS-PAGE analysis and probed for α-tubulin, β-tubulin and HA(12CA5). For Cin1p-β-tubulin association experiment we used strains JFY470 (p*GAL1-10 CIN1-*His_6*-HA CEN URA3)* and JFY471 (YCpGAL). For Pac2p-Cin1p association experiment we used strains LTY564 (p*GAL1-10 PAC2-*His_6*-HA CEN LYS2, YCpGAL*), LTY565 (p*GAL1-10 PAC2-*His_6*-HA CEN LYS2*, p*GAL1-10 CIN1 CEN URA3*), LTY566 (p*GAL1-10 PAC2-*His_6*-HA CEN LYS2,* p*GAL1-10 CIN1-HA*

CEN URA3), and LTY567 (p*CEN LYS2,* p*GAL1-10 CIN1-HA CEN URA3*). For Pac2p-α-tubulin association experiments we used strains LTY498 (Δ*cin1* p*GAL 1-10 PAC2-His$_6$-HA CEN URA3*), LTY597 (p*CIN1 GAL 1-10 PAC2-His$_6$-HA CEN URA3*), and JFY252 (Δ*cin1* YCpGAL).

Table 3-1. Strains and plasmids

Strain/plasmid	Genotype	Reference
Strains		
FSY183	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52* (pA5)	Weinstein and Solomon, 1990
FSY182	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52 Δtub1::HIS3 Δtub3::TRP1* (pRB539)	Schatz et al., 1988
FSY157	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52 Δtub1::HIS3 Δtub3::TRP1* (pRB624)	Schatz et al., 1988
JFY3	FSY183 plus pA5	This study
JFY4	FSY183 plus YCpGAL	This study
JFY5	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52 erl1-1* (pA5)	This study
JFY209	*MAT@ his3Δ200 leu2-3.112 lys2-801 ura3-52 Δcin1::hisG:URA3:hisG*	This study
LTY467	*MAT@ his3Δ200 leu2-3,112 lys2-801 ura3-52 Δpac2::hisG:URA3:hisG*	This study
JFY206	*MAT@ his3Δ200 leu2-3,112 lys2-801 ura3-52 Δcin1::hisG*	This study
JFY203	JFY206 with pA5	This study
LTY500	*MAT@ his3Δ200 leu2-3,112 lys2-801 ura3-52 Δpac2::hisG* (pA5)	This study
LTY576	FSY 183 with pGRH	This study
JFY252	JFY206 with YCpGAL	This study
JFY253	JFY206 with pGRH	This study
LTY503	*MAT@ his3Δ200 leu2-3,112 lys2-801 ura3-52 Δpac2::hisG* (pGRH)	This study
JFY236	*MAT@ his3Δ200 leu2-3,112 lys2-801 ura3-52 Δcin1::hisG TUB2-LEU2-GAL-TUB2* (pCT3)	This study
JFY238	*MAT@ his3Δ200 leu2-3,112 lys2-801 ura3-52 Δcin1::hisG TUB2-LEU2-GAL-TUB2* (p18C)	This study
LTY558	*MAT@ his3Δ200 leu2-3,112 lys2-801 ura3-52 Δpac2::hisG TUB2-LEU2-GAL-TUB2* (pRS313)	This study
LTY559	*MAT@ his3Δ200 leu2-3,112 lys2-801 ura3-52 Δpac2::hisG TUB2-LEU2-GAL-TUB2* (pLV63)	This study
JFY474	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52 Δcin1::hisG Δtub1::HIS3 Δtub3::TRP1* (pA1A5106)	This study
LTY479	*MATa his3Δ200 leu2-3,112 lys2-801 ura3-52 Δpac2::hisG Δtub1::HIS3 Δtub3::TRP1* (pTUB3 URA3 2u)	This study
JFY268	FSY157 with pJF10	This study
JFY269	FSY157 with YCpGAL	This study
JFY470	FSY182 with pJF15	This study
JFY471	FSY182 with YCpGAL	This study
JFY475	FSY 182 with pJF10 and pJF16	This study
JFY476	FSY 182 with pJF10 and pRS317	This study
JFY477	FSY 182 with YCpGAL and pJF16	This study
LTY564	FSY182 with pLV63 and YCpGAL	This study
LTY565	FSY182 with pLV63 and pJF10	This study
LTY566	FSY182 with pLV63 and pJF14	This study
LTY567	FSY182 with pRS317 and pJF14	This study
LTY498	JFY206 with pLV56	This study
LTY597	FSY183 with pLV56	This study
Plasmids		
YCpGAL	*CEN URA3*	
p18C	*CIN1 CEN URA3*	This study
pRS316	*CEN URA3*	Sikorski and Hieter, 1989
pA5	*GAL1-10 RBL2 CEN URA3*	Archer et al., 1996
pGRH	*GAL1-10 RBL2-HIS$_6$ CEN URA3*	Archer et al., 1998
pRB624	*tub1-724 CEN LEU2*	Schatz et al., 1986
pRB539	*TUB1 CEN LEU2*	Schatz et al., 1986
pJF10	*GAL1-10 CIN1 CEN URA3*	This study
pJF14	*GAL1-10 CIN1-HA CEN URA3*	This study
pJF15	*GAL1-10 CIN1-His$_6$-HA CEN URA3*	This study
pLV62	*GAL1-10 PAC2-His$_6$-HA CEN LYS2*	Vega et al., 1998
pLV56	*GAL 1-10 PAC2-His$_6$-HA CEN URA3*	Vega et al., 1998
pJF16	*GAL1-10 PAC2 CEN LYS2*	This study
pRS317	*CEN LYS2*	Sikorski and Hieter, 1989
pRS313	*CEN HIS3*	Sikorski and Hieter, 1989
pA1A5106	*TUB1 CEN URA3*	Kirpatrick and Solomon, 1994
pRB316	*TUB3 2μm URA3*	Schatz et al., 1986
pLV63	*PAC2 CEN HIS3*	This study

RESULTS

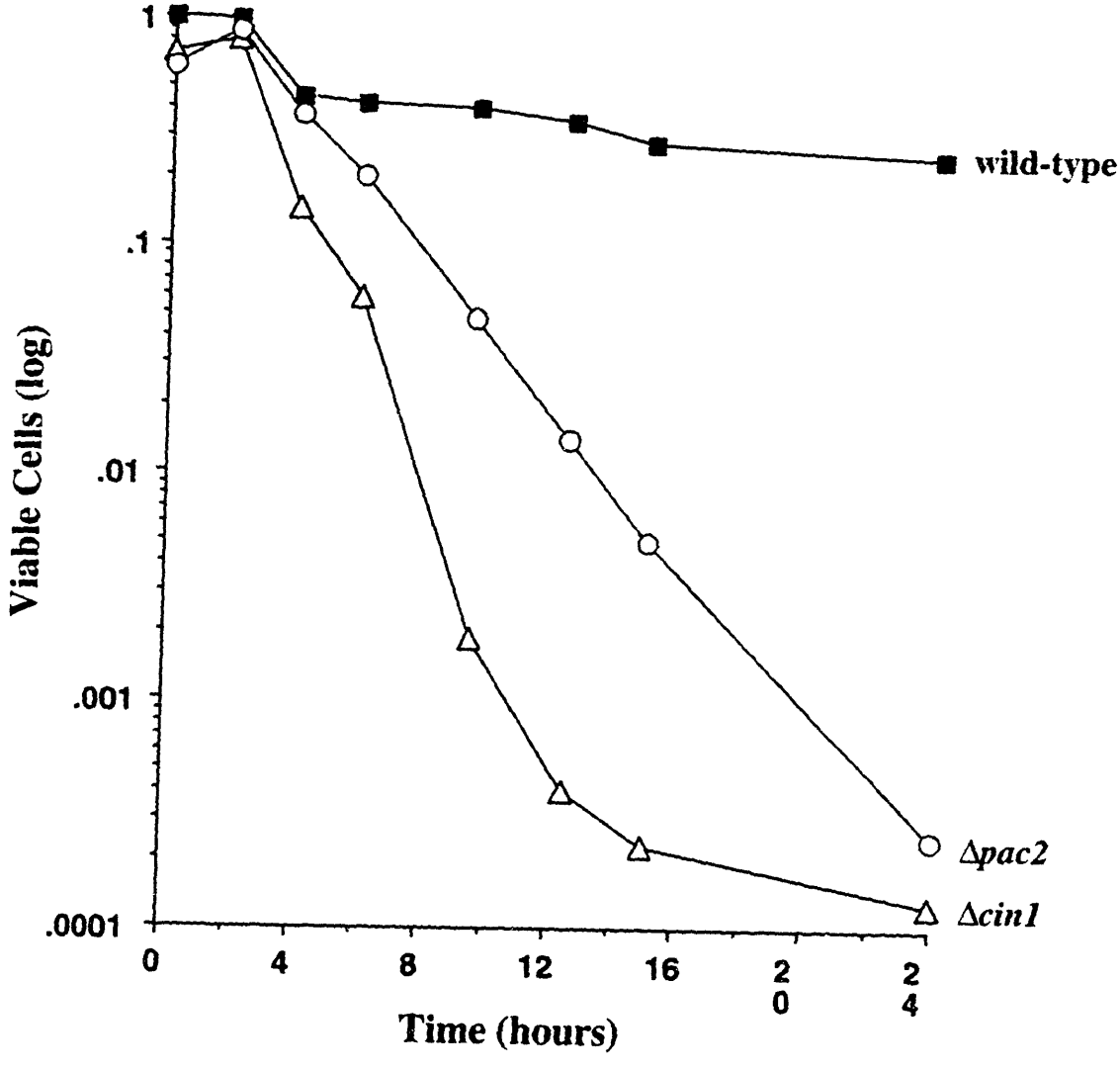
Mutations in the *CIN* genes are sensitive to Rbl2p overproduction

We searched for non-tubulin genes that affect heterodimer stability. Our strategy was based on the observation that excess Rbl2p kills *tub1-724* (Chapter Two and Archer et al., 1995), which encodes an α-tubulin with relatively weak affinity for β-tubulin (Chapter Two and Vega et al., 1998) . This synthetic lethality is probably explained by depletion of the heterodimer pool in mutant cells, since excess Rbl2p competes with and displaces the mutant α-tubulin protein.

Accordingly, we mutagenized wild-type haploid cells and screened for mutants that could not survive when the *GAL-RBL2* plasmid (pA5) was induced (see Experimental Procedures). This screen identified one mutant, *erl1-1* (for *e*nhancer of *R*bl2p *l*ethality), that was unable to live when overproducing Rbl2p. The *erl1-1* strain is cold-sensitive at 11° C and extremely sensitive to benomyl.

By several criteria, we demonstrated that the *erl1-1* mutation is an allele of *CIN1*. A library plasmid containing the entire *CIN1* open reading frame rescued both the benomyl phenotype of the *erl1-1* mutant and the lethality upon *RBL2* overexpression. To confirm that loss of *cin1* function confers the *erl* phenotype, we deleted the entire open reading frame of *CIN1* by integrative transformation, and tested the effect of *RBL2* overexpression. As shown in Figure 3-1, *Δcin1* cells overproducing Rbl2p start to lose viability ~4 hours after induction, and after ~12 hours

Figure 3-1. The lethality of RBL2 overexpression is enhanced in *CIN1* and *PAC2* nulls. *Δcin1* (open triangles Δ), *Δpac2* (open circles O), and wild-type strains (closed squares ■) containing a p*GAL-RBL2* plasmid were grown overnight in selective non-inducing media. At t = 0hr, Rbl2p overproduction was induced by addition of galactose to 2%. Cell viability is determined as the percentage of cells able to form colonies on glucose plates.


1
.1
.01
.001
.0001
Viable Cells (log)
0
4
8
12
16
20
24
Time (hours)
wild-type
Δpac2
Δcin1

fewer than 0.1% of the cells are viable. Finally, we confirmed that *erl1-1* is indeed an allele of *CIN1* by both complementation and linkage analysis (see Methods) with a *cin1* null allele originally characterized by Stearns and colleagues (Stearns et al., 1990).

Several previous results connect *CIN1* to microtubule function. It was first identified in genetic screens for mutations that result in chromosome instability (Hoyt et al., 1990) or supersensitivity to benomyl (Stearns et al., 1990). Mutations in *cin1* are synthetically lethal with loss of either *CIN8* - a mitotic motor (Geiser et al., 1997) - or of *PAC10* - which regulates the α- to β-tubulin ratio and which is itself synthetically lethal with *Δrbl2* (Alvarez et al., 1998; Geissler et al., 1998). Finally, cofactor D, a vertebrate homolog of Cin1p, participates in the *in vitro* mediated folding of β-tubulin (Tian et al., 1996).

The chromosome instability and drug sensitivity screens that first identified *CIN1* also identified *CIN2* and *CIN4* (Hoyt et al., 1990; Stearns et al., 1990). The phenotypes of the double and triple *CIN1, 2* and *4* mutants suggest that these three genes act as components of a complex or in a common pathway (Stearns et al., 1990). Although the *erl* screen did not identify mutations in *cin2* or *cin4*, we directly tested strains bearing null alleles of each (Hoyt et al., 1990; Stearns et al., 1990) for the *erl* phenotype. Over-expression of Rbl2p is lethal in both *cin2* and *cin4* null strains, although to a lesser extent than for *cin1* nulls (data not shown). This result supports the conclusion that the common microtubule-related functions of these three *CIN* genes is affected by Rbl2p levels.

Deletion of *PAC2* is sensitive to Rbl2p overproduction

Cin1p and Rbl2p are two of the four yeast homologues of vertebrate protein cofactors involved in a tubulin folding assay (see Introduction). We tested the other two components for interaction with excess Rbl2p. One of the components, Pac2p, is homologous to mammalian cofactor E (Hoyt et al., 1997). Figure 3-1 demonstrates that cells deleted for *pac2* rapidly lose viability upon over-expression of *RBL2*. *In vivo*, Pac2p binds to α-tubulin and its over-expression, like that of Rbl2p, kills *tub1-724* cells - probably by binding the α-tubulin of the unstable heterodimer and thus generating toxic levels of free β–tubulin (Chapter 2 and Vega et al., 1998) . The other homolog, Alf1p, is related to the vertebrate cofactor B which binds to α-tubulin (Tian et al., 1997). There is no effect of over-expressing *RBL2* in *Δalf1* cells (data not shown).

Under normal growth conditions *CIN1*, *PAC2*, and *RBL2* are not essential. In addition, pairwise combinations of *Δrbl2* with *Δcin1* or *Δpac2* are viable (unpublished results; Hoyt et al., 1997). Thus, these pairs of genes do not define an essential function. However, in the context of *RBL2* overexpression, *PAC2* and *CIN1* function become essential for viability.

Rbl2p-β-tubulin formation and microtubule depolymerization in *pac2* and *cin1* nulls overexpressing Rbl2p

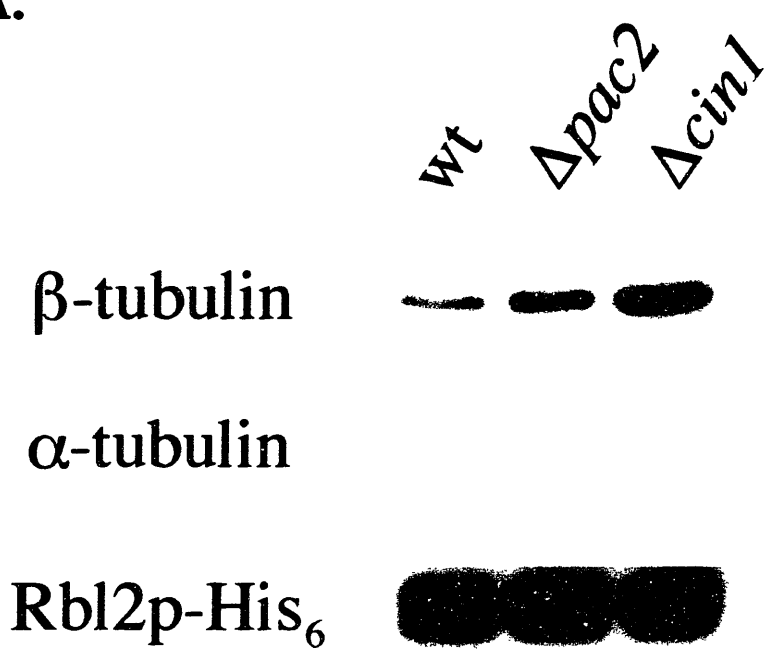
The effect of overexpressed Rbl2p in *cin1* or *pac2* mutants suggests that the functions of those two genes may affect the state of the tubulin heterodimer. We know

that the lethality of excess Rbl2p in *tub1-724* cells is accompanied by enhanced formation of Rbl2p-β–tubulin complex, and presumably concomitant depletion of heterodimer (Vega et al., 1998) . Therefore, we determined if the same reaction occurs in *Δcin1* and *Δpac2* mutant strains. Extracts were prepared from galactose grown *Δcin1*, *Δpac2* or wild type cells transformed with a plasmid encoding His_6-Rbl2p under the control of the galactose promoter. His_6-Rbl2p and bound proteins were specifically purified by incubation with nickel-agarose beads and elution with imidazole. Immunoblot analysis (Figure 3-2 (A, B) demonstrates that the amount of β-tubulin associated with the His_6-Rbl2p fraction was 2-5 fold higher in *Δpac2* and *Δcin1* cells. There is no significant binding of α-tubulin to His_6-Rbl2p in any of the strains.

In *tub1-724* cells, an additional consequence of *RBL2* overexpression is the loss of microtubule structures (Chapter two and Vega et al., 1998) . Figure 3-3 demonstrates that Rbl2p overexpression has the same consequence for both *Δcin1* and *Δpac2* cells. After Rbl2p overproduction for 3 hours, microtubule staining in wild-type cells is normal; 78% of the cells have intranuclear microtubules, 18% show a dot representing the spindle pole body, and 4% have no detectable staining. In contrast, only 25% of either *Δcin1* or *Δpac2* cells overexpressing Rbl2p have short or long spindles; the remainder have either no staining at all (28% for *Δcin1*, 38% for *Δpac2*) or single dots. Thus, a common feature of mutations that enhance Rbl2p

Figure 3-2. Enhanced β-tubulin binding in *CIN1* and *PAC2* nulls. (A) Protein extracts from *Δcin1*, *Δpac2* , and wild-type strains containing a p*GAL-RBL2-HIS*$_6$ plasmid were obtained from cells grown three hours in selective inducing media. The tagged Rbl2p and bound proteins were purified using nickel-agarose. Nickel eluates were analyzed by immunoblotting with antibodies to α-tubulin, β-tubulin and Rbl2p. (B) Quantitation of the Rbl2p-β-tubulin complex formed from *Δcin1*, *Δpac2* , and wild-type strains as in (A). The amount of Rbl2p and β-tubulin signal was quantitated by densitometry and normalized to Rbl2p signal. The values are expressed as fold increase above the wild-type control. Error bars represent the standard deviation of three independent trials.

A.


wt
Δpac2
Δcin1
β-tubulin
α-tubulin
Rbl2p-His6

B.

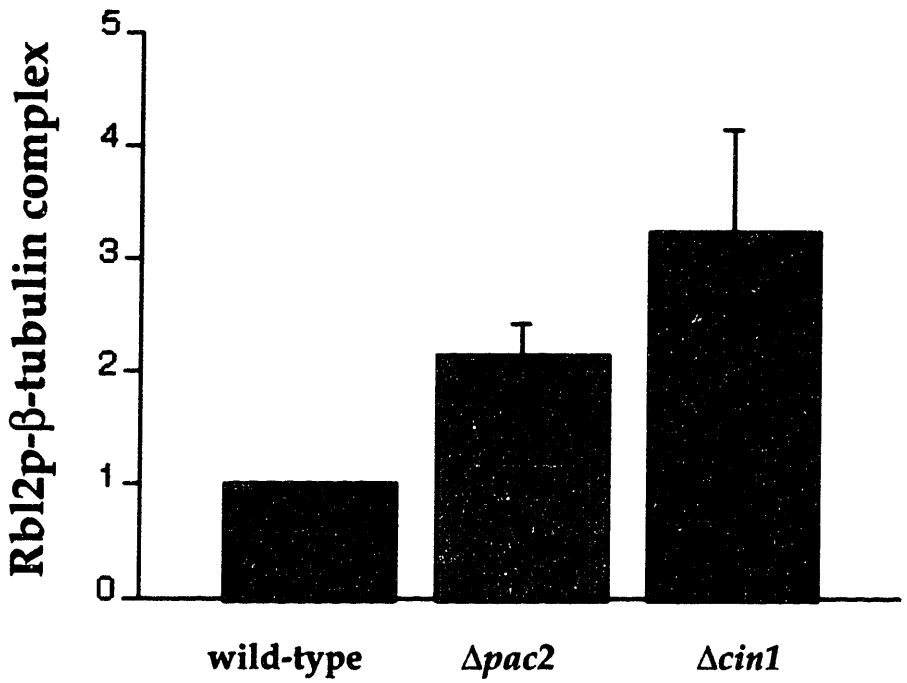
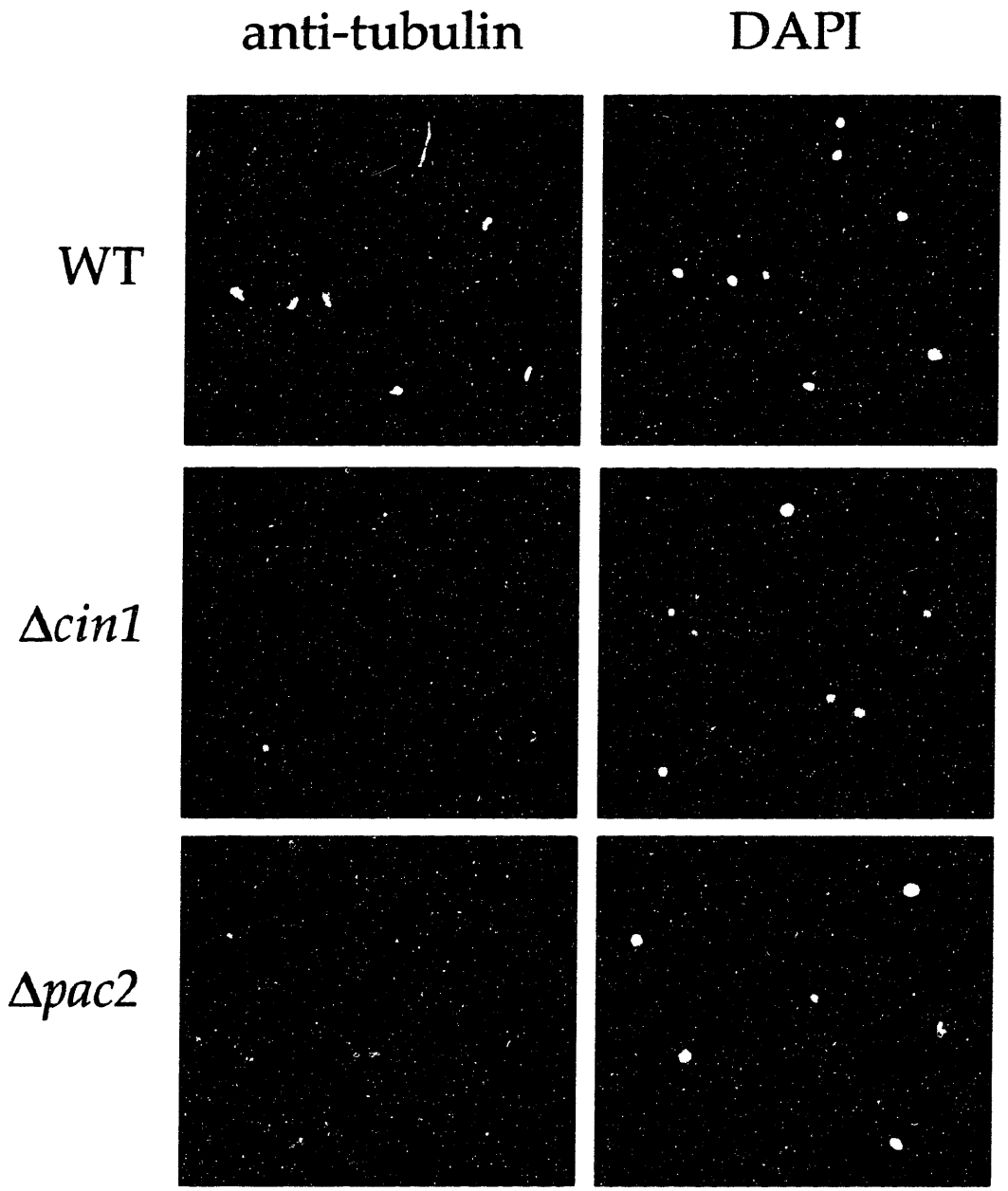

Rbl2p-β-tubulin complex
5
4
3
2
1
0
wild-type
Δpac2
Δcin1

Figure 3-3. Microtubule staining in *Δcin1* and *Δpac2* cells overexpressing *RBL2*. After three hours of *RBL2* overexpression, *Δcin1*, *Δpac2* and wild-type cells were processed for immunoflouresence. Cells were stained using an anti- β-tubulin antibody to detect microtubule structures and with DAPI to stain nuclei.

Rbl2p over-expression causes microtubule depolymerization in *erl* cells



overexpression lethality is the facilitation of Rbl2p-β-tubulin complex formation and the concomitant loss of microtubules.

Effects of *PAC2* and *CIN1* levels on β-tubulin lethality

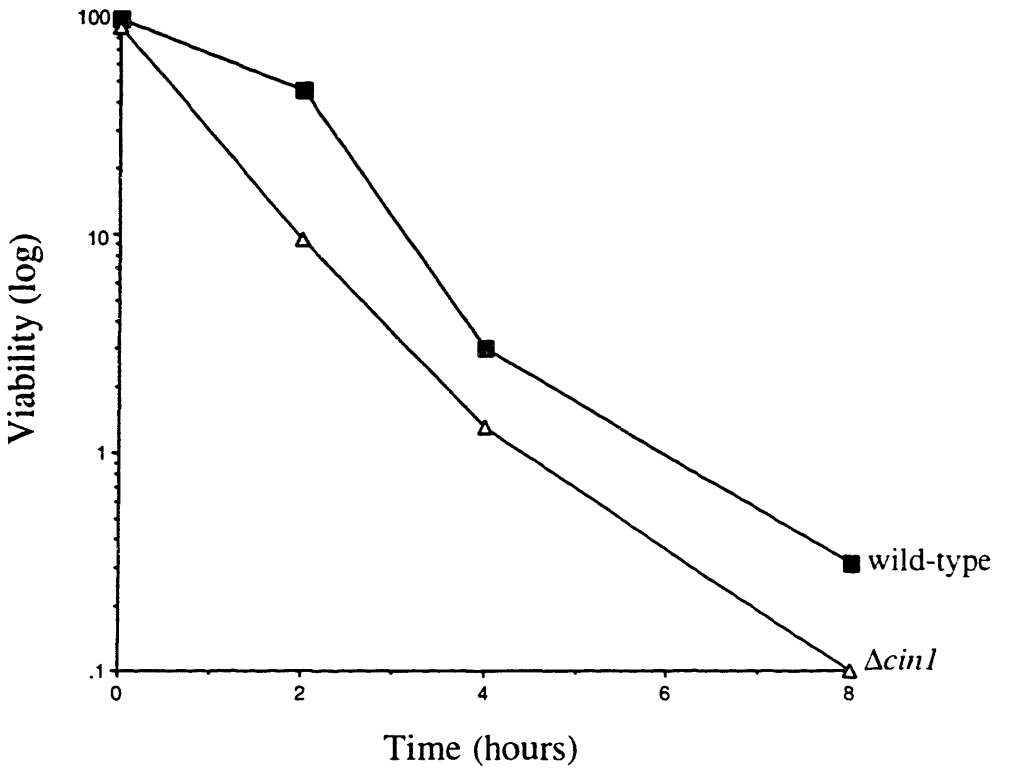
Sensitivity to β-tubulin overexpression is affected by both an excess of or a deficit in of two β-tubulin binding proteins, Rbl2p and α-tubulin (Archer et al., 1995). At one extreme, overexpression of either of these β-tubulin binding proteins dramatically decreases lethality of β-tubulin overexpression. The *in vitro* tubulin folding assay demonstrates that β-tubulin binds the mammalian homolog of Cin1p (cofactor D). It also provides indirect evidence for but does not demonstrate directly a complex containing β-tubulin, α-tubulin, cofactor D, and the mammalian homolog of Pac2p, cofactor E (Tian et al., 1997). Therefore, we tested whether overexpressed Cin1p or the combination of Cin1p and Pac2p could rescue cells from the lethality associated with excess β-tubulin. A diploid yeast strain (JAY47) that contains a third integrated copy of the β-tubulin gene, *TUB2* under the control of the galactose promoter grows normally on glucose, but only 0.01% of JAY47 cells can form colonies when plated to galactose. Co-overexpression of galactose promoted α-tubulin or Rbl2p raises plating efficiency on galactose to about 70% (Archer et al., 1995). However, overexpression of Pac2p or Cin1p, separately or together, does not rescue cells from the excess β-tubulin lethality (data not shown). Similarly, over-expression of *CIN1* does not rescue

the microtubule phenotypes of a *Δtub3* strain which has a constitutive excess of β- to α-tubulin (data not shown).

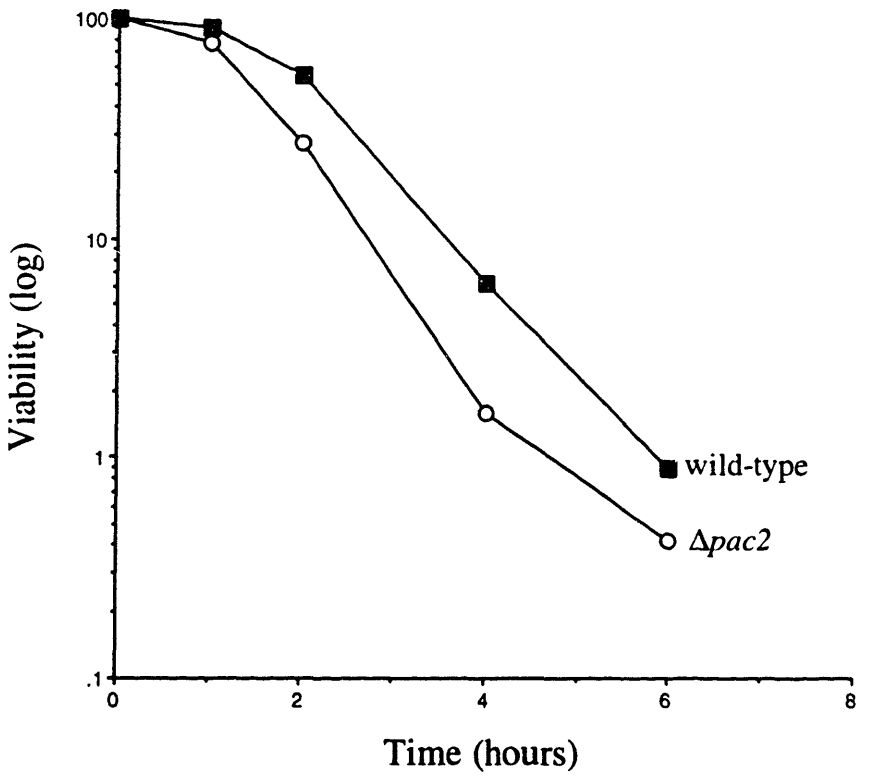
At the other extreme, *Δrbl2* cells are supersensitive to β-tubulin overexpression (Archer et al., 1995). We also assayed the effects of deleting *PAC2* or *CIN1* on cells' ability to survive excess β-tubulin poisoning. We made derivatives of wild type haploid cells containing an integrated *GAL-TUB2* allele and a deletion of either *PAC2* or *CIN1*. We induced β-tubulin overexpression with galactose and monitored the viability of the cultures. As seen in figure 3-4 (A)(B), cells deleted for either *PAC2* or *CIN1* are more sensitive to β-tubulin overexpression than are the control strains. This result suggests that, although overexpression of neither gene rescues cells from β-tubulin lethality, their function does participate in protection against excess β-tubulin lethality. Consistent with that conclusion, both *Δpac2* and *Δcin1*, as well as *Δrbl2*, are lethal in combination with a deletion in *pac10* (Alvarez et al., 1998). Pac10p affects the α/β tubulin ratio, and in *Δpac10* cells there is a constitutive excess of β-tubulin(Alvarez et al., 1998; Geissler et al., 1998). We conclude that the functions of *CIN1* and *PAC 2* become essential in the presence of such an imbalance in the tubulin polypeptides.

Figure 3-4. Deletions of *CIN1* or *PAC2* make cells more sensitive to β-tubulin overproduction. Haploid strains with an integrated copy of *GAL-TUB2* containing either deletions of *CIN1* (A) or *PAC2* (B) were grown and analyzed for viability as in Figure 3-1. (A): *Δcin1* cells with a p*CIN1-CEN* covering plasmid are represented by closed squares (■), and *Δcin1* cells with a control plasmid are represented by open triangles (Δ). (B): *Δpac2* cells with a p*PAC2-CEN* covering plasmid are represented by closed squares (■), and *Δpac2* cells with a control plasmid are represented by open circles (O). Data represent results from four independent determinations.

A.


100
10
1
.1
Viability (log)
0
2
4
6
8
Time (hours)
wild-type
Δcin1

B.


100
10
1
.1
Viability (log)
0
2
4
6
8
Time (hours)
wild-type
Δpac2

Absence of Cin1p or Pac2p is lethal with specific α-tubulin mutants

Previous work established that *RBL2* becomes essential in specific α-tubulin mutants (Archer et al., 1995). We asked whether *Δcin1* and *Δpac2* have similar interactions. Strains bearing *cin1* and *pac2* null alleles, and carrying a plasmid bearing wild-type α-tubulin (marked with *URA3*) as their major source of α-tubulin were transformed with one of 12 different *tub1* mutants (marked with *LEU2*) and plated to medium containing 5-FOA. *Δcin1* and *Δpac2* strains that require the wild type α-tubulin gene can not lose the plasmid marked with *URA3* and thus are unable to grow on 5-FOA. Five of the twelve α-tubulin mutations tested are synthetic lethal with both *Δpac2* and *Δcin1* (Table 3-2). Significantly, four of these five mutants are also synthetically lethal with *Δrbl2* (Archer et al., 1995). Several other α-tubulin alleles do not interact with *rbl2, cin1,* or *pac2.* The results suggest that *PAC2, CIN1* and *RBL2* affect related functions.

Overexpression of *CIN1* suppresses the phenotypes associated with *tub1-724*

Over-expression of either *RBL2* or *PAC2* in cells expressing Tub1-724p as their sole α-tubulin was previously shown to cause microtubule disassembly and cell death (Archer et al, 1995; Vega et al., 1998). This lethality is explicable because the mutant α-tubulin forms a weaker heterodimer. Thus, excess Rbl2p, a β-tubulin binding protein, or Pac2p, an α-tubulin binding protein (Chapter Two and Vega et al., 1998) , deplete the heterodimer in *tub1-724* cells below the level needed for viability.

Table 3-2. α-tubulin alleles synthetic lethal with nulls in *RBL2*, *CIN1*, and *PAC2*.

n.d. = not determined

	Viability		
	*Δrbl2**	*Δcin1*	*Δpac2*
tub1-724, -728, -738, -759	–	–	–
tub1-735	n.d.	–	–
tub1-704, -714, -744, -750	+	+	+
tub1-727, -730, -733, -741, -746, -758	n.d.	+	+

* (Archer et al., 1995)

Moreover, the formation of a Pac2p-α-tubulin complex (Chapter Two and Vega et al., 1998) likely produces free β-tubulin, which is toxic.

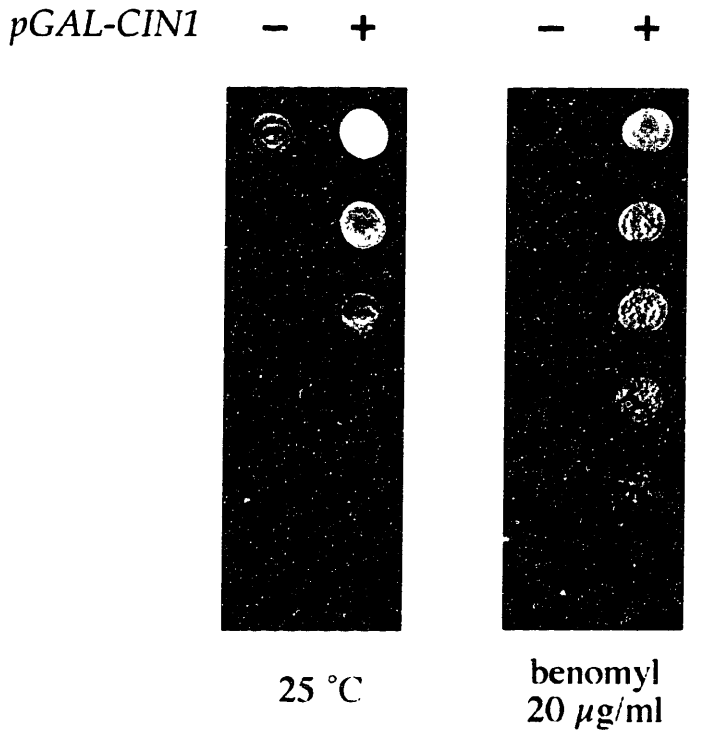
The effect of excess Cin1p in *tub1-724* cells yields a dramatically different result from that of Rbl2p or Pac2p. We transformed the plasmid pJF10, containing *CIN1* under control of the *GAL* promoter, into the *tub1-724* mutant strain, and monitored cell growth under various conditions. Cin1p overproduction suppresses both the lethality of the *tub1-724* mutant strain at 25°C (semi-permissive temperature), as well as the benomyl supersensitivity (Figure 3-5 A). The suppression activity is specific, since *CIN1* overexpression has no effect on the other α-tubulin alleles listed in Table 3-2. Furthermore, we find that excess Cin1p partially suppresses the deleterious consequences of overproduced Pac2p in *tub1-724* cells (Figure 3-5 B).

We show below that Cin1p is a β-tubulin binding protein. However, the suppression by excess Cin1p of *tub1-724* does not represent a β-tubulin sequestering activity, since excess Cin1p does not rescue either β-tubulin overexpressers or cells deleted for the minor α-tubulin, *tub3*.

The ability of excess Cin1p to prevent disruption of the mutant heterodimer by the α-tubulin binding protein Pac2p, may be due to a direct interaction between Pac2p and Cin1p that prevents the formation of a Pac2p-α-tubulin complex, or from the formation of another complex that overcomes Pac2p sequestration of α-tubulin. Biochemical experiments described below help to distinguish between these possibilities.

Figure 3-5. Overexpression of *CIN1* is able to rescue conditional phenotypes of the *tub1-724 mutant*. (A) Saturated cultures of *tub1-724* mutant cells containing either p*GAL-CIN1* or a YCpGAL control plasmid were serial diluted (one sixth dilutions for the 25°C plate, one fourth for the benomyl plate) and spotted to selective galactose plates containing 20 μg/ml benomyl and to selective galactose plates incubated at 25°C. (B) Saturated cultures of *tub1-724* strains containing two plasmids each: either p*GAL-CIN1* or YCpGAL control plasmid and either p*GAL-PAC2* or the pRS317 control plasmid were serial diluted (one fourth dilutions) and spotted to selective galactose plates.

A.

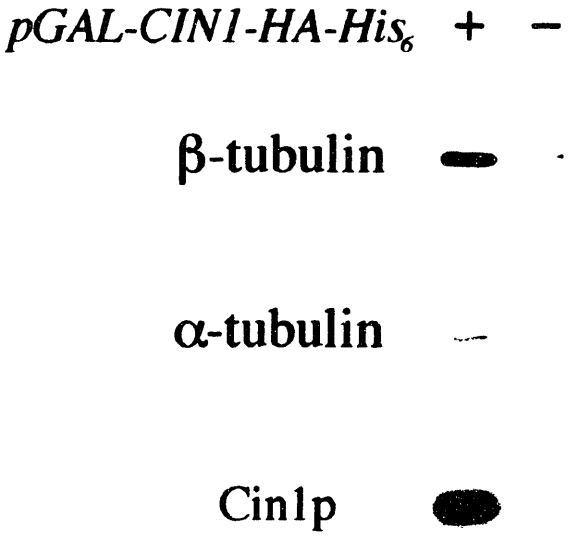


B.

Physical interactions of Cin1p

To assay for protein complexes between Cin1p and the tubulin polypeptides suggested by the *in vitro* work and the *in vivo* results above, we constructed a modified form of *CIN1* under control of the inducible *GAL* promoter, that contains both an HA epitope tag and a His_6-sequence at its carboxy terminus (pJF15). This allele of *CIN1* rescues the benomyl phenotype of *Δcin1* cells, and suppresses the *tub1-724* phenotypes like the unmodified *CIN1*. Extracts prepared from wild-type cells transformed with pJF15 or a *YCpGAL* control plasmid and grown for three hours in galactose were fractionated by nickel-agarose chromatography to purify the tagged Cin1p and proteins bound to it. The proteins were eluted and resolved by SDS-PAGE followed by immunoblotting. In five independent trials we found that β-tubulin specifically co-purifies with the tagged Cin1p. A representative blot is shown in Figure3-6. In contrast, there is no detectable enrichment of α-tubulin among the proteins eluted with Cin1p. Formation of the Cin1p-β-tubulin containing complex is independent of Pac2p (data not shown). This result suggests that Cin1p can bind directly or indirectly to β-tubulin but not α-tubulin *in vivo*, similar to Rbl2p. However, unlike Rbl2p, the Cin1p-β-tubulin interaction does not suppress the phenotypes of excess β-tubulin.

The *in vitro* assay of tubulin folding demonstrates a complex between the Cin1p homolog cofactor D and β-tubulin (Tian et al., 1997). Those experiments also infer a complex containing both Cin1p and Pac2p homologs along with both tubulin

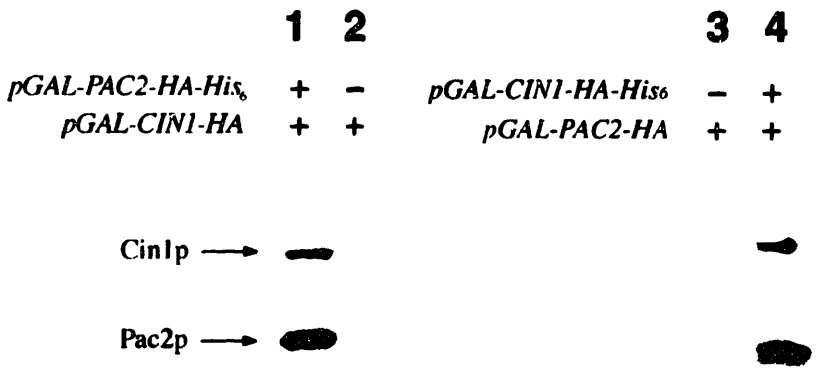

pGAL-CIN1-HA-His6 + −
β-tubulin
α-tubulin
Cin1p

polypeptides. To test for such a complex *in vivo*, we transformed cells with plasmids encoding various tagged forms of both *CIN1* and *PAC2* under control of the *GAL* promoter. For these experiments, we used either a *GAL-CIN1* construct that contains three HA epitopes in tandem at the extreme carboxy terminus of the open reading frame (pJF14) or *a Gal-CIN1-HA-HIS$_6$* construct. Both of these alleles are indistinguishable from wild type *CIN1* as assayed by overexpression in both *Δcin1* and *tub1-724* cells (data not shown). The *GAL-PAC2* construct contains three carboxy-terminal, tandem HA epitopes followed by a His$_6$ sequence (Vega et al., 1998) or a version of*GAL-PAC2 -HA* that lacks the His$_6$ tag. Figure 3-7 (A) shows that Cin1p and Pac2p co-purify from extracts of cells over-expressing both proteins.

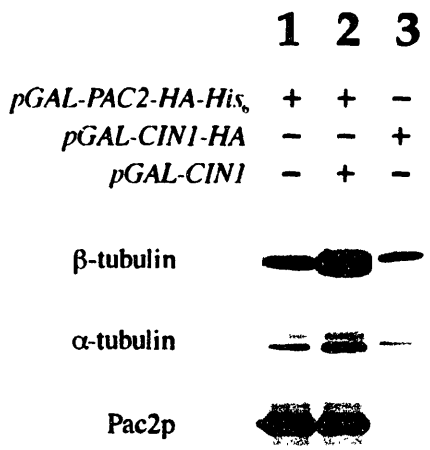
We find that small amounts of α and β-tubulin co-purify with the tagged Pac2p-HA-His$_6$ when it is overproduced; as shown in Figure 3-7 (B), the level of this complex increases when Cin1p is co-overexpressed in the same cells. The relative amount of co-purifying β-tubulin is significantly greater than the amount of co-purifying α-tubulin in both strains. The ability of overexpressed Pac2p-HA-His$_6$ to bind some β-tubulin is apparently due to an interaction with endogenous Cin1p; when Pac2p-HA-His$_6$ is overexpressed in *Δcin1* cells, neither tubulin polypeptide is associated with it. A representative blot from four independent trials is shown in Figure 3-7 (C).

Figure 3-7. Pac2p, Cin1p and tubulin. (A) FSY182 cells containing two plasmids as indicated: lane 1, p*GAL-PAC2-HA-His*$_6$ and p*GALCIN1-HA;* lane 2, pRS317 (control vector) and p*GAL-PAC2-HA-His*$_6$; lane 3, p*GAL-PAC2-HA* and YCpGAL (control vector); lane 4 p*GAL-CIN1-HA-His*$_6$ and p*GAL-PAC2-HA;* Cells were harvested after 4 hours of induction in galactose containing media. The tagged Pac2p or tagged Cin1p was purified and analyzed before. The results shown are representative blots from six independent trials where Cin1p co-purifies with His_6 tagged Pac2p (lanes 1, 2), and two independent trials showing that Pac2p co-purifies with His_6 tagged Cin1p (lanes 3, 4). (B) Enhanced binding of Pac2p to tubulin in the presence of excess Cin1p. FSY182 cells containing two plasmids as indicated: lane 1, p*GAL-PAC2-HA-His*$_6$ and YCpGALcontrol vector ; lane 2, p*GAL-PAC2-HA-His*$_6$ and p*GAL-CIN1;* lane 3, pRS317 control vector and p*GALCIN1-HA*; control were harvested after 4 hours of induction in galactose containing media. The tagged Pac2p was purified and analyzed as above. (C) Extracts from wild-type and *Δcin1* strains containing either p*GAL-PAC2-HA-His*$_6$ or YCpGAL control plasmid were obtained after four hours growth in inducing media. The tagged Pac2p and bound proteins were purified and analyzed as above.

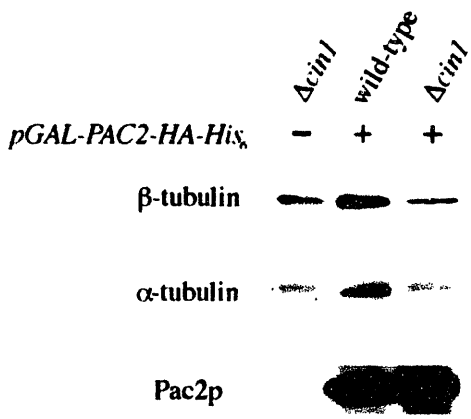
A.


1 2 3 4
pGAL-PAC2-HA-His6 + − pGAL-CIN1-HA-His6 − +
pGAL-CIN1-HA + + pGAL-PAC2-HA + +
Cin1p
Pac2p

B.


1 2 3
pGAL-PAC2-HA-His6 + + −
pGAL-CIN1-HA − − +
pGAL-CIN1 − + −
β-tubulin
α-tubulin
Pac2p

C.


Δcin1
wild-type
Δcin1
pGAL-PAC2-HA-His6 − + +
β-tubulin
α-tubulin
Pac2p

DISCUSSION

The data presented here identify genes that affect tubulin dimer formation *in vivo*. Mutations in these genes render cells sensitive to overexpressed Rbl2p, a β-tubulin binding protein that depleted tubulin dimers. Consistent with that finding, two of these mutants, *Δpac2* and *Δcin1*, are synthetically lethal with a mutant α-tubulin that destablizes heterodimers. Overexpression of Cin1p rescues that same mutant. Since Cin1p is not a stable ligand of the α-β tubulin heterodimer, the rescue is likely a consequence of Cin1p's ability to promote the formation of heterodimer rather than stabilize heterodimer. We also show that Pac2p and Cin1p interact with one another, in complexes that can contain the tubulin polypeptides. Taken together, the results demonstrate catalyzed tubulin heterodimerization *in vivo*. These findings demonstrate the extent to which activities of an *in vitro* tubulin folding assay described by Cowan and colleagues pertain to the *in vivo* situation. That these activities are not essential for tubulin assembly or function suggests that they may have other roles in cell physiology.

Consequences of Rbl2p overproduction in *cin1* and *pac2* nulls

Rbl2p over-expression in wild type cells confers only moderate phenotypes. However, induction of *GAL-RBL2* in *tub1-724* cells rapidly causes microtubule disassembly and cell death (Chapter Two and Archer et al., 1995). By several criteria, the Tub1-724p α-tubulin binds less tightly to β-tubulin than does wild type α-tubulin (Chapter Two and Vega et al., 1998) . That property allows excess Rbl2p to bind more

β-tubulin in the mutant than in wild type cells, leading to depletion of the heterodimer, loss of assembled microtubules and loss of viability.

These properties of *tub1-724* cells are shared by *Δcin1* and *Δpac2* cells. Induction of *RBL2* overexpression causes loss of microtubule structures and cell death in both strains. Strikingly, the Rbl2p-β-tubulin complex forms much more readily in these mutant strains than in wild type cells. These results suggest that the tubulin heterodimer in *Δcin1* and *Δpac2* cells is also destabilized relative to wild type. However, the underlying mechanism of this destabilization must differ from that of *tub1-724* cells, since neither Cin1p or Pac2p are stably associated with the bulk of tubulin heterodimers and therefore would not be expected to stabilize the heterodimer directly. Instead, it is more likely that these two proteins participate in reactions leading to heterodimer formation, and that their absence makes those reactions less favorable or less efficient. As a result, *Δcin1* and *Δpac2* cells might have lower levels of heterodimer and higher levels of free tubulin polypeptide chains. An excess of a β-tubulin binding protein could further deplete the pool of heterodimer. Alternatively, these mutants may have a diminished ability to convert Rbl2p-β-tubulin into heterodimer, an exchange reaction which occurs both *in vivo* and *in vitro* (Archer et al., 1998; Archer et al., 1995).

Effects of Cin1p and Pac2p levels in tubulin mutants

Null alleles of *cin1*, *pac2* and *rbl2* are lethal in combination with the same four mutant α-tubulins, suggesting that they affect related functions. Each of the relevant

tub1 mutations falls into class 1 - they are cold-sensitive and lose microtubules at the restrictive temperature (Schatz et al., 1988). However, not all class 1 mutants are lethal in combination with Δ*cin1*, Δ*pac2* or Δ*rbl2*. We have characterized the molecular defect in one of these mutations - *tub1-724* - as a weakened heterodimer. We rationalized the inability of *tub1-724* cells to live without Rbl2p as a consequence of excess β-tubulin released by dissociation of the weaker heterodimer combined with loss of the ability of Rbl2p to sequester excess β-tubulin. The results suggest that this subclass has a common defect exacerbated by mutations that affect heterodimer formation.

Similar to Rbl2p, the absence of Cin1p and Pac2p makes cells more sensitive to β-tubulin over-expression. However, it is unlikely that Cin1p and Pac2p act in exactly the same way as Rbl2p - by binding and sequestering β-tubulin. In particular, Rbl2p overproduction but not Cin1p overproduction suppresses the phenotypes associated with genetic conditions that produce an excess of β-tubulin - either *GAL-TUB2* or *Δtub3* strains (Archer et al., 1995; Hoyt et al., 1997). Conversely, overproduction of Cin1p suppresses the phenotypes of *tub1-724* cells, while overproduction of Rbl2p kills that mutant.

The conditional phenotypes of the *tub1-724* strains are due to the release of free β-tubulin by dissociation of the unstable mutant heterodimer (Chapter Two and Vega et al., 1998) . Why does excess Cin1p rescue these phenotypes but not the phenotypes associated with excess β-tubulin in *Δtub3* or β-tubulin over-producing

strains? A significant difference among these situations is that the *tub1-724* mutant contains a pool of undimerized α-tubulin, while the *TUB2* over-expressing strain and the *Δtub3* strain do not. This analysis suggests that Cin1p acts to promote the formation of the heterodimer.

Binding partners of Cin1p and Pac2p

Fractionation experiments performed using Cin1p and Pac2p have allowed us to characterize the complexes these proteins form *in vivo*. Here, we show that β-tubulin can co-purify with Cin1p in wild-type cells when Cin1p is overexpressed. That complex is detected in the presence or absence of Pac2p. In addition, we show that Cin1p is able to copurify with Pac2p when both proteins are overexpressed. The association of Cin1p with Pac2p also includes both α– and β- tubulin. Interestingly the amount of β-tubulin that copurifies is greater than the amount of α-tubulin. This may imply that there is a complex containing only Pac2p, Cin1p, and β-tubulin. Alternatively the α-tubulin present in this complex *in vivo* may be easily lost during purification.

We reported that over-expressed Pac2p binds α-tubulin but not β-tubulin when either tubulin is co-overexpressed (Vega et al., 1998) . We now find, using a substantially more sensitive assay, that both β-tubulin and α-tubulin are associated with Pac2p when it is over-expressed in wild type cells. The binding of both tubulin polypeptides to Pac2p is dependent on Cin1p in this assay. However, we do detect a diminished level of Pac2p-α-tubulin complex in *cin1* nulls co-overexpressing Pac2p and α-tubulin (L. Vega unpublished results). Therefore, it appears the Pac2p-α-

tubulin complex can form in the absence of Cin1p, but the binding is enhanced by Cin1p. Taken together, the results are consistent with interactions between Pac2p and both α-tubulin and Cin1p, which in turn can bind β-tubulin.

Tubulin assembly pathways, *in vivo* and *in vitro*

A series of *in vitro* experiments by Cowan and colleagues identified factors in addition to chaperones required for incorporation of β-tubulin and α-tubulin into exogenous heterodimer (Gao et al., 1992; Gao et al., 1993; Tian et al., 1996; Tian et al., 1997). In that assay system, β-tubulin released from the chaperone is bound independently by cofactors A or D, and α-tubulin is bound by either cofactor B or E. The β-tubulin released from cofactor A and the α-tubulin released by cofactor B fail to exchange into exogenous dimer directly. Instead, the pathway to heterodimer requires the α- and β-tubulin monomers to bind cofactors D and E, respectively. Cofactors D bound to β-tubulin and cofactor E bound to α-tubulin form a quaternary complex and finally, cofactor C mediates the release of the α–β tubulin heterodimer.

Four of these mammalian cofactors are homologous to yeast genes: Cofactor D shows 21% identity with Cin1p (Hoyt et al., 1997); cofactor E is 30% identical to Pac2p (Hoyt et al., 1997). Cofactor A is structurally and functionally homologous to Rbl2p (Archer et al., 1995), and cofactor B is 32% identical to Alf1p (Tian et al., 1997). In the *in vitro* assay, cofactors D and E are essential. However, none of the homologous yeast genes are essential, even in various combinations (Hoyt et al., 1997;

unpublished results). There may be redundant functions in yeast specified by genes as yet undetected, or the *in vivo* tubulin folding could follow an different pathway.

Some of the functional interactions we detect *in vivo* among these proteins are also consistent with the *in vitro* model. For example, the ability of excess Rbl2p to kill *Δcin1* or *Δpac2* cells is readily explained by the *in vitro* pathway (detailed above). Also, the ability of excess cofactor E (with cofactor B) to sequester α-tubulin from preexisting heterodimer (Tian et al., 1997) reflects the ability of excess Pac2p to kill *tub1-724 cells* (Chapter Two and Vega et al., 1998).

However, other results demonstrate differences between the *in vivo* and *in vitro* situations. Most important, suppression by excess Cin1p of a mutant α-tubulin with lowered heterodimer stability is not consistent with the *in vitro* model. The data suggest that Cin1p *in vivo* acts differently than cofactor D does *in vitro*. The ability of Cin1p to suppress the *tub1-724* mutant contrasts with the *in vitro* data that show cofactor D can interact with and disrupt the heterodimer forming a cofactor D-β-tubulin complex. Other evidence indicates that Cin1p has an activity that does not require stoichiometric Pac2p. First, overexpression of Cin1p alone is sufficient to suppress *tub1-724*. Second, overexpression of Cin1p is able to rescue the benomyl supersensitive phenotype of *Δpac2* strains (Hoyt et al., 1997). Therefore, it appears Cin1p does more than bring β-tubulin into a quaternary complex containing Pac2p and α-tubulin. The suppression mechanism in *tub1-724* cells may involve Cin1p presenting β-tubulin to α-tubulin and thus keeping the free β-tubulin from acting as a

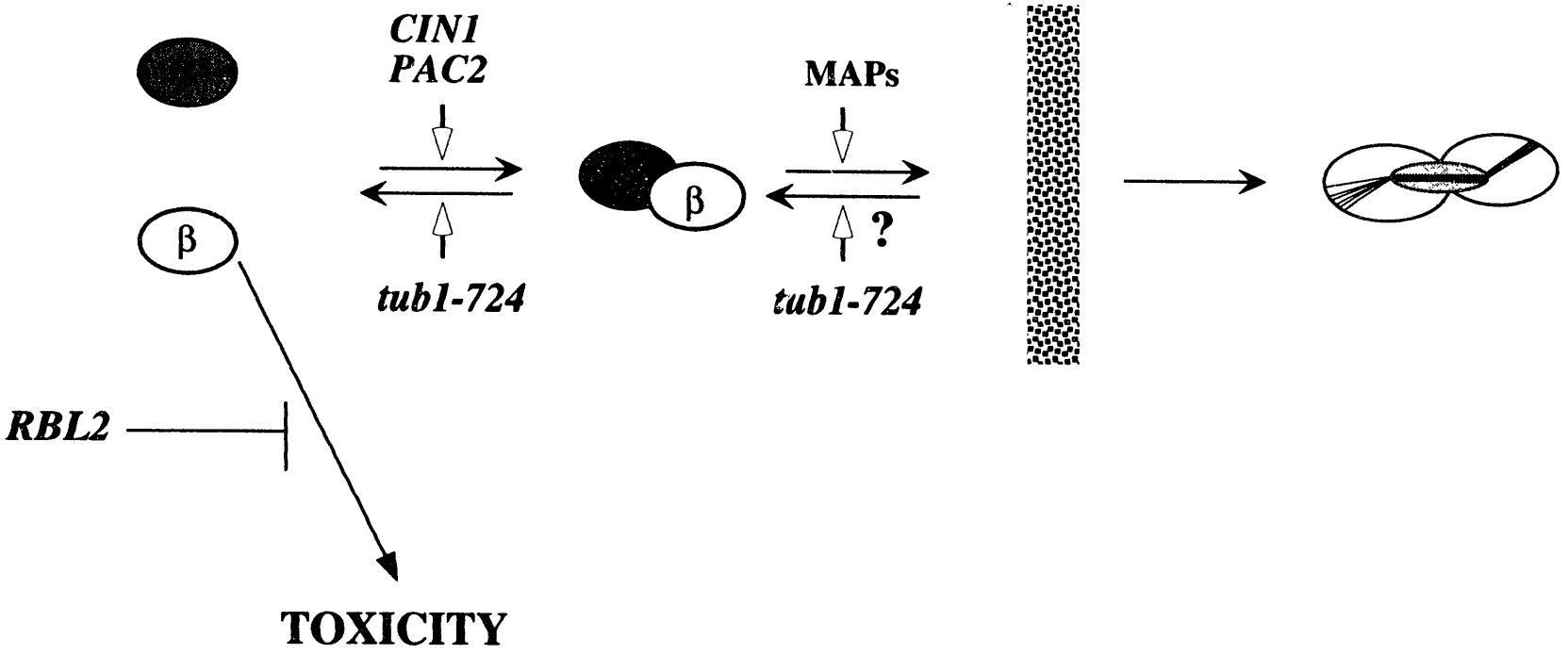
poison in the cell. Perhaps Cin1p provides the function of cofactor C in the *in vitro* system, given that no *S. cerevisiae* homolog of that gene exists.

Many but not all of the protein complexes we identified *in vivo* are predicted by the *in vitro* tubulin folding assay (Tian et al., 1996; Tian et al., 1997). The Cin1p β-tubulin complex demonstrated here, the Rbl2p-β-tubulin complex (Archer et al., 1995), and the Pac2p α-tubulin complex (Vega et al., 1998) , are all detected *in vitro*. However, our results demonstrate that the binding of α-tubulin to Pac2p is at least partially dependent on Cin1p *in vivo*. This may explain why the α-tubulin - cofactor E (Pac2p homolog) complex *in vitro* is detectable only after chemical crosslinking (Tian et al., 1997).

There are further differences with respect to the protein-protein complexes detected. The *in vitro* data suggest that β-tubulin can exchange directly between cofactor A (Rbl2p) and cofactor D (Cin1p). However, the very different activities of these β-tubulin binding proteins *in vivo* with respect to excess β-tubulin suggests that they either bind different forms of the protein or that the resulting complexes have different activities. We have also shown that Rbl2p can bind to β-tubulin both before and after it binds to α-tubulin (Archer et al., 1998); the second finding is inconsistent with the *in vitro* model. Finally, the *in vitro* data suggest that α-tubulin binds to cofactor B (Alf1p) or cofactor E (Pac2p) (Tian et al., 1997). However, unlike overexpressed Pac2p, we have found that excess Alf1p is not lethal when overexpressed in *tub1-724* cells (data not shown).

Figure 3-8. Genes that affect tubulin dimer formation *in vivo.* The *tub1-724* mutation affects the stability of the heterodimer directly and render cells sensitive to overexpressed Rbl2p. The properties of *tub1-724* cells are shared by *Δcin1* and *Δpac2* cells. Since neither Cin1p nor Pac2p is associated with the bulk of tubulin heterodimers, Cin1p's ability to rescue *tub1-724* mutant cells is likely a consequence of Cin1p's ability to promote the formation of heterodimer rather than stabilizing heterodimer directly. Pac2p and Cin1p interact with one another in complexes that can contain the tubulin polypeptides. Taken together, *in vivo* Cin1p and Pac2p work together to promote tubulin heterodimerization.

Managing Tubulin Polypeptides *in vivo*



Conclusion

The experiments presented here demonstrate that proteins which interact with individual tubulin polypeptides can influence the formation of heterodimer *in vivo*. Especially in the case of Cin1p, the relationship between protein activity and expression levels suggests that this protein acts catalytically to promote α-β-tubulin complex formation. Such an activity will require coupling to a highly exergonic step, in order to make the reaction act as if it were unidirectional. A candidate for that coupling factor is Cin4p, which has a predicted GTP binding motif (Hoyt et al., 1997) and which likely acts either in the same pathway or in a complex with Cin1p.

The *in vivo* data also demonstrate differences and similarities between the activities of tubulin interacting proteins *in vivo* and the activities of their vertebrate homologs *in vitro*. Most striking is the fact that the proteins are not essential *in vivo*. Screens for genes involved in a parallel pathway redundant with the one defined by these proteins have not been successful, raising the possiblity that the primary and sufficient tubulin heterodimerization pathway is uncatalyzed. In that circumstance, the activities described here may become important only under special conditions - for example, in mediating fluctuations in the pool of unassembled tubulin heterodimer and its dissociation products.

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CHAPTER FOUR:

Identification and characterization of overexpressed cDNAs that confer benomyl resistance

INTRODUCTION

Yeast microtubules are sensitive to microtubule depolymerizing drugs, such as benomyl and nocodazole. The consequence of drug treatment is the failure of microtubule mediated processes such as nuclear division, nuclear migration and nuclear fusion (Delgado and Conde, 1984; Jacobs et al., 1988). Thus, altered sensitivity to benomyl is a phenotype associated with impaired microtubule function in *S. cerevisiae* and is often used as evidence that a gene product is involved in a microtubule related process. A number of mutations defining several complementation groups are known to be supersensitive to benomyl (Ben^S). Both α-tubulin genes, *TUB1* and *TUB3,* as well as the β-tubulin gene, *TUB2*, can be mutated to confer a Ben^S phenotype (Reijo et al., 1994; Schatz et al., 1988; Stearns and Botstein, 1988).

In addition to the tubulin encoding genes, other components of the mitotic apparatus may be mutated to benomyl hypersensitivity. But only a few genes, other than the tubulin genes, have been identified that confer supersensitivity to very low concentrations of benomyl (10 ug/ml) a concentration of benomyl that has little effect in wild-type cells. Extreme sensitivity to benomyl seems to be correlated with mutations in genes that affect tubulin assembly. These genes include *CIN1, 2* and *4* which were identified in a genetic screen for benomyl supersensitive mutants (Stearns et al., 1990). These three genes were also identified in an independent screen for chromosome instability mutants (Hoyt et al., 1990). In addition, mutants in *pac2* are super-sensitive to very low

concentrations of benomyl and have defects in microtubule functions (Hoyt et al., 1997). *PAC2* is required in cells deleted for *cin8*, which encodes a kinesin-related protein that participates in anaphase (Geiser et al., 1997).

Other benomyl hypersensitive mutants include genes whose products have been implicated in a mitotic checkpoint control which monitors spindle formation and requires that microtubules be intact for mitosis to proceed. The *MAD* (mitotic arrest defective) genes and the *BUB* (budding uninhibited by benomyl) genes were isolated as mutants that fail to arrest when challenged with high concentrations of benomyl (Hoyt et al., 1991; Li and Murray, 1991). Indeed mutations in almost every aspect of microtubule assembly have been identified that confer a Ben^S phenotype.

In contrast, mutations in *S. cerevisiae* that confer resistance to benomyl (Ben^R) are rare. Most of them map to *TUB2;* the gene encoding β-tubulin (Reijo et al., 1994; Thomas et al., 1985). In addition to mutations in β-tubulin, *CDP1* nulls which require centromere binding factor I (Cbf1p) for viability also show increased benomyl resistance. *cdp1* cells display unusually long cytoplasmic microtubules and show increased frequencies of chromosome loss (Foreman and Davis, 1996). Alleles of*SAC3*, isolated as a suppressor of *act1-1*, are also benomyl resistant (Bauer and Kolling, 1996).

We found that overexpression of either α-tubulin or Rbl2p in wild-type cells confers a reproducible, benomyl resistant phenotype (Archer et al., 1995; Schatz et al., 1986). α-tubulin is a component of the tubulin heterodimer and

RBL2 is a β-tubulin monomer binding protein *in vivo* (Archer et al., 1995). α-tubulin and Rbl2p share another feature: overexpression of either gene rescues the effects of β-tubulin overexpression, microtubule depolymerization and cell death (Archer et al., 1995; Weinstein and Solomon, 1990). We reasoned that we might identify other β-tubulin binding proteins by screening for gene products whose overexpression confers increased resistance to benomyl and nocodazole but not to unrelated compounds. Alternatively, overexpressed genes that confer increased resistance to benomyl might be general stabilizers of microtubules that can overcome the destabilizing effects of benomyl and excess β-tubulin. We designed a genetic screen in wild type cells for galactose-induced cDNAs that confer resistance to benomyl.

MATERIALS AND METHODS

Strains, Plasmids, and Media

All yeast strains are derivatives of FSY185 (Weinstein and Solomon, 1990). We used standard methods (Sherman, *et al.*, 1986; Solomon, *et al.*, 1992). We used a yeast cDNA (CEN) library from pool 10A (Liu et al., 1992). For pLV6, a 3.7 kb fragment containing the entire *ADE3* gene flanked by Not I sites was generated by P.C.R. using the primers 5' primer -(D41524) 5'ATAAGATAGCGGCCGCTAAAGGATCCGGAGTACTTACGTGAGC and 3' primer-(D41484)-5'AAGGAAAAGCGGCCGGTGGTTATAGATTTGGATACTTG and ligated into the SK- Bluescript vector. pLV8 and pLV10 contain the Not I ADE3 fragment inserted into the Not I site of pA5 or p4C respectively. pLV127 was constructed by P.C.R. of the 3' end of pBRO5 (pGAL-LBO1) using the 5' primer -(D50266) 5'AAATCCTTAAGGAGGTACCCATACGACGTCCCAGACTACGCTTAGACGAA CTATTTGAAACC which included a single HA epitope and the 3' primer (D50267) 5'TAGACGAACTCTTTGAAACCAATT. The P.C.R. product digested with Afl II-Not I and cloned into the Afl II-Not I site of pBRO5. pET15 containing pGAL-LBO2 with a triple HA epitope tag, was constructed by using P.C.R. The 5' primer, 5'-AAAATTGTTGAGATTGATAATCCCAGCATTTTGGGTGATTTCACAAGGGAAGA TCGCGCGCGCTGACCGGCTATATCAATGCACCTAAAT introduced a Not I site at the 3'- end of *LBO2* coding sequence and the 3' primer (D52016) 5'-

GAATTGGAGCGCCACCGCGGTGGCGACCGCCC was to the vector but deleted a Not I site on the pRS316-GAL1 vector. The 111 bp triple HA epitope cassette from B2385 (Fink Lab) was then cloned into the Not I site. pLV21 was constructed by subcloning a 2.3 kb EcoR I fragment containing the entire LBO2 genomic sequence from pLV17 into the EcoR I site of the pCT3 vector. pLV15 contains the 5' and 3' flanking region flanking the *LBO2* ORF. The 5' flanking sequence of *LBO2* was amplified by P.C.R. of genomic DNA using the 5' primer (D50983)-KOBPAAT 5' AGCGTGACGTCCAGGAACATTGGTATTCTTATCATGTTGAG and the 3' primer (D50981) 5'-GAAGATCTTTCTTATTCTTGTTAACTTCGTCCT and cloned into the AatII-Bgl II site of pNK51; the resulting plasmid was digested with Sal I-BamHI and 3' sequence of *LBO2* ,amplified using 5' primer (D50982) 5'-CGGGATCCGACCGGCTATATCAATGCACCTAAATTCAGAACA and the 3' primer 5'-ACGCGTCGACAGTCAGACACCTATCTAACTTTCATTAAATTATC was inserted into the Sal I-BamH I. A similar approach was used to construct *LBO1* disruption construct, pLV19. The 5' flanking region of *LBO1* was amplified by P.C.R. using the primers (D500265) 5'-AAGATGACGTCGATCTTTCTCATTCCTTCATTTAAAGTTCG and (D50626) 5'-GAAGATCTTGTAGTTATAATGGAAGGAGGGGGTG and inserted into the Aat II-Bgl II site of pNK51 to make pLV14. The 3' flanking region of *LBO1* was amplified by P.C. R. amplified using 5'CGCGGATCCACGAACTATTTGAAACCAATTTTTTTCAATATAGATTTTGGC and

ACGCGTCGACCCGCCATAAACTTGCAAAGCAGCATTGGACATAGTTATAC and cloned into the BamH I -Sal I site of pLV14 to make pLV19.

pLV25(GAL- YDR066C) containing the *LBO2* homologue under the control of the galactose promoter was constructed by P.C.R. using 5' primer 5'ACGCGTCGACGGAGGCATTCTGGAAAAATTGCAGC3' the 3' primer was 5'ACATCGGCCGTACGCCAAACGGTTGACACTATTCAG3'. The P.C.R. product was digested with Sal I-Eag I and ligated into the Sal I-Eag I of pRS316-GAL1.

pLV28, disrupts the entire coding sequence of YDR066C. A 430 bp of 5' YDR066C flanking sequence with an additional Xba I site added was amplified by P.C.R. using the 5' (D61131) primer- 5' ACCGTGCGATATATTGCAAGGACAGCC and the 3' (D61130) primer- 5'TGCTCTAGATATGGCTTGCGTTGCTCCTGTCC and 658 bp of 3' YDR066C flanking sequence with Xma I and Sac I sites added was amplified by using the primers 5' (D61129) primer 5'TCTCCCCGGGTAGGTAGTATAATTAAATCGTCTTCATCAGG and the 3' primer (D61128) primer-5' TCAAAGAGCTCTATGGTTTGTTCGGTCAATTAACAAGGGC and by P.C.R.

pLV29 containing the YDR066C 5' and 3' UTR around *LEU2* in JH-L2 was constructed by cloning the 5' UTR P.C.R. product of YDR066C digested with HinDIII and Xba I and the 3' UTR P.C.R. product digested with SacI and XmaI into JH-L2.

Sectoring Assay

We transformed *ade2,ade3* yeast with either a *CEN-URA3-ADE3-GAL1-RBL2* plasmid or with a *CEN-URA3-ADE3-GAL* control plasmid and plated cells to low adenine glucose plates, low adenine galactose plated and to low adenine galactose plates containing 30 ug/ml benomyl at 30°C. Growth was monitored by visual inspection.

Screen

Wild-type diploid cells containing were transformed with a *URA3* marked cDNA library (Liu et al., 1992). We transformed pool 10A into FSY185 and obtained (X). We grew the transformants in selective glucose media to saturation and plated approximately 4.0 X 10^4 transformants onto SC galactose plates containing 26, 30, and 35 ug/ml. We made a slurry of galactose survivors and re-plated these cells onto SC -ura glucose. We tested 534 benomyl survivors for plasmid dependence by selecting for loss of the *URA3* plasmid on 5-FOA, then checking for loss of benomyl resistance. After isolation of the library plasmid, we identified plasmids containing *TUB1, TUB3* or *RBL2* by a combination of restriction digests, colony hybridization, and DNA sequencing. The isolated plasmids were re-transformed into FSY185 and checked for their ability to confer survival on galactose benomyl plates.

Quantitation of LBO Phenotype

Diploid cells containing galactose promoted *RBL2*, *TUB1*, *LBO1*, *LBO2* or *YCpGAL* were plated to, galactose plates and to galactose plates containing various concentrations of benomyl 0-50 ug/ml. We determined the ratio of the number of colonies that grew on the various galactose benomyl plates relative

to the galactose plate without benomyl. Alternatively diploid strains containing the indicated plasmid were grown to saturation overnight in SC -ura glucose liquid media. The cultures were serial diluted in 96 well dishes, and spotted onto SC -ura galactose plates containing various concentrations of benomyl and to SC -ura glucose plates incubated at 30°C.

Construction of KO constructs

The 5' flanking sequence of *LBO2* was amplified by P.C.R. of genomic DNA using the 5' primer (D50983) and the 3' primer (D50981) and cloned into the AatII-Bgl II site of pNK51; the resulting plasmid was digested with Sal I-BamHI. The 3' sequence of *LBO2* ,amplified using 5' primer (D50982) and the 3' primer 5'-ACGCGTCGACAGTCAGACACCTATCTAACTTTCATTAAATTATC was inserted into the Sal I-BamH I site to generate pLV15. pLV15 was digested with Pvu II and Sal I to release was the disruption fragment and the fragment was isolated (Quiax II from Quiagen) after electrophoresis on a 1% agarose gel. This DNA was then transformed into FSY185 to create a disruption of the entire *LBO2* open reading frame. The disruption was confirmed by Southern blot analysis of the diploids and of their haploid segregants, and by the phenotypic analysis of the haploid segregants. A similar approach was used to construct *LBO1* disruption construct, pLV19. The *LBO1* 5' (600 bp) and 3' (300 bp) UTR was amplified using the polymerase chain reaction and inserted around the Bgl II-BamHI site of *hisG-Ura3- hisG* of pNK51 using the primers described above. The 4.5 kb *LBO1* disruption fragment was released by digesting pLV19 with BstXI and Sal I. The purified knockout fragment was used to transform FSY185

and transformants were selected on sc -ura. As before, putative heterozygotes and haploid segregants were confirmed by Southern blot analysis. To knock out the YDR066C coding region we digested pLV28 containing 5' and 3' UTR flanking YDR066C around the *LEU2* gene in JH-L2 with ApaI and SacI to release the knockout fragment. We purified this fragment as before and used it to transform FSY185. The disruption was confirmed by Southern Blot analysis of the diploids and of their haploid segregants.

Viability measurements and Immunofluorescence

Cell containing the indicated plasmids were grown overnight in SC -ura raffinose media. Log phase cells were then induced with 2% galactose and at various time points aliquots of cells were taken and counted using a hemacytometer. Known numbers of cells were then plated to SC -ura glucose plates. Cell viability was measured as the percent of cells counted able to form colonies on the SC -ura glucose plates. At time points indicated cells were fixed for immunofluorescence in 3.7% formaldehyde. Anti β-tubulin staining was done with #206 (Bond *et al.*, 1986) at 1/2000 in phosphate buffered saline containing 0.1% bovine serum albumin.

To measure the effect of high temperature on *Δlbo2* , cells containing the indicated plasmids were grown overnight in SC -ura glucose media at 30°C. At the T=0 the cells were shifted to 37°C or were maintained at 30°C. At various time points aliquots of cells were taken and counted using a hemacytometer. Known numbers of cells were then plated to SC -ura glucose plates. Cell viability was measured as the percent of cells counted able to form colonies on

the SC -ura glucose plates at 30°C. For some experiments, cells were fixed for immunofluorescence in 3.7% formaldehyde and stained with anti β-tubulin antibodies as described above. For other experiments the bud size distribution of fixed cells was determined for the indicated time points. The size of the bud was determined as unbudded cells, small/medium budded cells (the size of the bud was less than the size of the mother), large budded cells (the cells were close to or equal the size of the mother.

FACS Analysis

Cells were grown under the desired conditions to early log phase. For each analysis, between 5 x10^6 to 1 x10^7 cells were used. Cells were pelleted in IEC (1000 rpm). Cells were fixed in 66.5% EtOH at 4°C overnight. Fixed cells were pelleted as before washed in 5 mls of 50 mM NaCitrate (pH7.4). After washing, the cells were pelleted again and resuspended in 1 ml of 50 mM NaCitrate (pH7.4) plus 0.25 mg/Ml RNase A and incubated for one hour at 50°C. Proteinase K is added to a final concentration of 1 mg/ml and the cell are incubated for an additional hour at 50°C. Finally, 1 ml of 50 mM NaCitrate (pH7.4) containing 16 ug/ml of propidium iodide is added and the cells are incubated overnight at 4°C wrapped in tin foil. The samples were sorted by FACS analysis.

Immune techniques

Immunoblots: We used standard procedures (Solomon, *et al.*, 1992). For ^{125}I detection we blocked blots for 15-30 min. in TNT, 0.05% Tween. Primary antibodies were incubated overnight in TNT, 0.05% Tween at 1/3500 (#206 or

#345) or at 1/1000 (12CA5) (Babco) and then washed 5 (5 min. each) in TNT, 0.05% Tween. Bound antibody was detected by ^{125}I Sheep Anti-mouse Ig (SAM) (NEN).

Immunoprecipitations - Antibodies 206 or 345 were affixed to Affigel-10 beads (BioRad). Yeast strains grown up at 30°C. Total protein was harvested by glass bead lysis in PME (0.1M Pipes, 2mM EGTA, 1 mM magnesium chloride, pH 6.9) plus protease inhibitors (Solomon, *et al.*, 1992) and added to antibody beads for a one hour incubation with rotation at 4°C. We washed the beads eight times with PME + protease inhibitors. Bound proteins were eluted by boiling is SDS sample buffer and resolved by SDS-PAGE analysis. Protein A sepharose beads were used to immunoprecipitate HA tagged proteins. To prepare beads, protein A beads were blocked in 10% BSA, 0.05 M Tris pH 8.0, 0.1% NaN_3 for 1 hour at 4°C then washed 7X with PME + protease inhibitors. For 12CA5, 40 ul of antibody was added to 200 ul protein extract prepared as above. After one hour 300 ul of the protein A in PME was added to the 12A5 containing extract and incubated for an additional hour with rotation at 4°C. The Beads were then washed as above and bound proteins were eluted by boiling is SDS sample buffer and resolved by SDS-PAGE analysis.

RESULTS

Plasmid Segregation and Benomyl Resistance

We tried to identify cDNAs that confer a Ben^R phenotype directly by picking colonies that grew on galactose benomyl containing plates. However, the background was very high, many of the colonies that arose were not reproducibly benomyl resistant. The reason for the high background remains unclear. To make the screen feasible, we required improved methods for detecting the Ben^R colonies from the background colonies.

Based on previous work from others, we expected that cells resistant to the effects of benomyl would segregate a *CEN* plasmid more faithfully than the background colonies that are able to grow on benomyl. In *S. cerevisiae* the frequency of chromosome loss is about 1 in 10^5 per cell division (Guthrie and Fink, 1991). For *CEN* plasmids, the frequency is higher--1% plasmid loss per generation (Guthrie and Fink, 1991). However, in the presence of benomyl the frequency of chromosome loss and plasmid loss increases (Jacobs et al., 1988).

To test whether overexpression of Rbl2p could reduce benomyl induced plasmid loss, we used the *ADE2*, *ADE3* sectoring assay in *S. cerevisiae*. *ade2*, *ade3* mutant cells are white. If *ADE3* is present in an *ade2* mutant background, the cells accumulate a pigmented metabolite, and are red. Loss of the *ADE3* plasmid gives rise to a white sector on the red colony background (Hieter et al., 1985; Elledge and Davis; 1988).

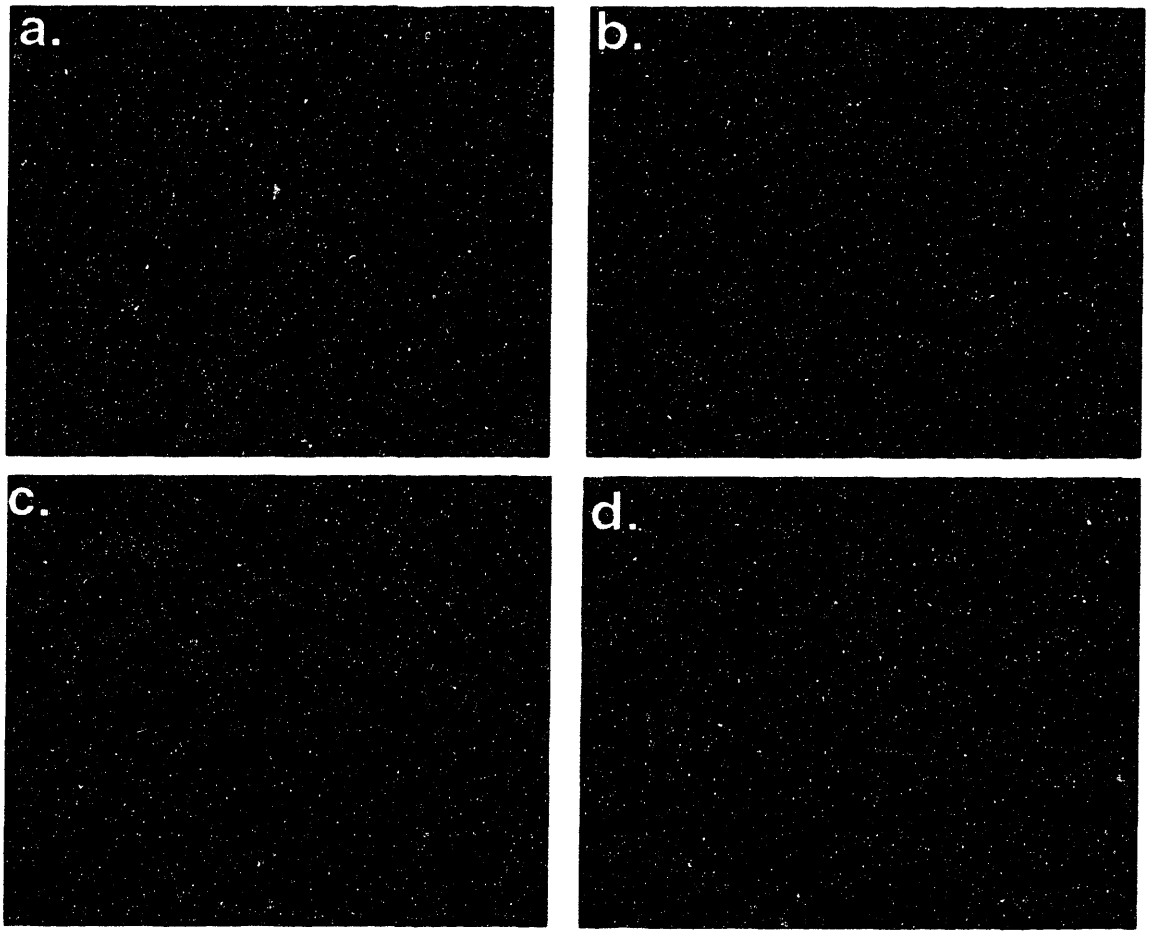
We transformed *ade2,ade3* yeast with either a *CEN-URA3-ADE3-GAL1-RBL2* plasmid (figure 4-1 C, D) or with a *CEN-URA3-ADE3-GAL1* control plasmid (figure 4-1 A, B) and plated cells to low adenine glucose plates (A, C) and to low adenine galactose plates containing 30 ug/ml benomyl (B, D). As shown in figure 4.1 cells containing a *CEN-URA3-ADE3-GAL* control plasmid lost the plasmid early in colony growth: most of the colonies on the benomyl containing plates are white (B). In contrast, the *CEN-URA3-ADE3-GAL1-RBL2* plasmid was selectively maintained when cells were plated to galactose-benomyl plates (D). This increased rate of plasmid loss was benomyl dependent. At lower concentrations of benomyl, the plasmid was maintained in both the experimental and the control (data not shown). These experiments demonstrate that, despite the high background, it is possible to identify over-expressed genes that confer benomyl resistance.

Revised screen

There is no *ADE3* -marked yeast cDNA library available that would enable us to have a color assay for plasmid maintenance in the presence of benomyl; however, we can use any library marked with a selectable marker to identify benomyl resistant colonies that maintained the library plasmid by plating the benomyl survivors to selective plates.

We transformed the pRS316-GAL1 cDNA library (Liu et al., 1992) into wild-type cells. Transformants were selected in liquid media containing glucose as the carbon source but lacking uracil. To select and enrich for galactose induced cDNAs that conferred benomyl resistance, we plated the transformants

Figure 4-1. Overexpression of Rbl2p increases plasmid maintenance on benomyl plates. *ade2, ade3* cells containing a *CEN-URA3-ADE3-GAL* control plasmid (A, B) or *CEN-URA3-ADE3-GAL-RBL2* (C, D) were plated to low adenine glucose plates (A, C) and to low adenine galactose plates containing 30 ug/ml benomyl (B, D) and incubated at 30°C. Colonies were visually inspected for the ability to sector.


a.
b.
c.
d.

onto plates containing galactose, benomyl and uracil. The cells were allowed to form colonies. From our previous experiment, we knew that the colonies that grew up at this point could be background survivors as well as *bona fide* Ben^R survivors. We expected colonies containing galactose regulated cDNAs that confer Ben^R to maintain the *URA3* library plasmid.

We scraped the survivors off of the benomyl plates and we plated a slurry of these cells to ura- plates to select for the library plasmid. This would enable us to distinguish between background growth that had lost the plasmid and Ben^R colonies that maintained the library plasmid. We picked Ura+ colonies from individual plates to characterize further.

To determine if any of the Ura+ cells contained library plasmids that confer a plasmid dependent Ben^R phenotype, we picked individual colonies and restreaked these cells onto plates containing uracil to allow the *URA3* plasmid to be lost. Then we selected for absence of the *URA3* plasmid by growing the cells on 5-FOA plates. We compared colonies derived from the same original benomyl survivor with and without the *URA3* containing plasmid on benomyl plates by serial dilution. Yeast cells requiring the *CEN* library plasmid to grow on galactose-benomyl plates were identified. A summary of the results of this screen are shown in Table 4-1.

Table 4-1 LBO Screen Results

Number of Cells Plated to Galactose Benomyl	4.0×10^4
Number of Colonies Tested for Ben^R	534
Number of Putative Plasmid Dependent Ben^R	18
Number of Plasmids that Retranformed	5

cDNA indentity	Number of isolates
TUB1	1
RBL2	1
LBO1	1
LBO2	2

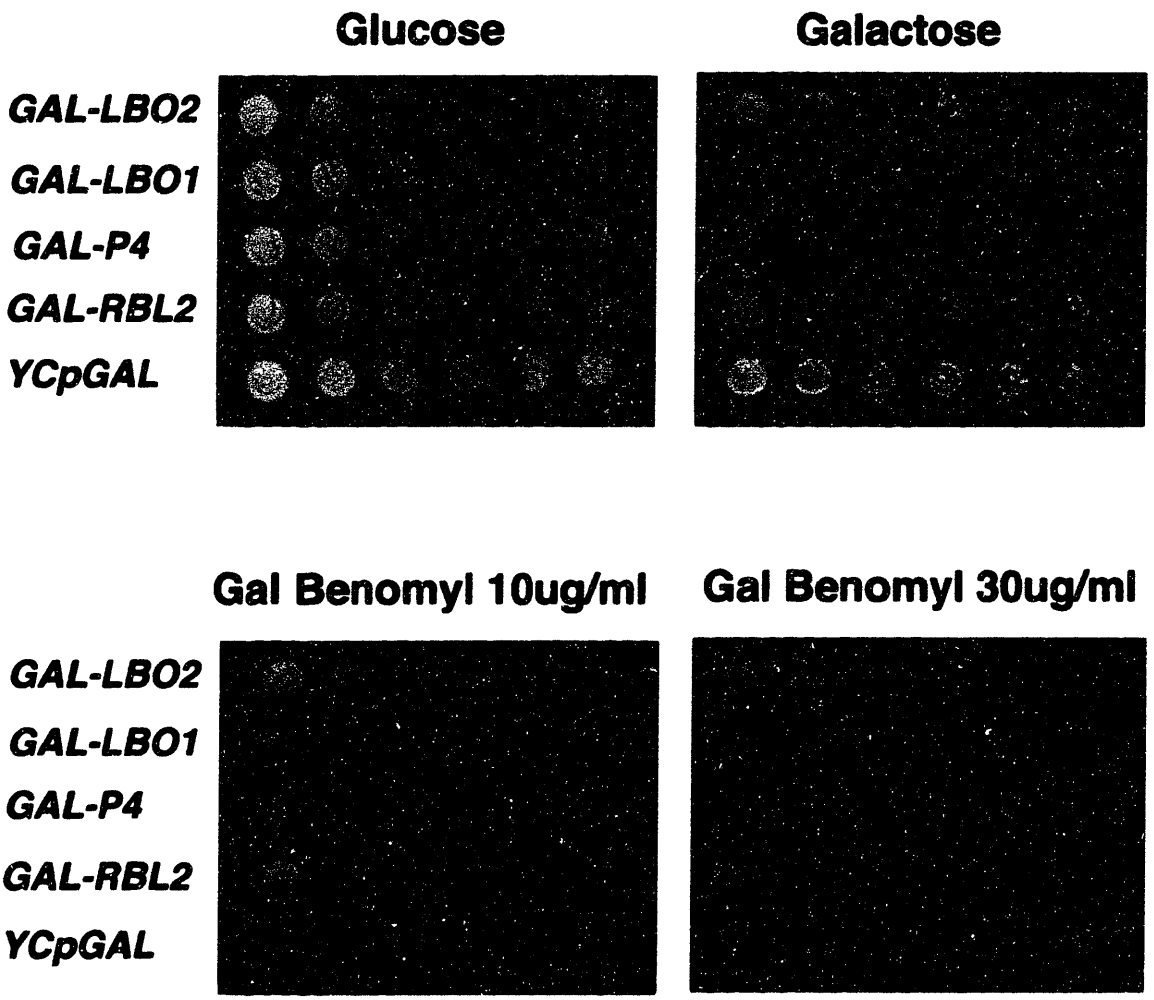
As expected we found plasmids encoding Rbl2p and one of the α-tubulins (Tub1p) but not the other. We also identified two additional cDNA's which allow cells to *L*ive on *B*enomyl when *O*verexpressed *LBO*'s *1* and *2*. An example of wild type cells retransformed with the *LBO 1* or *LBO2* cDNA is shown in figure 4-2. Gal-P4 in figure 4-2 is an example of a cDNA that failed to increase benomyl resistance upon retransformation. That we only identified one isolate for each of the cDNA's that conferred benomyl resistance, suggests that our screen was not saturated.

Gene products that act to suppress the benomyl sensitivity of wildtype cells might do so in various ways. Suppressors could include gene products involved in transport of the drug. For example, expression of the *C. albicans* genes CDR1 and BENr, which encode potential drug efflux pumps, confers a multiple drug resistance phenotype in *S. cerevisiae* (Cannon et al., 1998; Ben-Yaacov, et al. 1998). We previously showed that neither Rbl2p nor α-tubulin overexpression is capable of conferring a multiple drug resistance phenotype (Archer et al., 1995). We tested whether yeast cells overexpressing *LBO1* or *LBO2* for resistance to cycloheximide and ethidium bromide and found that they did not confer resistance to these other compounds (data not shown).

Quantitation of the LBO phenotype

Cells containing extra α-tubulin or extra Rbl2p show both quantitative and qualitative differences relative to control cells when plated to benomyl containing plates--more colonies form and these colonies are larger than colonies that grow up on control plates. To characterize the ability of *LBO1* and

Figure 4-2. Overexpression of LBOs confer increased benomyl resistance. Wild-type diploid cells were retransformed with plasmids containing *GAL-LBO2, GAL-LBO1, GAL-P4, GAL-RBL2 or YCpGAL*. Serial (four-fold) dilutions of saturated cultures were plated to SC-ura: glucose, galactose and galactose benomyl as indicated and incubated at 30°C.

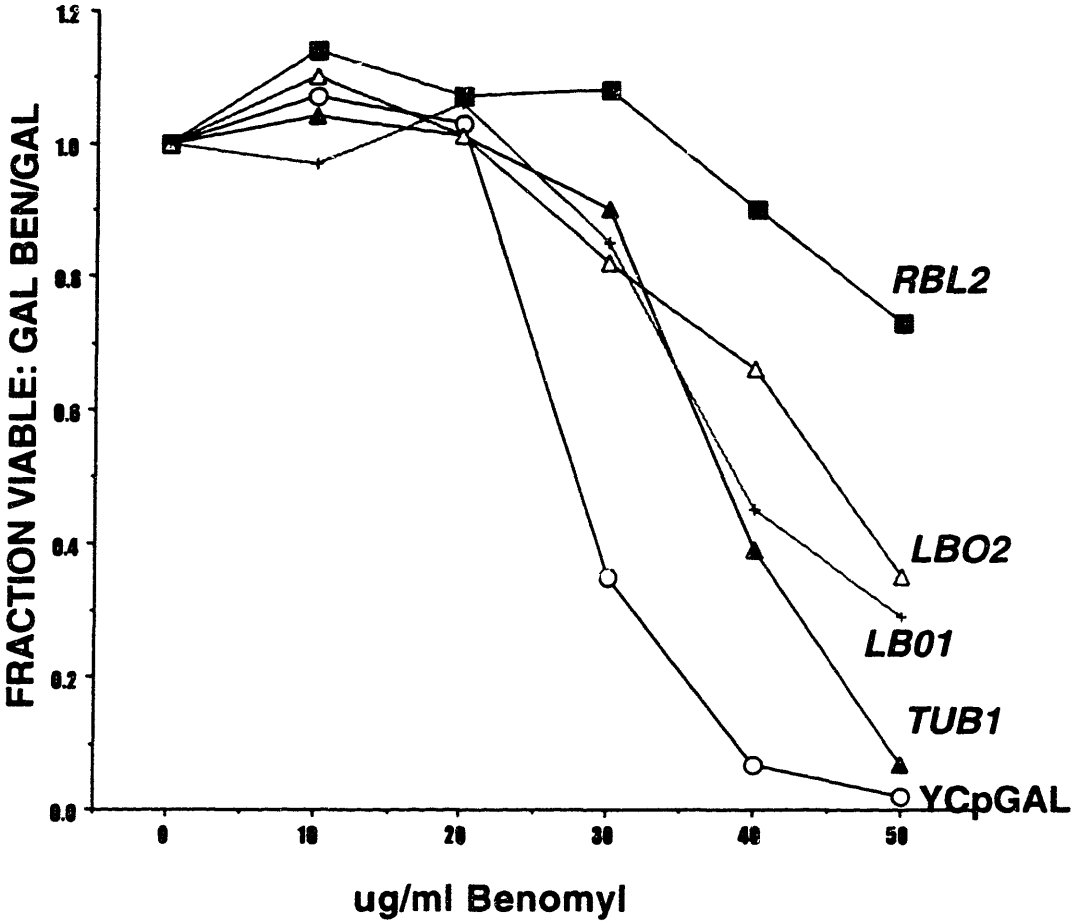

Glucose
Galactose
GAL-LBO2
GAL-LBO1
GAL-P4
GAL-RBL2
YCpGAL
Gal Benomyl 10ug/ml
Gal Benomyl 30ug/ml
GAL-LBO2
GAL-LBO1
GAL-P4
GAL-RBL2
YCpGAL

LBO2 to increase the benomyl resistance of wild-type yeast cells we performed a quantitative plating experiment. Diploid cells containing galactose promoted *RLB2*, *TUB1*, *LBO1*, *LBO2* or *YCpGAL* were plated to, galactose plates and to galactose plates containing various concentrations of benomyl. We determined the ratio of the number of colonies that grew on the various galactose benomyl plates relative to the galactose plate without benomyl. As shown in figure 4-3 overexpression of Rbl2p was the best at protecting cells from the effects of benomyl even at the highest concentrations of benomyl. *LBO's 1*, and *2* were slightly better than *TUB1* at the highest concentrations (50 ug/ml) of benomyl.

Overexpression of LBO's in benomyl supersensitive mutants

We were interested to know whether we could identify mutants in which the overexpression of *LBO* genes did not increase benomyl resistance. Such a situation would suggest that the benomyl resistant phenotype depended on the presence of the wild-type function of the mutant gene product. We tested whether the overexpressed LBO genes also increase the benomyl resistance of various mutants stains that are supersensitive to benomyl. In all the backgrounds tested -*Δtub3*, *Δcin1*, *Δcin2*, *Δrbl2*, and *tub2-590* - the *LBO* were able to confer increased resistance benomyl (data not shown).

Figure 4-3. Quantification of the *LBO* phenotype. Wild-type diploid cells containing *GAL-LBO1, GAL-LBO2, GAL-TUB1, GAL-RBL2* or *YCpGAL* were plated to galactose plates and to galactose plates containing increasing concentrations of benomyl. The ratio of the number of colonies that grew on the various galactose benomyl plates relative to the galactose plate was determined.


FRACTION VIABLE: GAL BEN/GAL
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
ug/ml Benomyl
RBL2
LBO2
LB01
TUB1
YCpGAL

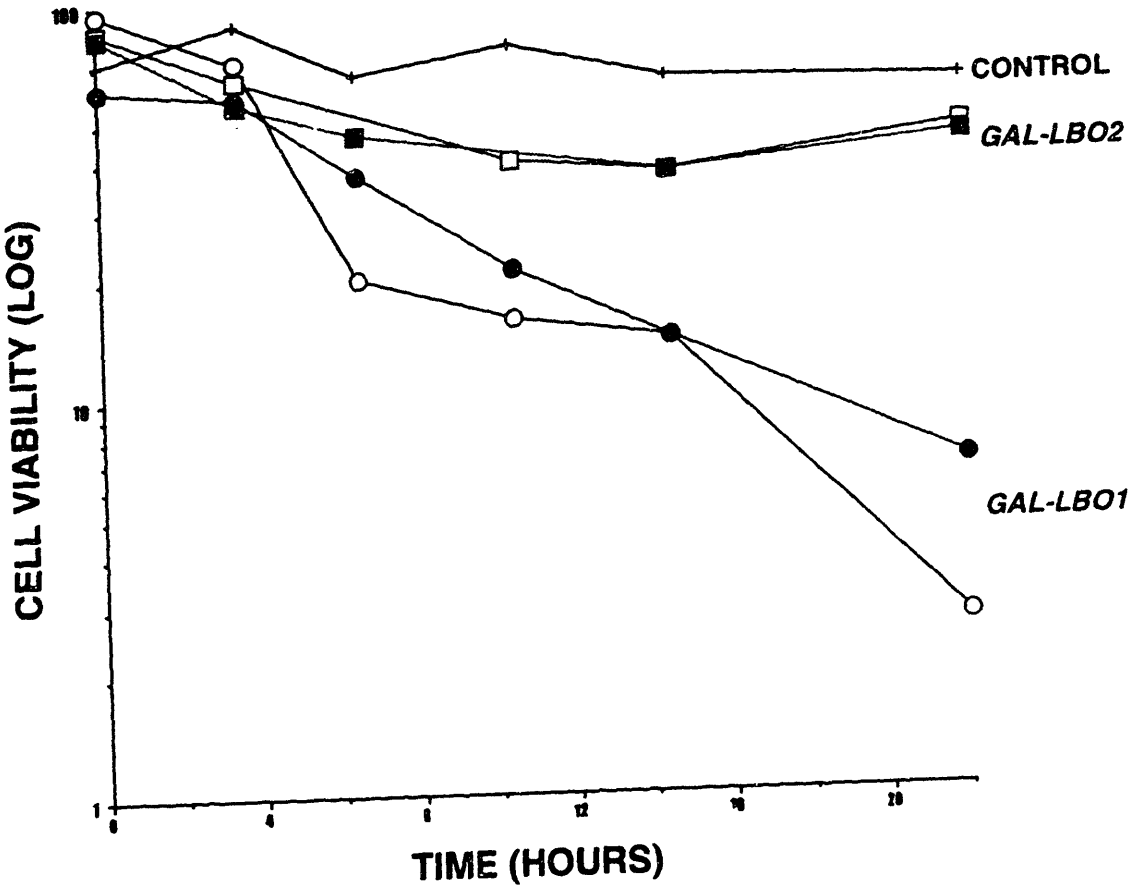
Overexpression of LBO's in wild type cells.

cDNAs encoding proteins that interact along the length of microtubules or at the ends of microtubules might stabilize the microtubules and confer resistance to microtubule depolymerizing drugs. For example, a *KAR3-lacZ* hybrid protein, containing the microtubule binding domain of a kinesin heavy chain related protein that is essential for karyogamy in *S. cerevisiae*, stabilized pre-formed cytoplasmic microtubules to nocadazole mediated depolymerization (Meluh and Rose, 1990). A similar observation was made for cells expressing a fusion protein of another gene, *CIK1* (Chromosome Instability and Karyogamy) (Page and Snyder, 1992). Thus, proteins that bind to microtubules may confer at least a transient resistance to benzimidazole-mediated microtubule depolymerization. Perhaps the presence of microtubule-associated proteins on the yeast microtubule alters the binding capacity for the drug. In the absence of benomyl, we might expect overexpressed proteins that hyperstabilize microtubules would cause a growth phenotype in wild type cells. For example, the cells may not be able to transit though the cell cycle since hyperstable microtubules may interfere with microtubule dynamics.

We examined the effect of galactose induced overexpression of *LBO1* and *LBO2* in wild-type yeast cells at 30°C. Yeast cells containing *GAL-LBO1*, *GAL-LBO2* or containing *YCpGAL* were induced with 2% galactose at 30°C. At various times the cells were counted and a dilution plated to non-inducing plates to determine viability. As shown in figure 4-4, overexpression of *LBO1* is toxic. The viability of the culture is down to about 3% after 20 hours. In contrast

Figure 4-4. Viability of cells overexpressing *LBO*s.

Diploid cells containing *YCpGAL* (+),*GAL-LBO1* (circles), or*GAL-LBO2* (squares), were grown overnight in selective non-inducing media at 30°C. At T=0 overexpression of the indicated gene was induced in log phase cultures by the addition of 2% galactose. At various time points aliquots of the cells were taken and counted by hemacytometer. Known numbers of cells were plated to SC -ura glucose plates and incubated at 30°C. Cell viability was measured as percent of counted cells able to form colonies on SC -ura glucose plates. Two separate *GAL-LBO1* and *GAL-LBO2* transformants were monitored.


CONTROL
GAL-LBO2
GAL-LBO1
CELL VIABILITY (LOG)
TIME (HOURS)

overexpression of *LBO2* is only moderately toxic in wild-type cells: the viability is ~50% after 20 hours in galactose. However, we found that anti-tubulin immunofluorescence of cells overexpressing either *LBO1* or *LBO2* was indistiguishable from control cells. We could not detect increased amounts of microtubule polymerization. This suggests that, the growth defect in cells overexpressing *LBO1* or *LBO2* is probably not due to a gross hyperstablization of the microtubule cytoskeleton. Perhaps a more sensitive test will be necessary to determine if the microtubules in cell overexpressing *LBO1* or *LBO2* are hyperstabilized.

Identification of *LBO1*

We established the identity of the *LBO1* plasmid by dideoxy sequencing of the insert cDNA. The *LBO1* cDNA is 3.85 kb and the full length cDNA encodes a previously identified yeast protein *SCP160* for *S*acchromyces *C*ontrol of *P*loidy (Wintersberger and Karwank, 1992). The carboxy-terminus of Scp160p contains 14 K homology (KH) domains (Castiglone Morelli et al., 1995; Delahodde et al., 1986; Siomi et al., 1993). KH motifs are evolutionary conserved and have been shown to bind to RNA *in vivo* [Dejgaard, 1996 #1908; Urlaub, et al., 1995]. SCP160p shares significant homology, particularly within the KH motifs, with the conserved vertebrate RNA binding protein, vigilin (Webber et. al, 1997).

LBO1/SCP160 nulls

Strains deleted for *SCP160* are viable; however, Wintersberger and co-workers (Wintersberger et al., 1995) reported that deletion of parts of the

SCP160 gene resulted in decreased viability, abnormal morphology and increased DNA content of the mutant strains. We disrupted the complete open reading frame encoded by *SCP160* in wild type diploids. Diploids containing a disruption allele at the correct chromosomal location were identified by Southern blot analysis and were sporulated and dissected on YPD at 30°C. As shown in figures 4-5 (A, B) cells deleted for the entire coding sequence of *LBO1/SCP160* are viable. We tested tetrads for various conditional phenotypes -temperature sensitivity at 15°C, 18°C, 25°C, 37°C and benomyl sensitivity- and found that no conditional phenotype co-segregated with *Δlbo1/scp160::URA3.*

Marker analysis on 40 complete tetrads showed 2:2 segregation of URA+:ura- in 37 of 40 tetrads examined. Three of the tetrads analyzed were 1:3 for URA+: ura3-. 39 of the 40 tetrads showed the correct 2:2 segregation for ADE+:ade-. As show in figure 4-5 (C), Southern blot analysis on the tetrads showed 2:2 segregation of the *Δlbo1/SCP160* disruption allele in 3 of the 4 tetrads tested. One of the four segregants (4b) from tetrad #4 was heterozygous for the disruption allele even thought this tetrad showed normal 2:2 Ura+:ura- segregation. This particular tetrad was also the only one that showed aberrant segregation of ADE+: ade-.

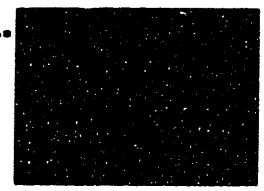
Localization of Lbo1p-Scp160p

We generated an allele of *LBO1/SCP160* tagged at the carboxy terminus with a single HA epitope tag. The tagged construct is as effective as the untagged version in conferring benomyl resistance. As shown in figure 4-6, the epitope tagged protein runs as a single band of about 160 kD on SDS

Figure 4-5. Cells deleted for *LBO1/ SCP160* are viable. Diploid cells heterozygous for the*Δlbo1/scp160*::*hisG-URA3-hisG* disruption allele were sporulated in 1% potassium acetate, dissected on YPD and incubated at 30°C for 2 days. (A) and (B) are two independent heterozygous diploids. (C) Southern blot analysis of 4 of the tetrads generated from the above dissection. Δ indicates the *Δlbo1/scp160*::*hisG-URA3-hisG* allele; **WT** indicates the wild-type *LBO1* allele.

Δlbo1/LBO1

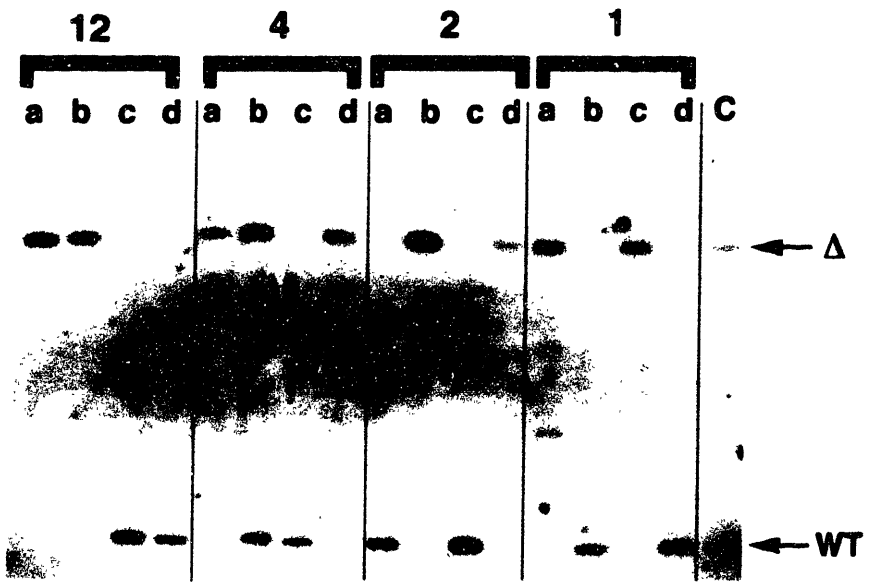
A.



B.

YDP 30 ℃

C.



PAGE gels from extracts of cells overexpressing Lbo1p/Scp160p-HA (G). This protein band not present in extracts from uninduced cells (R) . By immunofluorescence microscopy we found that Lbo1p/Scp160p-HA the localizes to the nucleus and to what appears to be the ER (data not shown). However, we did not compare this localization with a nuclear or ER marker since our result is similar to the localization that has been previously reported (Wintersberger et al., 1995). We tested whether overexpressed Lbo1p/Scp160p-HA shows any direct interaction with either α-or β-tubulin. However, co-immunoprecipitation experiments with the anti-tubulin monoclonal antibodies or with the anti-HA failed to show any direct interactions with tubulin (data not shown).

LBO2

The *LBO2* sequence is 0.96 kb in size and encodes a novel yeast ORF, with a predicted molecular weight of 22.6 kD and a predicted pI of 4.81. *LBO2* protein sequence contains a P-loop motif commonly found in ATP/GTP binding proteins. In addition, *LBO2* sequence shares 65% similarity and 46% identity with another hypothetical yeast ORF, YDR066C (BLAST).

Charaterization of *Δlbo2* cells

To determine the loss of function phenotype of *LBO2* we generated an *LBO2* disruption construct by P. C .R. that deletes the entire predicted open reading frame of YER139C. The 5'- and 3'- flanking region of *LBO2* was cloned around *hisG-URA3-hisG* in pNK51. Diploids transformants were selected on SC -ura and putative heterozygotes were analyzed by Southern blot. We

Figure 4-6. SDS PAGE analysis of epitope tagged Lbo1p/Scp160-HA. Two independent strains containing *GAL-LBO1/SCP160-HA* were grown overnight in SC -ura raffinose media. 2% galactose (G) was added to log phase cultures to induce Lbo1p/Scp160-HA or 2% raffinose (R) was added to the control culture. Total protein was harvested by glass bead lysis in PME plus protease inhibitors then boiled in SDS sample buffer. Samples were run on 7.5% SDS PAGE, transferred to nitrocellulose, probed with the monoclonal antibody 12CA5 and detected with I^{125} SAM (NEN). A single band of about 160 Kd is detected.

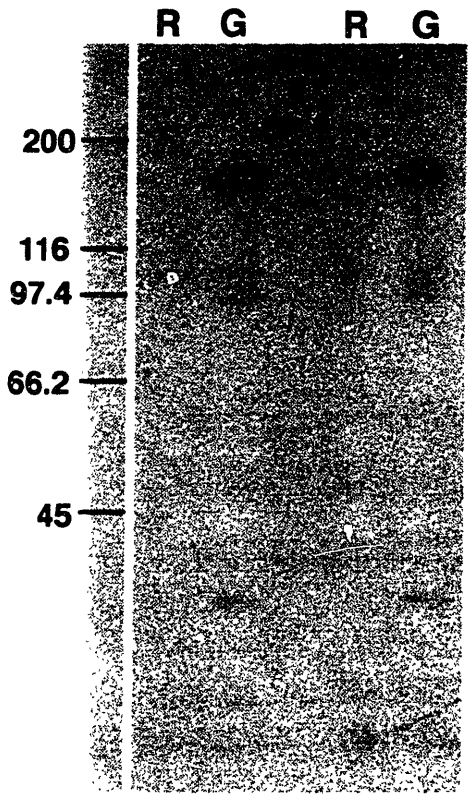
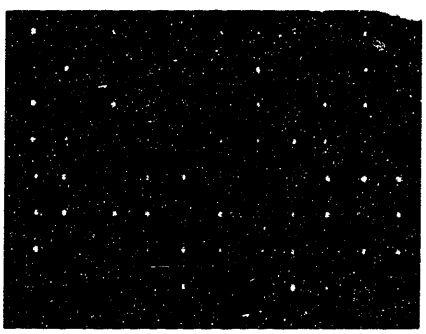

R G R G
200
116
97.4
66.2
45

Figure 4-7. Cells deleted for *LBO2* are viable. Diploid cells heterozygous for the*Δlbo2*::*hisG-URA3-hisG* disruption allele were sporulated in 1% potassium acetate, dissected on YPD and incubated at 30°C for 2 days. Three independent heterozygous diploids were tested however only one diploid is shown.

Δlbo2/LBO2



YPD 30°C

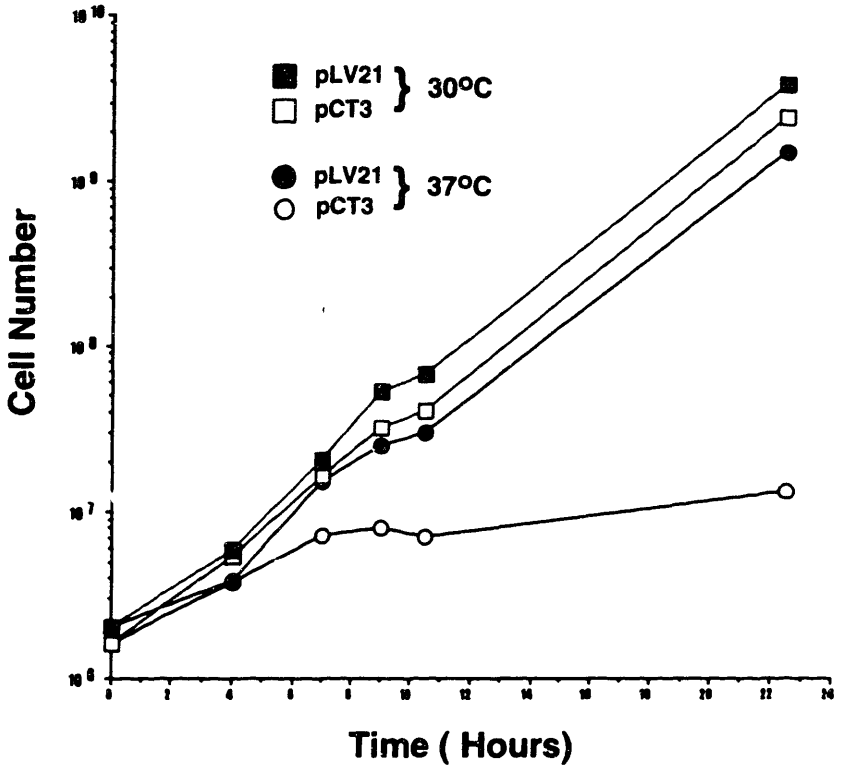
sporulated and dissected diploids containing the disruption allele at the correct chromosomal location. Tetrad analysis showed that *Δlbo2* cells are viable at 30°C (shown in figure 4-7).

However, as shown in figure 4-8, we found Lbo2p function is required for growth at 37°C . *Δlbo2* containing a control plasmid (pCT3, open figures) or covered with a low copy genomic plasmid containing *LBO2* (pLV21, closed figures) were grown at 30°C (squares) or were shifted to37°C (circles). We counted cell number and plated cells to monitor the viability of the culture at various times. Figure 4-8 (A), shows that the cell deleted for *LBO2* stop dividing after about 2 doublings at this temperature. Moreover, prolonged incubation of *Δlbo2* cells at 37°C results in loss of viability. As shown in figure 4-8 (B), after 20 hours at 37 °C only about 0.1% of the *Δlbo2* cells are viable (open squares). During the course of our analysis Smith et al., (1996) performed a systematic functional analysis of the genes on yeast chromosome V by genetic footprinting. They reported that Ty1 transposon insertions in YER139C reduced the fitness of *S. cerevisiae* at elevated temperatures (Smith et al., 1996).

To determine if cells deleted for *LBO2* arrest at a specific point in the cell cycle we quantitated the bud size distribution of *Δlbo2* cells or wild-type cells after a 9 hour shift to the non-permissive temperature. As shown in figure 4-9 (B), we found that the percentage of unbudded cells increased 2-fold for *Δlbo2* cells relative to the control when held at the non-permissive temperature for 9

Figure 4-8. Lbo2p is required for growth at 37°C. (A) *Δlbo2* haploid cells containing a genomic copy of *LBO2* on a *CEN* plasmid, pLV21 (filled figures), or with a control plasmid, pCT3 (open figures), were grown overnight in SC -ura media. At T=0, the cultures were shifted to 37°C (circles) or were maintained at 30°C (squares). Cell number was monitored by hemacytometer counts. At 37°C cells deleted for *LBO2* stop dividing after 2 doublings. (B) At various time points after shifting to 37°C aliquots of the *Δlbo2* haploid cells covered with pLV21(filled squares) or with a control plasmid, pCT3 (open squares) were taken and counted by hemacytometer. Known numbers of cells were plated to SC -ura glucose plates and incubated at 30°C. Cell viability was measured as percent of counted cells able to form colonies on SC -ura glucose plates.

A.


pLV21
pCT3
30°C
pLV21
pCT3
37°C
Cell Number
Time (Hours)

B.

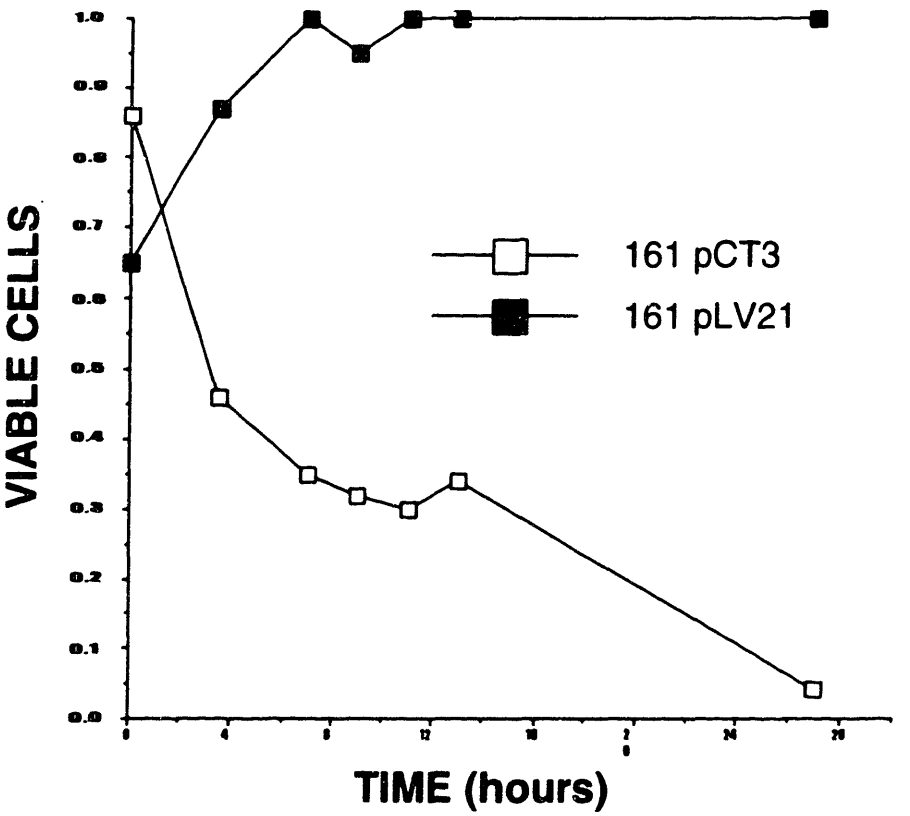
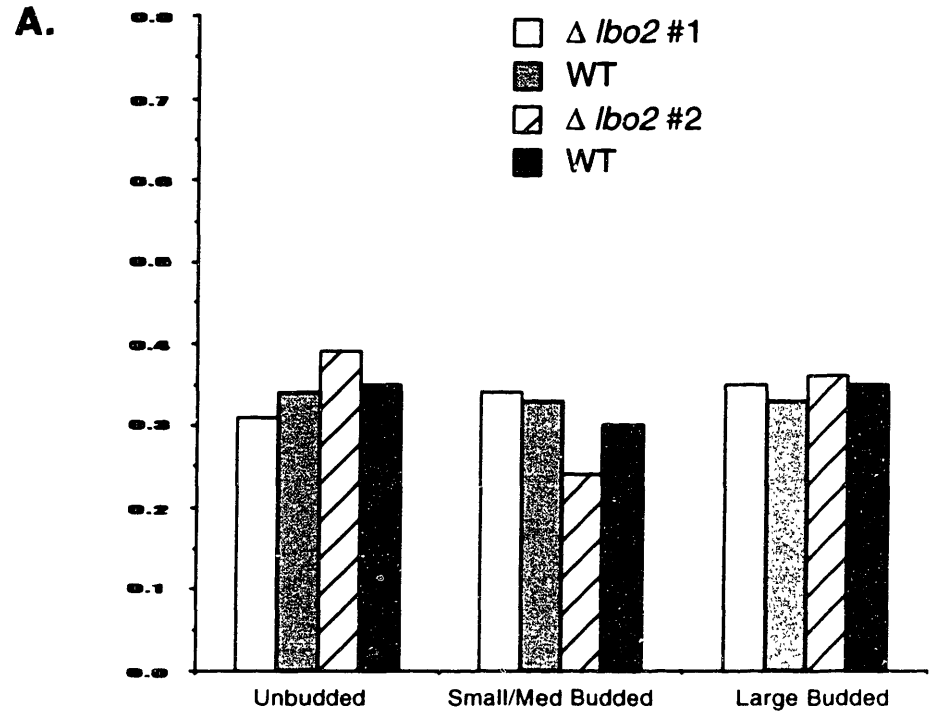
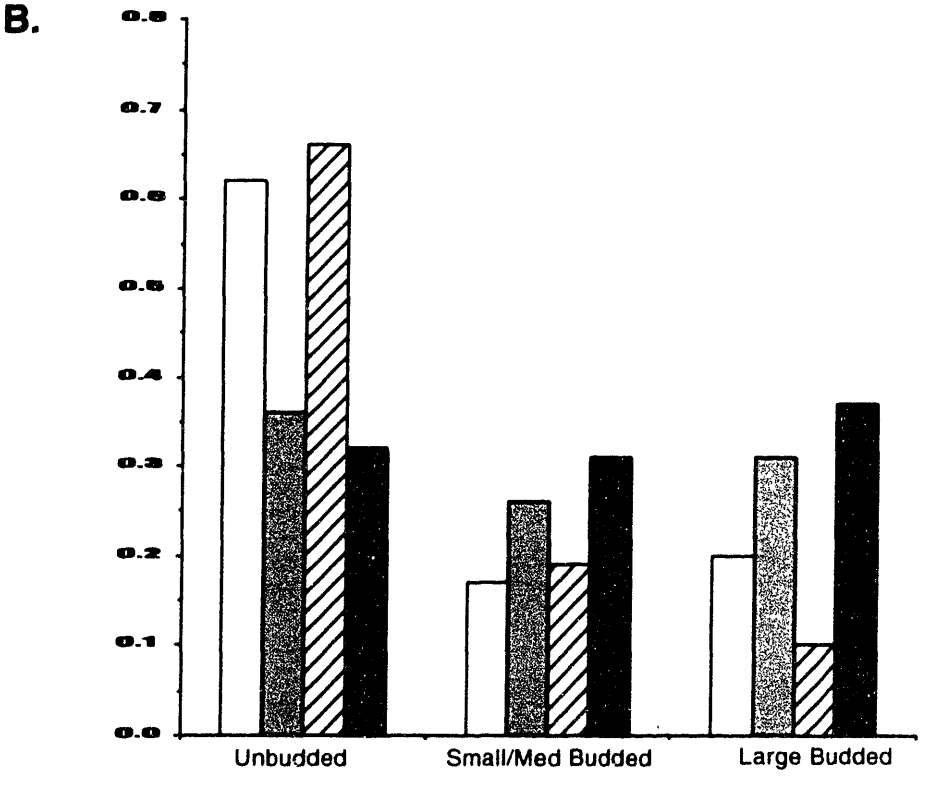

VIABLE CELLS
161 pCT3
161 pLV21
TIME (hours)

Figure 4-9. Incubation at 37°C alters the bud size distribution of *Δlbo2* cells. Two independent *Δlbo2* haploids (LTY161, LTY162) covered with pLV21 or with the pCT3 control plasmid were grown at 30°C (A) or at 37°C for 9 hours (B). We determined the bud size distribution of cells by visual inspection and we processed cells for FACS analysis as described in materials and methods. After 9 hours at 37°C *Δlbo2* cells containing pCT3 showed an increase in the percent unbudded cells. (A): *Δlbo2* #1, (n=119); WT #1, (n=98); *Δlbo2* #2, (n=154); WT #2 (n=127). (B): *Δlbo2* #1, (n=122); WT #1 (n=125); *Δlbo2* #2, (n=150); WT #2 (n=122).


A.
Δ lbo2 #1
WT
Δ lbo2 #2
WT
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Unbudded
Small/Med Budded
Large Budded


B.
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Unbudded
Small/Med Budded
Large Budded

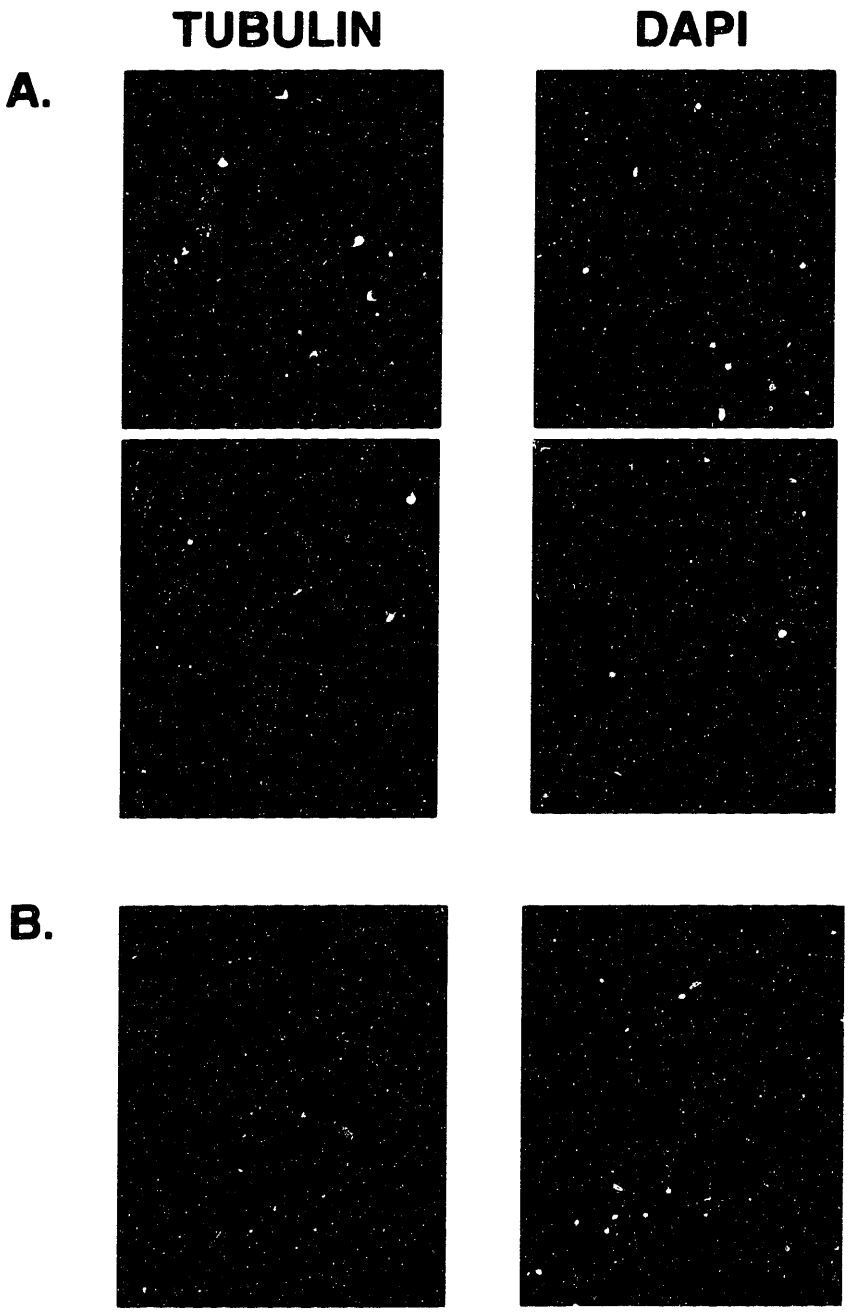
hours. However, we found that the percentage of cells in G1/S did not increase when measured by F.A.C.S. analysis (data not shown).

To test whether cells deleted for *LBO2* also have a defect in microtubule assembly, we performed anti-tubulin immunofluorescence analysis of *Δlbo2* cells shifted to 37°C for 9 hours. As shown in figure 4-10 (A), the unbudded *Δlbo2* show very elaborate cytoplasmic microtubule arrays for unbudded cells. By DAPI staining the DNA in *Δlbo2* cells appears enlarged and somewhat disorganized at 37°C. In contrast wild-type cells do not show this phenotype when subjected to these same conditions (figure 4-10 (B)). The microtubules of wild type, unbudded cells usually look like dots or small asters.

Localization and Immunoprecipitation experiments with Lbo2p-HA.

As previously mentioned, we found that overexpression of *LBO2* is toxic in yeast cells (figure 4-4); however, we failed to find any gross alteration of the microtubules in *LBO2* overexpressing cells by immunofluorescence. Because of the elaborate microtubule arrays in *Δlbo2* cells we were interested to see if the wild-type Lbo2p co-localized with microtubules or whether Lbo2p showed any discrete localization *in vivo*. Therefore, we generated both a galactose-promoted and a genomic-promoted version of Lbo2p with a triple HA epitope tag at the extreme carboxy terminus. We tested genomic Lbo2p-HA constructs for the ability to complement the temperature sensitivity of *Δlbo2* cells at 37°C and the GAL-promoted constructs for the ability to confer benomyl resistance when overexpressed. We found that both the genomic and the galactose

Figure 4-10. *Δlbo2* cells exhibit aberrant microtubule morphologies at 37°C. Anti β-tubulin immunofluorescence and DAPI staining of *Δlbo2* cells containing pCT3 (A) or covered with pLV21 (B) after 9 hours at 37°C. *Δlbo2* cells appear enlarged and show aberrant tubulin staining.


TUBULIN
DAPI
A.
B.

tagged versions of Lbo2p-HA were fully functional (data not shown). As shown in figure 4-11, Lbo2p-HA runs as a single band above the 31 kd marker. Thus, Lbo2p-HA runs slightly larger on SDS PAGE than the predicted molecular weight of Lbo2p (22.6 kd) plus the triple HA tag (~4 kd). We used both the GAL-promoted and the Genomic promoter versions of Lbo2p for immunolocalization experiments. Neither version of Lbo2p-HA showed any discrete localization *in vivo*; rather, Lbo2p-HA showed diffuse staining in both the nucleus and the cytoplasm (data not shown).

Rbl2p binds to β-tubulin monomer and its overexpression like, Lbo2p, confers benomyl resistance in wild-type cells (Archer et al., 1998; Archer et al., 1995). However, Rbl2p does not give a discrete localized signal by immunofluorescence microscopy; thus, it does not appear to co-assemble into microtubule polymer *in vivo* (Archer et al., 1995). We tested whether Lbo2p, like Rbl2p, showed any physical interaction with the individual tubulin chains by co-immunoprecipitation with the anti-tubulin monoclonal antibodies or with anti-HA. However, we did not find any direct interaction of Lbo2p with either α or β-tubulin (data not shown).

YDR066C is an *LBO2* homologue by sequence but is not a functional homologue.

LBO2 shares sequence similarity with YDR066C, a hypothetical yeast ORF of 196 amino acids. As shown in figure 4-12, YDR066C shares 46% identity to Lbo2p over 105 amino acids (BLAST). Unlike *LBO2*, YDR066C does

Figure 4-11. SDS PAGE analysis of epitope tagged Lbo2p-HA.

Yeast strains containing GAL-LBO2-HA or YCpGAL (control) were grown overnight in SC -ura raffinose media. 2% galactose was added to log phase cultures to induce the expression of Lbo2p-HA. Total protein was harvested by glass bead lysis in PME plus protease inhibitors then boiled in SDS sample buffer. Samples were run on 7.5% SDS PAGE, transferred to nitrocellulose, probed with the monoclonal antibody 12CA5 and detected with I^{125} SAM (NEN). A band of about 35 Kd is detected.

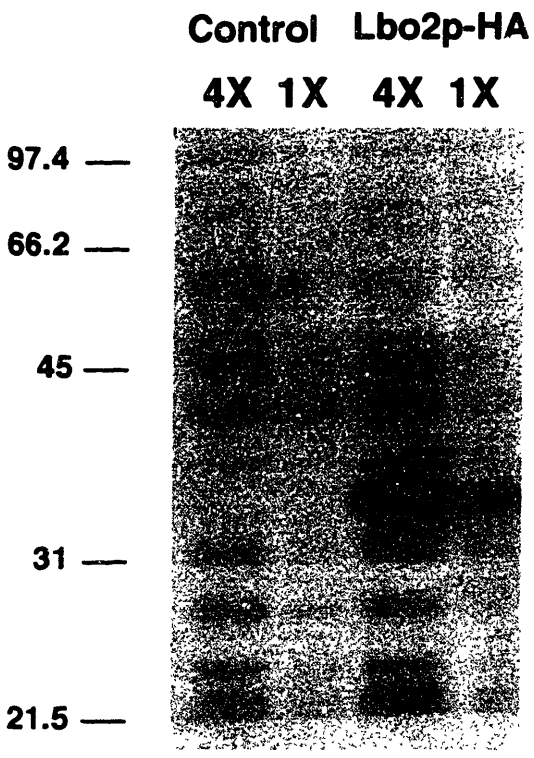

Control
Lbo2p-HA
4X 1X 4X 1X
97.4
66.2
45
31
21.5

Figure 4-12. Lbo2p is homologous to a hypothetical yeast ORF, YDR066C. Sequence alignment of Lbo2p and YDR066Cp shows that they share 46% identity over their entire length.

```
LB02. pep       MATIEDIKETALIPFQKHRQLSMHEAEVITLEIIGLLCDSECKDEKTLKY 50
                :  T..  I:..  L :  Q H QLS:  EA : I.  . I:::L : S  C. : E: TLKY
YDR066C. pep    IITTTFIQKVILGSHQLHEQLSIVEARMIESAIVSMLTESFCENEQTLKY 52

LB02. pep       LGRFLTPDMYQDLVDERNLNKRCGYPLCGKS-PERIRDPFSMNDTTKKFL 99
                L: R: L: P   Y  D: : : . R.  : K  CGYPLC  KS  : E.  . D  F
YDR066C. pep    LARLLSPMSYMDVINARRGKKICGYPLCYKSAAENSSDGF---------- 92

LB02. pep       LENNPYAYL-SHYCSKFHFRCSQFYQVQLSDEALFARTGVHLFEDPEQDK 148
                        : :  S  YC: : : H  : CS  :     QLS: . : L  . R  GVHL  .    :  :
YDR066C. pep    -------F IHSMYCNNYHSKCSLYLMRQLSQTPLHERRGVHLTSYINLEF 135

LB02. pep       HDIDFKVTLFEELLREKASEEDIKSLISGLKKLGLNPDSGTTEKDDTELE 198
                : D:  : . V: L: EEL:  . . . :  : . : KSLI: : : K. L. : :  .  .  .  .
YDR066C. pep    DDM-YSVSLLEELVGSEVPIDTVKSLITSFKDLEFDDTYKNEPLPLDVYF 184

LB02. pep       DDLSKWLAQIKI 210
                : : L: .  .
YDR066C. pep    GQLTTDEETCIE 196
```

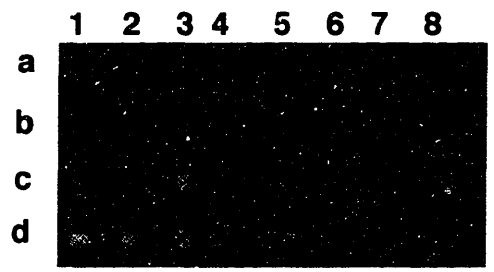
not contain a P-Loop motif. In addition YDR066C has mitochondrial energy transfer protein signature motif (DATABASE).

To test whether the loss of function phenotype of YDR066C was similar to that of *Δlbo2* cells we disrupted the ORF encoded by YDR066C. Briefly, we cloned 5' and 3' flanking region of YDR066C, 225 and 658 bp respectively, around *LEU2* in pJH-L2. The disruption fragment was transformed into a wild-type diploid and transformants were selected on SC leu-. Putative heterozygotes were checked by Southern blot analysis. Diploids containing the disruption allele at the correct chromosomal location were sporulated and dissected on YPD at 30°C. We found that cells deleted for YDR066C are viable (data not shown and figure 4-13). The tetrads were serially diluted and plated to different growth conditions to test for sensitivity to benomyl, various temperatures, and auxotrophies. We were unable to detect any growth phenotype for the conditions tested. In addition, we tested whether *Δlbo2, ΔYDR066C* double mutants were viable. As shown in figure 4-13 the double mutant is viable. We found that double mutant cells show the same temperature sensitivity as the single *Δlbo2* mutant (data not shown).

In order to determine if the protein encoded by YDR066C was also a functional homologue of Lbo2p we cloned the YDR066C sequence under the control of the galactose promoter. We transformed wild-type cells with pGAL-YDR066C and plated cells to galactose benomyl plates. We found that

Figure 4-13. *Δlbo2*; *ΔYDG066C* mutants are viable. *+/Δlbo2::URA3 hisG*, *+/ΔYDG066C::LEU2* double heterozygous diploid cells were sporulated in 1% potassium acetate, dissected on YPD and incubated at 30°C for 2 days. (**x**) indicates double mutant cells.

Δlbo2; YDR066C X *LBO2; ΔYDR066C*



YPD 30 °C

× = double mutant

galactose overexpression of YDR066C did not confer increased benomyl resistance in wild-type cells. Since we did not have a functional assay for YER066C it was not possible to determine if the protein encoded by construct was expressed.

DISCUSSION

Our laboratory showed that overexpression of the β-tubulin binding proteins, α-tubulin or Rbl2p confers increased resistance to benomyl in wild-type cells (Archer et al., 1995; Schatz et al., 1986). We have screened for other cDNAs that allow cells to Live on Benomyl when Overexpressed (*LBO*). Gene products with this property may provide valuable information about microtubule assembly and perhaps about the response of animal cells to chemotherapies.

In this chapter, we describe the identification and initial characterization of two such genes *LBO1* and *LBO2*. Like Rlb2p and α-tubulin, overexpression of either *LBO1* or *LBO2* in wild-type cells confers increased resistance to microtubule depolymerizing drugs but not to other unrelated compounds. We found that in the absence of benomyl overexpression of either gene product is slightly toxic. However, we were unable to identify a defect in microtubule assembly in cells overexpressing either *LBO1* or *LBO2*. Further analysis will be necessary to determine the mechanisms by which cells overexpressing *LBO* gene products confer increased resistance to microtubule depolymerizing drugs. Unlike Rbl2p and α-tubulin which we know interact with β-tubulin, we did not find that either *LBO1* or *LBO2* can interact with the individual tubulin chains by co-immunopreciptiation.

Neither *LBO1* nor *LBO2* are essential under standard conditions; however, the *LBO2* gene product is required for viability at high temperature

(this study and (Smith et al., 1996). We found that cells deleted for *LBO2* stop dividing after about two doublings at the restrictive temperature. *Δlbo2* cells accumulate as unbudded cells and have aberrant microtubule staining. Further analysis will be necessary why Lbo2p is required for viability at elevated temperatures.

LBO1 and KH domain proteins

Lbo1p/Scp160 shares homomlogy to a family of conserved proteins containing KH domains (Siomi et al., 1993). Some KH domain containing proteins are clinically important. These include: the FMR1 protein, involved in human fragile X syndrome and Nova-1, an autoantigen in paraneoplastic opsoclonus myoclonus ataxia (POMA), a disorder associatd with breast cancer and motor dysfunction (Buckanovich et al., 1993; Buckanovich et al., 1996; Burd and Dreyfuss, 1994; Siomi et al., 1993). In some cases KH domain proteins have been shown to bind to RNA *in vivo* and *in vitro* [Dejgaard, 1996 #1908; Urlaub, et al., 1995; Burd, 1994 #1911; Buckanovich, 1997 #1915] however, the details of how KH domain containing proteins interact with RNA are not known. And KH domains have been shown to be involved in the regulation of RNA synthesis and RNA metabolism (Buckanovich and Darnell, 1997; Burd and Dreyfuss, 1994). Interestingly, koc mRNA, encoding a KH domain containing protein overexpressed in cancer, was found to be highly overexpressed in various human cancer cells (Mueller-Pillasch et al., 1997). However, the precise role of koc in human tumor cells is unknown. It will be interesting to determine if Lbo1p/Scp160p is also an RNA binding protein *in vivo*. Work by Weber and

colleagues suggests that Scp160 binds various types of nucleic acids non-specifically including ribohomopolymers; rRNA, ssDNA and dsDNA (Weber et al., 1997). Perhaps Lbo1p/Scp160 can interact with and stabilize specific RNAs. For example, it may be that excess Lbo1p/Scp160p (either specifically or non-specifically) binds and stabilizes *TUB1* or *TUB3* mRNA which in turn leads to more α-tubulin and increased benomyl resistance. Recent work in our laboratory by Adelle Smith and Margaret Magendantz suggests that perhaps the α-tubulin message is unstable in certain genetic backgrounds. We are now testing whether excess *LBO1* causes an increase in α-tubulin levels *in vivo*.

Parallels between benomyl and excess β-tubulin

Both excess β-tubulin and benomyl lead to loss of microtubules, large budded cell-cycle arrest and cell death. This parallel is supported by the consequences that changes in the level of either Rbl2p or α-tubulin have for both of these microtubule poisons. Overexpression of either gene rescues cells from the effects of β-tubulin overexpression (Archer et al., 1995; Weinstein and Solomon, 1990) and deletion of Rbl2p renders cells more supersensitive to excess β-tubulin (Archer et al., 1995). Extra Rbl2p or α-tubulin produced by the galactose induction confers resistance to benomyl, while the absence of Rbl2p or a modest decrease in the α-tubulin levels produced by disruption of the *TUB3* gene renders cells more sensitive to the drug (Archer et al., 1995; Schatz et al., 1986). The mechanisms by which extra α–tubulin or extra Rbl2p mediate

resistance to benomyl is not still not clear. However, the benomyl resistant phenotype together with the other interesting properties of α-tubulin and Rbl2p may indicate that resistance to benomyl occurs via a microtubule related process.

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CHAPTER 5:

Future Directions

Prospects for further studies

-How does free β-tubulin poison cells.

Many experiments in our laboratory and in this thesis stem from the observation made by Brant Weinstein when he was a graduate student that free excess β-tubulin poisons microtubule assembly and results in cell lethality. We have used the activity of free β-tubulin to examine cellular mechanisms controlling microtubule assembly. However, we still do not understand the mechanism by which β-tubulin kills cells.

Despite many attempts by several people in the laboratory we have been unable to identify the lethal target of excess β-tubulin. The original RBL screen (Rescue Beta-tubulin Lethality) developed by Julie Archer and continued by Pablo Alvarez employed high copy suppression of a galactose-promoted β-tubulin gene. This approach did not identify the lethal target of β-tubulin but rather led to the identification of Rbl2p, a β-tubulin monomer binding protein. Adelle Smith and Kate Compton tried to identify mutations that suppress β-tubulin lethality in cells overexpressing β-tubulin from the galactose promoter. However, they found that in each of several cases the ability to suppess β-tubulin lethality does not segregate in genetic manner. Kate Compton is trying to understand the epigenetic phenomenon involved in rescue of β-tubulin overexpression. Finally, Alice Rushforth has tried to identify biochemically components that bind to overexpressed β-tubulin. By using mass spectroscopy analysis she has identified candidate tubulin binding proteins,

including some hsp70 family members. But none of the proteins identified thus far are specific for β-tubulin over α-tubulin; rather, these proteins are enriched in cells overexpressing either tubulin chain.

Perhaps an analysis of genetic interactors with *tub1-724* will uncover other genes that affect β-tubulin lethality. We showed (in Chapter Two and (Vega et al., 1998)) that the cold sensitivity of *tub1-724* is semi-dominant in cells heterozygous for the mutant allele of α-tubulin. The semi-dominant cold sensitive phenotype in these cells is suppressed by overexpression of Rbl2p. This result argues that these cells die due to the free β-tubulin produced by dissociation of the mutant heterodimer or by newly translated β-tubulin which is unable to dimerize with the mutant α-tubulin at the restrictive temperature. Our work suggests that suppressors of the conditional phenotypes of *tub1-724* heterozygous cells may be useful to identify genes that affect heterodimer formation or that affect β-tubulin lethality.

For example, we could create wild type haploid cells that contain a plasmid copy of *tub1-724* on a genomic promoter (*CEN* or 2um). If, as we suspect, these cells have a significant cold sensitive phenotype we could mutagenize these cells and look for mutations in genes that either enhanced or suppressed the conditional phenotypes. This would allow us to look at cells with very modest levels of free β-tubulin. It is likely that genes that are involved in heterodimer formation would be identified in this manner.

In addition, we could perform a high copy suppressor screen to identify genes that when overexpressed are able to rescue *tub1-724/TUB1* and/or the *tub1-724*

haploid phenotype. From this type of analysis we could identify genes that interact with the β-tubulin monomer and/or that promote heterodimer formation such as *RBL2*, *CIN1*, and α-tubulin. We might also be able to identify the essential target of β-tubulin. By overexpressing the essential target of β-tubulin, the modest levels of free β-tubulin generated as a result of dissociation of the mutant heterodimer would no longer be in excess over the target. This screen has the advantage over the original RBL screen that the amount of free β-tubulin should be substantially less than the free β-tubulin generated from the galactose promoter. Thus, the kinetics of β-tubulin lethality should be slower, allowing the cells more time to establish suppression. In addition, depending on the library used, the target gene product could be available prior to shifting the cells to the non-permissive temperature that generates free β-tubulin. Quantitatively, the ability of a gene product to rescue acute β-tubulin poisoning from *GAL-TUB2* may differ from its ability to rescue the chronic amounts of free β-tubulin in *tub1-724/TUB1* cells. For one thing, it may be that the essential target of β-tubulin is itself toxic or lethal when it is highly overexpressed. Because the levels of free β-tubulin are less in the *tub1-724* mutant cells than in cells containing a galactose promoted *TUB2*, we may screen for high copy suppressors using more moderate levels of overexpression (2um library).

It may be useful to mutagenize β-tubulin (or the HIS6 tagged β-tubulin) to identify mutant β-tubulin alleles that are unable to poison microtubule assembly. If we

do this in a *tub2-590* strain we can quickly eliminate mutants that fail to express the β-tubulin using dot blot western analysis of protein extracts. Cells that express the non-poisonous β-tubulin can then be screened for the ability to interact with α-tubulin in the α/β heterodimer by co-immunopreciptiation with β-tubulin antibodies. In addition we can use indirect immunoflourescence with anti-β-tubulin to detect whether these β-tubulin alleles are still competent to incorporate into microtubules. It will be interesting to see if we can recover any alleles of β-tubulin that are no longer able to poison microtubule assembly but that are still able to interact with α-tubulin either at the level of α-β heterodimer polymer or formation. Using the available crystal structure for tubulin we would map residues important for β-tubulin poisoning onto the β-tubulin structure. We could then mutagenize cells containing the benign form of β-tubulin and try to identify mutant genes that can restore the excess β-tubulin lethality.

Are we looking at folding?

Another interesting question is; what is the fate of the dissociated tubulin in *tub1-724*? Is the free monomer degraded or is there a system to recycle the unpartnered tubulin chains into new heterodimer? Perhaps one clue comes from Jim Fleming's work on overexpression of *CIN1* in *tub1-724* cells. Overexpression of Cin1p, which he shows to be a β-tubulin binding protein, suppresses the phenotypes of *tub1-724* cells but has no effect in either *Δtub3* or *GAL-TUB2* overproducing strains. A significant difference among these situations is that the *tub1-724* mutant cells

contain a pool of α-tubulin large enough to bind all of the free β-tubulin, while the *TUB2* over-expressing strain and the *Δtub3* strain do not. That excess Cin1p rescues the *tub1-724* mutant phenotype suggests that the dissociated heterodimer can be recovered at least to some extent.

We can look at the *in vitro* tubulin folding assay (Tian et al., 1997) from the perspective of these data. The *in vitro* assay for tubulin folding requires the addition of exogenous heterodimer. The output of this assay is the ability of the newly folded tubulin chain to exchange into pre-existing heterodimer. Perhaps the "folding" reaction is not assaying folding but rather factors that are important for exchange of newly folded subunits with pre-existing heterodimer. Cofactors D and E are required in this assay, however, in *S. cerevisiae* the corresponding genes, Cin1p and Pac2p, are non-essential and have relatively minor phenotypes in wild-type cells. We and others have identified situations in which some of the *S. cerevisiae* homologues of the mammalian co-factors become essential: in the cold, with microtubule depolymerizing drugs, with mutations that affect α to β tubulin ratios, or in combination with mutations in tubulin encoding genes (including *tub1-724*). We think that *tub1-724* cells require *CIN1*, *PAC2* and *RBL2* for viability because the heterodimer is less stable in these cells. Under these circumstances, a salvage pathway to recycle unpartnered tubulin chains may be essential. In wild-type cells this pathway is not important because most of the heterodimer remains intact. Cold temperatures, or microtubule depolymerizing drugs, may affect the stability or the structure of the tubulin heterodimer. In our analysis we found several alleles of α-tubulin that are lethal in combination with

deletions in *PAC2*, *CIN1*, and *RBL2*. All of the alleles fall into the class of mutant α-tubulins that arrest with no microtubules at the non-permissive temperature. It will be of interest to see if any of these α-tubulin mutants also affect heterodimer formation and/or stability.

We presume that heterodimer breathing occurs *in vivo*. Yet to our knowledge no one has tested whether this occurs. We have in the laboratory reagents to test whether heterodimer exchange occurs --antibodies to two distinct forms of β-tubulin. By using a strain that contains wild-type versions of both *TUB1* and *TUB2* and in addition contains an inducible copy of *tub2-590* we can test whether newly synthesized monomers can exchange into pre-existing heterodimer. The cellular tubulin can be radiolabelled with S^{35}methionine under non-inducing conditions. After chasing the with cold methionine we could briefly induce *tub2-590* expression. Antibody #339 is specific for *tub2-590* , and can be used to immunoprecipitate *tub2-590* heterodimer. We could determine whether the newly synthesized *tub2-590* is able to associate with the labeled α-tubulin. If we find that heterodimer exchange occurs *in vivo*, we could look at genetic configurations that affect this exchange. Or look for a change in the rate of exchange. For example, we could look in cells deleted for Cin1p or overexpressing Cin1p to see if Cin1p affects this process.

<u>How does Rbl2p rescue cells from β-tubulin lethality</u>?

We know much about the properties of Rbl2p. However, the mechanism by which Rbl2p rescues cells from β-tubulin poisoning remains unclear. Preliminary work by Julie Archer, shows that the percentage of cells rescued by Rbl2p depended on the

amount of Rbl2p. The plating efficiency of β-tubulin overexpressing cells containing Rbl2p under the control of the galactose promoter is an order of magnitude higher that cells containing a genomic copy or Rbl2p. Kate Compton has extended this analysis and has found that more subtle changes in Rbl2p levels affects the ability of β-tubulin rescue by Rbl2p.

The ability of Rbl2p to rescue excess β-tubulin lethality likely requires binding of β-tubulin *in vivo*. Kate Compton has found that *in vivo*, only a small amount of Rbl2p is complexed to β-tubulin in cells overexpressing both proteins. Only in *tub1-724* cells does she find a large pool of the Rbl2p-β-tubulin complex. Kate Compton has preliminary evidence which suggests that Rbl2p appears to be unstable *in vivo*, when it is not complexed to β-tubulin. She also finds that rescue of β-tubulin overexpression by Rbl2p requires that Rbl2p must be co-translated with β-tubulin, perhaps because the unpartnered Rbl2p is degraded.

The question remains, how does Rlb2p act to rescue cells from free β-tubulin? Rbl2p might be part of a buffering system that prevents accumulation of significant, toxic amounts of undimerized β-tubulin. We know that even a slight excess of β-tubulin affects essential functions. Those imbalances can be generated by perturbations in expression levels or as a consequence of α-β tubulin dissociation such as in cells expressing the mutant α-tubulin, *tub1-724*. We are investigating whether cells encounter such imbalances under normal circumstances as well . A recent comprehensive study of cell-cycle regulated genes in S. cerevisiae shows that

tubulin message may be cell cycle regulated (Spellman et al., 1998). This study found that *TUB2* message peaks in G2. In addition, they found *TUB1* also peaks in G2 albeit to lower levels than *TUB2*, and *TUB3* showed little to no difference throughout the cell cycle (Spellman et al., 1998). We are interested to know whether Rbl2p levels increase in response to alterations in the ratio of α to β tubulin. Will Chen and I are looking at whether Rbl2p is upregulated in cells with alterations in tubulin levels or in response to microtubule depolymerizing drugs. It will be interesting if indeed Rbl2p levels are upregulated *in vivo*. Perhaps, Rbl2p activity might be important during α-β heterodimer breathing or during heterodimer degradation.

What does benomyl do to microtubules *in vivo*.

Mutations in many of the genes that affect microtubule function *in vivo*, are supersensitive to the microtubule depolymerizing drug, benomyl. In many organisms, mutations that confer resistance to benomyl map to the β-tubulin encoding gene. Since both β-tubulin overexpression and benomyl toxicity are rescued by overexpression of either α-tubulin or Rbl2p (Schatz et al 1988; Archer et al 1995), we hypothesized that perhaps benomyl caused the α-β heterodimer to dissociate and release free β-tubulin. Some experiments not described in this thesis suggest that benomyl does not cause the heterodimer to fall apart in wild-type cells (unpublished results). However, it is still possible that the interaction of benomyl with tubulin causes a more subtle effect on the conformation β-tubulin.

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APPENDIX

Publications

Cell, Vol. 82, 425–434, August 11, 1995.

Rbl2p, a Yeast Protein That Binds to β-Tubulin and Participates in Microtubule Function In Vivo

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Summary

Genetic configurations resulting in high ratios of β-tubulin to α-tubulin are toxic in S. cerevisiae, causing microtubule disassembly and cell death. We identified three non-tubulin yeast genes that, when overexpressed, rescue cells from excess β-tubulin. One, *RBL2*, rescues β-tubulin lethality as efficiently as does α-tubulin. Rbl2p binds to β-tubulin in vivo. Deficiencies or excesses of either Rbl2p or α-tubulin affect microtubule-dependent functions in a parallel fashion. Rbl2p has functional homology with murine cofactor A, a protein important for in vitro assays of β-tubulin folding. The results suggest that Rbl2p participates in microtubule morphogenesis but not in the assembled polymer.

Introduction

Cytoskeletal structures are constructed from a few basic polymers that are notable for the stringent and detailed conservation of their ultrastructure. Those polymers occur, however, in arrays with a wide range of geometries and functions. For example, microtubule organizations differ dramatically among cell types. Even in a single cell type, the microtubule arrays can vary in form and extent of assembly during development or upon passage through the cell cycle. An unresolved issue is an understanding of how cells specify the quantitative and qualitative variations in cytoskeletal assembly.

Regulation of microtubule assembly could occur at any of several places along the pathway. Divergent domains in the primary sequence of tubulin subunits could be crucial (Fuller et al., 1987). The amount of the individual subunits (Cleveland et al., 1981) and folding of the polypeptides to form assembly-competent dimers (Yaffe et al., 1992) may also be important. A variety of experiments demonstrate that activities that nucleate microtubule assembly (Oakley et al., 1990) and that stabilize microtubules by binding along their lengths (Caceres and Kosik, 1990; Dinsmore and Solomon, 1991) can contribute to microtubule function. The precise role and detailed mechanism of action of each of these factors are not yet well understood, nor is their contribution to the regulation of microtubule structure.

Genetic approaches provide valuable tools to identify important steps and essential components of morphogenetic pathways in vivo. A standard tool is the analysis of interacting mutations. An early and successful application of this sort of analysis is crucial to our understanding of phage assembly. Isolation of second-site revertants of mutant components identified interacting structural partners such as genes *1* and *5* in bacteriophage P22 (Jarvik and Botstein, 1975). For microtubules, second-site revertants of tubulin mutants identified γ-tubulin, a presumably ubiquitous and essential component of the microtubule-organizing center (Zheng et al., 1991), as well as proteins that may act along the length of microtubules (Pasqualone and Huffaker, 1994). This approach has been particularly useful in identifying genes that affect actin assembly in yeast (Adams and Botstein, 1989; Adams et al., 1989). These suppression events are likely to represent physical interactions.

An alternative genetic approach to a qualitative suppression analysis derives from quantitative considerations. The assembly of complex structures can require coordinated participation of multiple elements, some at intermediate steps and some in the final product. Again, phage studies demonstrate that successful assembly of complex structures may be sensitive to the relative levels of those components and require precise stoichiometries; an abnormal stoichiometry can lead to formation of aberrant and poisonous intermediates. For example, amber mutations in the T4 tail fiber gene *18* result in a lowered expression level of product, and mature phage progeny are not produced. Suppressors of this defect include amber alleles of interacting components (tail base plate genes) that result in lower, balanced levels of expression of the two components (Floor, 1970). This interaction is interpretable if one considers that the two gene products ordinarily interact and that a deficit in one of them leaves the other free to form otherwise forbidden interactions that lead to defects in assembly. Normal assembly, then, depends not on the absolute level of the gene products but rather a balance of components (Floor, 1970; Sternberg, 1976). The same sort of reasoning explains the requirement for balanced expression of histone proteins to produce normal chromosome segregation in the yeast Saccharomyces cerevisiae (Meeks-Wagner and Hartwell, 1986).

The details of tubulin expression in yeast present an opportunity to apply this analysis to microtubule assembly (Weinstein and Solomon, 1992). Genetic configurations that result in an increase in the ratio of β-tubulin to α-tubulin relative to wild-type cells are either toxic or lethal (Burke et al., 1989; Katz et al., 1990; Schatz et al., 1986). When β-tubulin is overproduced using an inducible galactose promoter on a 2μ (multicopy) plasmid, cells lose their microtubules within 1.5 hr, as assayed by immunofluorescence. Only 1% of the cells are viable after 4 hr, at which time the β-tubulin protein levels have only increased 2- to 4-fold. In contrast, the galactose-mediated induction of α-tubulin on a high copy plasmid does not affect microtubule assembly and becomes modestly toxic only after many hours and much higher levels of expression. However, restoration of the balance between α- and β-tubulin levels, by simultaneous overexpression, rescues both the

microtubule and cell lethality phenotypes associated with excess β-tubulin (Weinstein and Solomon, 1990).

It is not clear why β-tubulin in the absence of its normal partner, α-tubulin, affects microtubules and, presumably as a result, causes cell death. It may compete with αβ-tubulin heterodimers for growing ends of microtubules or for microtubule-associated proteins. It also may poison the nucleation site: shortly after the microtubules disappear in cells overexpressing β-tubulin, small foci of anti-β-tubulin but not anti-α-tubulin staining appear near the nucleus (Weinstein and Solomon, 1990); those dots colocalize with spindle pole body staining, using the anti-90 kDa spindle pole body component described by Rout and Kilmartin (1991) (M. Magendantz and F. S., unpublished data). By sequestering stabilizing factors or blocking nucleation sites, β-tubulin polypeptides may preclude native microtubule structure.

To identify proteins that interact with β-tubulin, we designed a screen to find genes whose products suppress the lethality associated with β-tubulin overexpression. Our rationale was that the overproduction of the target of β-tubulin, or more generally any β-tubulin-binding protein, would titrate the excess polypeptide and so allow polymer assembly and cell viability. We have identified three genes encoding proteins other than α-tubulin whose overexpression suppresses excess β-tubulin toxicity. One of them, here called *RBL2* (for rescues excess β-tubulin lethality), encodes a protein that rescues the excess β-tubulin phenotype as efficiently as does α-tubulin. Rbl2p is a β-tubulin-binding protein (the second identified, after α-tubulin). Its properties in vivo are similar to those of α-tubulin, and its levels affect microtubule functions. Rbl2p is a structural and functional homolog of cofactor A, a protein identified as necessary for an in vitro assay of tubulin folding (Gao et al., 1993, 1994). The results are consistent with an activity for Rbl2p in microtubule assembly at a step after folding but before dimerization.

Results

A Screen for Non-Tubulin Components of the Microtubule Assembly Pathway

To identify gene products that interact with β-tubulin, we screened for cDNAs that when overexpressed allowed cells to grow in the presence of excess β-tubulin. JAY47 is a diploid strain into which we integrated a third copy of the *TUB2* gene under control of the galactose promoter. This strain is indistinguishable from its wild-type parent in glucose, but in galactose it rapidly loses microtubule staining, arrests as large-budded cells, and dies with a half-life essentially identical to strains bearing pGAL-TUB2 on a 2μ plasmid (Weinstein and Solomon, 1990). We transformed a pGAL1-10-promoted yeast cDNA library (Liu et al., 1992) into JAY47 and selected colonies that were able to survive on plates with galactose as their sole carbon source (see Experimental Procedures). We isolated the plasmids from the suppressed JAY47 cells and sequenced the cDNA inserts.

The suppressing cDNAs encoded both of the yeast α-tubulins, Tub1p and Tub3p, and three other proteins. We have named the non-tubulin genes *RBL1*, *RBL2*, and *RBL3*. We evaluated their effectiveness as suppressors by comparing the number of colonies that arise on galactose (inducing) plates versus those on glucose (noninducing) plates (Table 1). For JAY47 cells containing a control plasmid, that ratio is 0.01%. By this assay, *RBL2* is as good a suppressor as either α-tubulin gene, *TUB1* or *TUB3* (Table 1); 70% of the *RBL2*-suppressed JAY47 cells can form colonies on galactose. The colonies are robust and uniform in size. Both *RBL1* and *RBL3* confer intermediate values of suppression (1% and 3%, respectively), and in both cases there is some variability in the size of the colonies. These characteristics of *RBL* suppression argue against a model in which there is a constant probability of death at each cell division, since that circumstance would predict a high percentage of colonies when growing on galactose, although small in size. An alternative explanation is that cells plated in galactose could face an early event at which the suppressed state can be established and thereafter maintained. In this sense, the effectiveness of the suppressors reported in Table 1 is a measure of their ability to establish suppression at early times.

The Sequences of the *RBL*s Suggest Different Functions

We cloned the genomic version of each of the *RBL* cDNAs and determined that each represented its full-length transcript. *RBL1* bears no homology to any sequence available in the database. The sequence of *RBL3* was entered in the database during the course of this study under the names *TIF3* (Altmann et al., 1993) and *STM1* (Coppolecchia et al., 1993). The gene product of *RBL3* is similar to human translation factor eIF4-B, although a direct assay of initiation activity is not yet available. Rbl2p is 32% identical and 61% similar at the level of predicted amino acid sequence to mouse cofactor A (Figure 1). Cofactor A is a necessary but not sufficient component required for α- and β-tubulin release from the chaperone t-complex polypeptide 1 (TCP-1) in a form competent for exchange into exogenous bovine tubulin dimer (Gao et al., 1993, 1994).

Table 1 *RBL1*, *RBL2*, and *RBL3* Suppress JAY47 Lethality

Plasmid	Number of Isolates	Colonies on Galactose / Colonies on Glucose
YCpGAL	NA	0.0001
TUB1 and *TUB3*	95	0.7
RBL1	1	0.01
RBL2	31	0.7
RBL3	1	0.03

Of 8.1 × 10^5 JAY47 cells containing the pGAL cDNA library plated on galactose medium, 950 survived to grow into colonies. Of these, 194 were plasmid dependent, and we have isolated 146 of these plasmids. The number of isolates column lists the representation of *TUB1* and *TUB3* and *RBL1*, *RBL2*, and *RBL3* among the plasmids recovered. The remaining 18 fail to suppress when retransformed into JAY47. Upon retransformation of the *TUB* and *RBL* plasmids, we determined their extent of suppression by plating cells to galactose (inducing) and glucose (noninducing) plates and comparing colony-forming units.

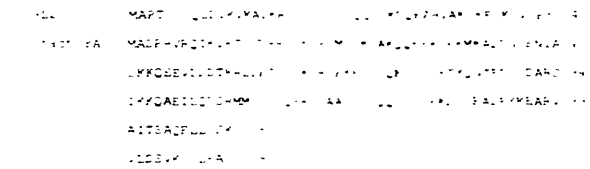


Figure 1. Yeast Rbl2p and Murine Cofactor A Are 32% Identical

Comparison of predicted complete amino acid sequences of Rbl2p and cofactor A by the Genetics Computer Group program BESTFIT. Sequences are 32% identical and 61% similar across their entire lengths.

Effect of Overexpressing *RBL* Genes on Tubulin Levels

A potential mechanism for suppression of β-tubulin lethality is diminished accumulation of excess β-tubulin polypeptide due to effects at any point in its synthesis or on its stability. None of the *RBL*s appear to act in this manner. Protein samples harvested from galactose-induced JAY47 cells suppressed with each of the *RBL* plasmids contain an increased level of β-tubulin relative to noninduced cells, as judged by Western blot (Figure 2A). The result suggests that overproduced *RBL* gene products act by rendering the excess β-tubulin protein nontoxic to the cells. The α-tubulin levels remain constant for *RBL1* and *RBL2*, but increase modestly in cells with *RBL3* (Figure 2B). However, in wild-type cells, overexpressing *RBL3* does not increase steady-state levels of α-tubulin (data not shown), so we do not know whether it represents a direct effect on α-tubulin synthesis

Our preliminary characterization suggests that the three *RBL* genes may act in quite different ways. We have chosen to focus on *RBL2*, which is as effective a suppressor as a previously known β-tubulin-interacting gene, *TUB1*.

Specificity of Genetic and Physical Interactions between Rbl2p and Tub2p

Excess Rbl2p does not act as a general suppressor of lethality resulting from the overexpression of other cytoskeletal genes. In particular, overexpression of Rbl2p does not rescue cells overexpressing either *ACT1* (encoding actin) or *TUB1* (α-tubulin; data not shown). This specificity and the similarity between the efficiency of suppression displayed by Rbl2p and α-tubulin suggest that Rbl2p may interact physically with the β-tubulin polypeptide.

The specificity of the genetic interaction is recapitulated by the results of immunoprecipitations from cells overexpressing Rbl2p and either α- or β-tubulin. We prepared total cell protein extracts from cells overexpressing both *RBL2* and either *TUB2* (JAY286) or *TUB1* (JAY381). We see Rbl2p expression increase by approximately 30-fold when induced behind a galactose promoter (data not shown). Each extract was incubated with antibodies against α- or β-tubulin or against Rbl2p, and the resulting precipitates were analyzed by immunoblots with antibodies against all three proteins (Figure 3). The antibodies against each of the tubulin polypeptides bring down the other chain with high efficiency. The results also demonstrate that approximately 5%–10% of total Rbl2p coimmunoprecipitates with β-tubulin when both are overexpressed in the same cells. In contrast, only 0.5% or less coprecipitates with anti-α-tubulin antibodies when those

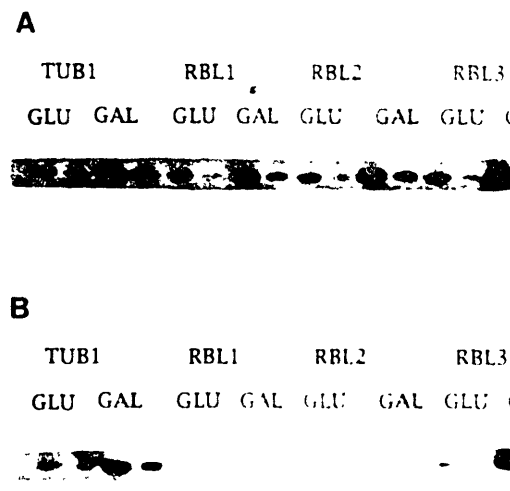


Figure 2 Levels of β- and α-Tubulin in Suppressed JAY47 Cells

JAY47 cells (diploids with an integrated pGAL-TUB2) containing pGAL-TUB1, pGAL-RBL1, pGAL-RBL2, or pGAL-RBL3 CEN plasmids were plated to galactose or glucose plates We harvested colonies from galactose plates after 2 5 days or from glucose plates after 1 5 days and prepared total protein extracts Samples representing 2× and 1× loads normalized to cell number were analyzed on 7 5% SDS–polyacrylamide gels After transfer to nitrocellulose, β-(A) and α-tubulin (B) levels were assessed by Western blot using the polyclonal antibodies 206 and 345, respectively

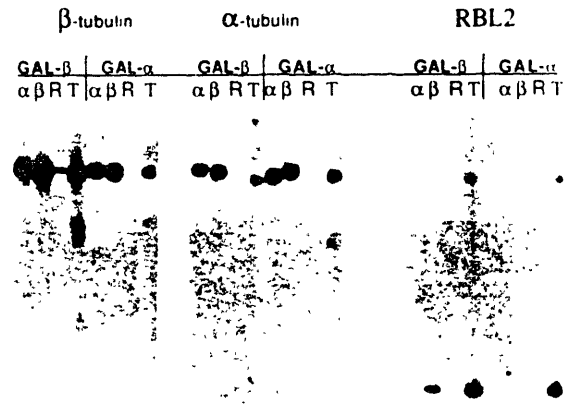


Figure 3. Rbl2p Coimmunoprecipitates with β-Tubulin

Cells containing inducible *RBL2* (CEN plasmid) and either inducible *TUB2* (JAY286) or *TUB1* (JAY381) on 2μ plasmids were grown in raffinose and then shifted to 2% galactose for 8 hr. Total protein extracts and relevant immunoprecipitates were analyzed by immunoblotting after resolution on three parallel 12% polyacrylamide gels. β-Tubulin, α-tubulin, and RBL2 indicate the antibodies used for blotting; GAL-β and GAL-α are the strains co-overexpressing either Rbl2p and β-tubulin or Rbl2p and α-tubulin, respectively. Lanes contain protein precipitated with anti-α-tubulin (α), anti-β-tubulin (β), or anti-Rbl2p (R). Lane T contains total cell protein, representing one-fourth the material in the immunoprecipitates

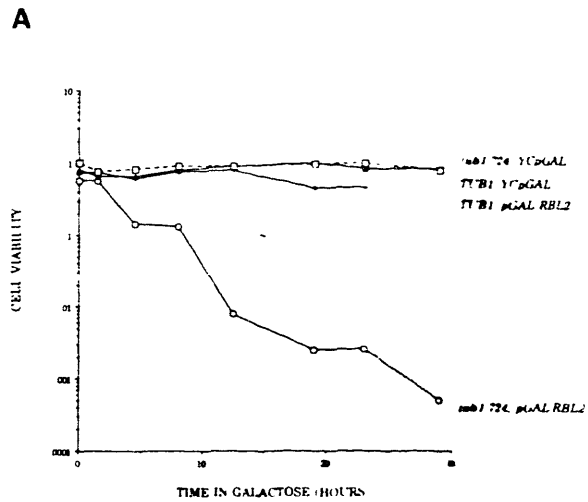


Figure 4 Synthetic Interaction of *RBL2* Overexpression with *tub1-724*

(A) Haploid cells contain two plasmids each either *TUB1* or *tub1-724* on a CEN plasmid as their only source of α tubulin and either inducible *RBL2* or YCpGAL (control) CEN plasmid These strains were grown overnight in selective raffinose media at 30°C At t = 0 hr, galactose was added to 2% Cell viability equals the number of colonies arising on glucose plates divided by cell number counted in a light microscope (B and C) At t = 5 hr in galactose, *tub1-724* cells containing either control (B) or *pGAL-RBL2* (C) plasmids were fixed and processed for immunofluorescence with anti-β-tubulin antibody 206 In control cells, there are a variety of tubulin staining patterns In cells overexpressing Rbl2p, large-budded cells contain little or no localized staining

two proteins are overexpressed. The same specific association is apparent when anti-Rbl2p antibodies are used. Much more β-tubulin than α-tubulin is present in anti-Rbl2p precipitates from the respective overproducing strains. We precipitate the same specific complex, although with lower efficiency, in strains overexpressing only Rbl2p but none of the tubulin genes. In strains not overexpressing Rbl2p, we fail to detect coimmunoprecipitation, probably because endogenous levels of Rbl2p are so low.

We can detect no colocalization of Rbl2p with assembled microtubule structures in cells. In both wild-type cells and in strains overproducing Rbl2p, antibodies against the protein do not give a discretely localized signal by immunofluorescence microscopy (data not shown). Instead, anti-Rbl2p antibodies do stain Rbl2p-overexpressing cells very brightly, suggesting that the antibodies can recognize cellular Rbl2p after fixation. We conclude that the failure to detect a discrete signal probably reflects a diffuse localization of the protein. Therefore, the apparent association of Rbl2p and β-tubulin is likely to occur with unassembled tubulin chains rather than assembled microtubules.

Microtubule Defects Are Sensitive to the Level of Rbl2p

The overproduction of Rbl2p in wild-type cells leads to a modest loss of viability. After 10 hr of induction, about 80% of the cells are not viable, but the effect levels off at that point. However, overexpression of Rbl2p in some backgrounds with compromised microtubules greatly enhances this lethality. For example, we previously described a panel of α-tubulin mutants (Schatz et al., 1988), several of which are conditional lethals that arrest with no microtubules at low temperature and are supersensitive to the microtubule-depolymerizing drug benomyl at permissive temperatures. Overexpression of Rbl2p at permissive temperature in one such mutant strain, *tub1-724*, causes rapid and nearly complete cell death (Figure 4A). One other *tub1* allele, *tub1-728*, shows a similar loss of viability when Rbl2p is overexpressed, while several *tub1* alleles show no such interaction (Table 2). This lethal interaction also causes a dramatic loss of microtubules. Figure 4 also shows immunofluorescence micrographs of *tub1-724* in the absence (Figure 4B) or presence (Figure 4C) of excess Rbl2p for 5 hr.

The phenotype of *RBL2* overexpression is recapitulated by *RBL2* null alleles. *RBL2* is not essential for mitotic growth, but it has a synthetic lethal phenotype in combination with four *tub1* alleles, but not with four others (Table 2). Two of those four alleles that do interact genetically with the Δ*RBL2* null, *tub1-724* and *tub1-728*, are the ones that enhance the lethality of excess Rbl2p.

Table 2. Synthetic Lethality of *RBL2* Overexpression and Null Strains

Allele	*RBL2* Overexpression	Δ*RBL2*
tub1-724 and *tub-728*	–	–
tub1-736 and *tub-759*	+	–
tub1-704, tub-714, tub-744, and *tub-750*	+	+
tub1-727, tub-730, tub-733, tub 741, tub-746, and *tub-758*	+	ND

Ability of mutants to grow at permissive temperatures upon induction of pGAL-RBL2 or in *RBL2* nulls. ND, not determined.

That both excess and absence of the *RBL2* gene product affect viability and probably microtubule assembly in these different genetic backgrounds suggests that it acts as a structural rather than catalytic element in microtubule assembly. In addition, the allele specificity of the interaction with mutant *tub1* alleles indicates that the combinatorial defect represents a more proximal functional interaction than simply two defects in unrelated processes.

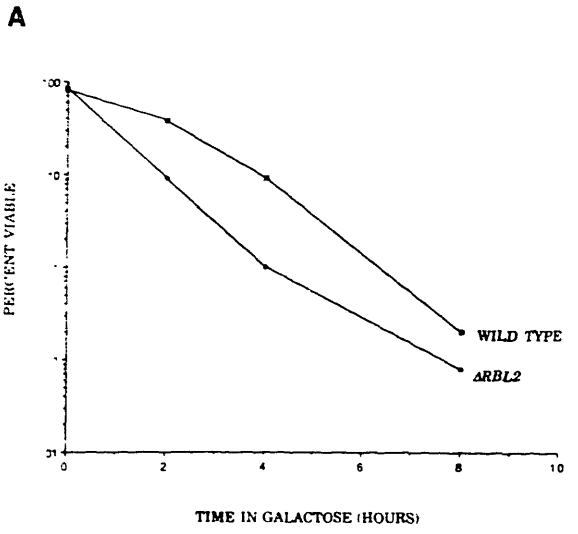
The Stoichiometry of Rbl2p to Tubulin Is Critical

As noted above, any genetic configuration that results in an excess of β-tubulin over α-tubulin is toxic. Changes in the level of Rbl2p expression affect the phenotypes associated with changes in α-to-β tubulin ratios. Overexpression of Rbl2p suppresses excess β-tubulin lethality; similarly, when overexpression of β-tubulin is induced in strains bearing a deletion in *RBL2*, the cells lose viability with more rapid kinetics than strains wild type for *RBL2* (Figure 5A).

In the converse direction, extra Rbl2p also rescues the phenotypes produced by creating a modest deficit in α-tubulin. Strains bearing a deletion of the quantitatively minor α-tubulin gene, *TUB3*, are viable but benomyl supersensitive (Schatz et al., 1986). The enhanced sensitivity to this microtubule-depolymerizing drug is suppressed by a modest increase in Rbl2p (Figure 5B). This result fulfills our expectation that excess β-tubulin is lethal because of its stoichiometry relative to α-tubulin rather than its absolute level. Therefore, Rbl2p levels appear to compensate for the defects associated with either too much β-tubulin or too little α-tubulin.

Rbl2p Levels Affect Cellular Sensitivity to the Microtubule-Depolymerizing Drug Benomyl

In a sense, the phenotypes of excess β-tubulin mimic those of benomyl; both lead to loss of microtubules, cell cycle arrest as large-budded cells, and death. This parallel is supported by the consequences that changes in the level of either Rbl2p or α-tubulin have on either poison. Extra Rbl2p or α-tubulin (Schatz et al., 1986) produced by galactose induction confers resistance to benomyl (Figure 6A). Conversely, the absence of Rbl2p renders cells more sensitive to the drug (Figure 6B). A modest decrease in α-tubulin levels produced by disruption of the *TUB3* gene also produced supersensitivity to benomyl (Schatz et al., 1986).



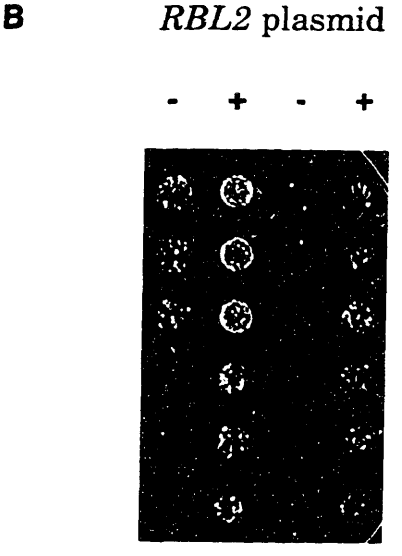


Figure 5. Phenotypic Consequences of Altered Stoichiometry between Rbl2p and Tubulin

(A) Effects of Rbl2p levels on sensitivity to β-tubulin overexpression. Haploid cells with an integrated copy of inducible *pGAL-TUB2* (derivatives of JAY47) either wild type for *RBL2* or bearing a null allele were grown and analyzed for viability as described in Figure 4.

(B) Effects of excess Rbl2p on the benomyl sensitivity of cells with a deficit in α-tubulin. Δ*TUB3* haploids (FSY21) containing either control (minus) or genomic *RBL2* (plus) on a CEN plasmid were serially diluted on plates containing 10 μg/ml benomyl. Dilutions were by halves, beginning at 10^6 cells per milliliter in the first two columns and at 10^7 cells per milliliter in the second two columns.

The phenotypes of *RBL2* overexpression do not appear to be manifestations of multidrug resistance because they do not include resistance to several other drugs, such as cycloheximide and ethidium bromide (data not shown). This phenotype is further remarkable because it is uncommon; enhanced resistance to this drug suggests a degree of specificity for microtubule function not inherent in enhanced sensitivity.

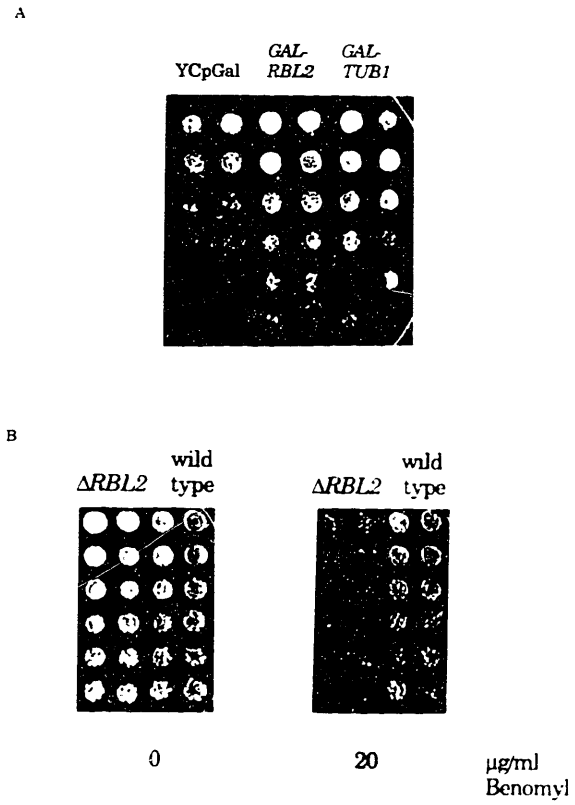


Figure 6 Levels of Rbl2p Affect Growth on Benomyl, a Microtubule-Depolymerizing Drug

(A) Diploids cells containing control (YCpGAL), pGAL-RBL2, or pGAL-TUB1 CEN plasmids were serially diluted on selective galactose plates with 20 μg/ml benomyl. Dilutions were by halves, beginning at 10^7 cells per milliliter.

(B) *ΔRBL2* haploid cells containing control or genomic *RBL2* CEN plasmids were serially diluted as in (A) on 0 and 20 μg/ml benomyl plates.

Rbl2p Is Important for Meiosis

Although *RBL2* is not essential for mitosis, it is necessary for cells to complete sporulation and meiosis successfully. We made diploids that were heterozygous or homozygous for *ΔRBL2*. Both grew normally. However, the *ΔRBL2* homozygotes sporulate abnormally; they produce asci with only slightly reduced efficiency, but substantially fewer of those asci contain four spores (Table 3). Those spores vary significantly in size and are arranged in a disorderly fashion. In contrast, the heterozygotes sporulate comparably to wild-type diploids. We examined this defect with an assay for haploid spores, using the difference in color between *ADE2* and *ade2* colonies. We sporulated strains that were heterozygous at the *ADE2* locus. Colonies from either *ADE2* haploids or unsporulated *ADE2/ade2* diploids are white; cells bearing only the *ade2* allele are red. We recovered no red colonies from the homozygous *RBL2* nulls. The same strain containing *RBL2* on a low copy plasmid produced red colonies at an efficiency indistinguishable from wild type (35%–40%).

A Functional Homology between Rbl2p and Murine Cofactor A

The predicted protein sequence of Rbl2p is approximately 30% identical to mouse cofactor A across their entire lengths (see Figure 1). Cofactor A is thought to participate in chaperonin-mediated folding of β-tubulin in vitro (see Discussion). To determine the relationship between Rbl2p and cofactor A, we expressed mouse cofactor A in yeast. Like excess Rbl2p, overexpression of this sequence in yeast cells confers substantial resistance to excess β-tubulin lethality (Figure 7A); the efficiency of suppression is approximately 5%, compared with 70% for the yeast protein. In addition, murine cofactor A suppresses the benomyl supersensitivity associated with deletions of *RBL2* (Figure 7B). These results suggest that cofactor A performs overlapping functions with Rbl2p.

Discussion

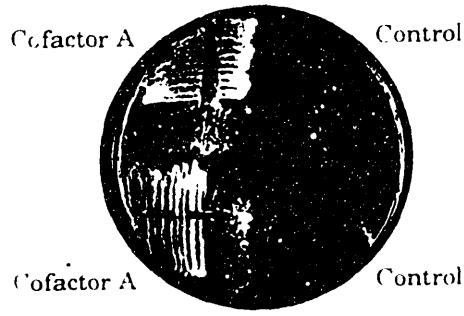
We identified Rbl2p in a screen for proteins that, when overexpressed, protect cells from the deleterious effects of β-tubulin overexpression. We envisioned at least two possible sorts of suppressing elements that would answer this screen. One might be a protein with which excess β-tubulin interacts to cause microtubule disassembly, which might include associated proteins or nucleating elements, but also might include tubulin itself. For example, γ-tubulin acts as a nucleator of microtubules and interacts genetically with β-tubulin (Oakley and Oakley, 1989). However, overexpression of the presumptive yeast γ-tubulin *TUB4* (L. Marschall and T. Stearns, personal communication) does not rescue β-tubulin lethality (data not shown). Another suppressor might be a protein with which undimerized β-tubulin interacts normally, as part of the morphogenetic pathway. The interaction between actin mono-

Table 3 *ΔRBL2/ΔRBL2* Cells Have a Defect in Sporulation and Meiosis

Strain	Plasmid	Percent Asci	Spores per Ascus				
			Four	Three	Two	One	Zero
Wild type	None	71	69	9	9	2	11
ΔRBL2/ΔRBL2	*RBL2*	56	47	13	12	4	24
ΔRBL2/ΔRBL2	Control	49	18	24	18	12	28

After 4 days in sporulation media (1% potassium acetate [pH 7]) at room temperature, each population was scored for the number of spores within asci. We designated cells as asci based on their rounded shape and thickened cell coat and counted the number of spores contained within. The category of zero spores per ascus met the criteria for an ascus but looked either empty or murky inside. The strains are either wild type (FSY185) or *ΔRBL2/ΔRBL2* homozygotes containing either a *RBL2* genomic or control CEN plasmid. We counted >300 cells for each.

A



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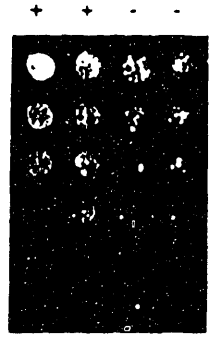

Figure 7 Overexpression of Murine Testes Cofactor A Has Phenotypes Reminiscent of Rbl2p in Yeast

(A) JAY47 cells containing pGAL cofactor A (CEN plasmid) are suppressed relative to those containing control CEN plasmid on galactose plates

(B) *ΔRBL2* haploids transformed with pGAL cofactor A (plus) or control (minus) CEN plasmid serially diluted on galactose benomyl (25 μg/ml) plates by halves, beginning at 10 cells per milliliter

mers and profilin may serve as a precedent (Magdolen et al., 1993). The experiments described above suggest that Rbl2p may be in the second class of potential suppressors. The suppression and immunoprecipitation data argue for an intimate interaction between Rbl2p and β-tubulin The binding of Rbl2p to β-tubulin probably explains the suppression of excess β-tubulin by Rbl2p The aggregate genetic evidence suggests that Rbl2p acts along the microtubule assembly pathway But the fact that Rbl2p cannot be detected in the end product of this pathway, assembled microtubules, implies that it participates in some intermediate along the pathway

Scaffolding Proteins and Surveillance Functions

Assembly of bacteriophage proceeds through intermediates that can contain scaffolding proteins, elements that participate in the maturation of the particle, but which are not incorporated into the final structure For instance, the prohead of P22 grows around a core of gp8 molecules that are later replaced by DNA while the coat proteins expand to arrange themselves as the head (King et al., 1973). Some properties of Rbl2p suggest that it may act as a scaffolding protein in microtubule assembly. For example, the stoichiometry of a scaffolding protein would be predicted to be important for ensuring proper assembly. Although Rbl2p is not essential for mitosis, aberrant levels of the protein are deleterious when microtubule integrity is impaired: genetic backgrounds bearing mutant α-tubulins or in the presence of benomyl. However, there is no evidence that scaffolding proteins are required in the formation of microtubule polymer from an αβ heterodimer. No intermediates involving transiently associated proteins have been identified, and, at least in vitro, tubulin heterodimers can self-assemble. Perhaps, then, Rbl2p acts between the synthesis of the β-tubulin polypeptide and its incorporation into active dimer. The physical interaction with β-tubulin and the specificity of the suppressor activity of excess Rbl2p for β-tubulin are consistent with Rbl2p binding to the β-tubulin monomer. Alternatively, Rbl2p could act transiently during remodeling of microtubules among different organizational states. In any of these sites of action, Rbl2p might mediate progress through the assembly process, like a phage scaffolding protein.

Another possibility is that the screen that identified Rbl2p represents its function in cells: Rbl2p might act as a β-tubulin ligand to suppress deleterious interactions under aberrant conditions in which β-tubulin monomers occur. Such a surveillance function could quite adequately be executed by α-tubulin itself. However, significant excesses of α-tubulin are deleterious and do not persist (Katz et al., 1990, Weinstein and Solomon, 1990). In addition, a role for a β-tubulin-binding protein may become more important under special circumstances, for example, when the α-tubulin gene product is compromised in mutant *tub1* strains

Functional Similarities between Rbl2p and α-Tubulin

We find striking similarities between the properties of Rbl2p and α-tubulin in vivo (Figure 8). The ability of Rbl2p, like α-tubulin, to bind β-tubulin is itself strong evidence of function. An excess of either protein suppresses β-tubulin lethality and confers resistance to the microtubule-depolymerizing drug benomyl. Deficiencies of either enhance sensitivity to benomyl (Schatz et al., 1986). Extra Rbl2p can actually compensate for a quantitative defect in α-tubulin: cells lacking the minor α-tubulin gene are supersensitive to benomyl, but are rescued by genomic *RBL2* on a low copy plasmid. Finally, the phenotypes of α-tubulin mutants are strongly affected by levels of Rbl2p. In sum, Rbl2p is a β-tubulin-binding protein in vivo, and that binding is detectable both physically and functionally.

The benomyl resistance could be explained if that drug acts by promoting dissociation of the αβ dimer to release free β-tubulin that poisons the microtubules in the cell. However, two other suppressors of excess β-tubulin, *RBL1* and *RBL3*, fail to confer benomyl resistance. Another possibility is that the binding of benomyl to tubulin creates toxic drug–dimer complexes analogous to those that may

	LEVEL OF Rbl2p OR α-TUBULIN EXPRESSION		
	DEFICIENCY	NORMAL COMPLEMENT	EXCESS
Condition:			
NORMAL	< *	=	< *
tub1	< ^	=	< ^
α-OVEREXPRESSION	< ^	=	> *
tub3	n.d.	=	> *
BENOMYL	< *	=	> *

Figure 8 Alterations in the Level of Rbl2p or α-Tubulin Have Similar Effects on Cell Growth Under a Variety of Conditions

The lesser-than symbol denotes inhibition of growth by a change in the level of Rbl2p relative to the normal complement; the greater-than symbol represents better growth. The asterisk denotes those circumstances in which changes in the level of α-tubulin have the same effects as Rbl2p. The carat indicates those experiments not done (n.d.) with α-tubulin.

be the active species in inhibition of microtubule assembly by colchicine (Skoufias and Wilson, 1992). These toxic complexes might be sequestered by the activity of excess Rbl2p or α-tubulin.

The meiotic requirement for Rbl2p may be due to a greater reliance on events that are in fact common to both meiosis and mitosis. Cells undergoing mitosis even in the absence of Rbl2p may be safely above a threshold for an essential component, for example, an assembly-competent tubulin dimer or a Rbl2p functional homolog, whereas meiosis may change that threshold so that the contribution of Rbl2p becomes required. Cells may be more sensitive to free β-tubulin during meiosis. Alternatively, Rbl2p may perform an essential meiosis-specific function. We note that although cofactor A mRNA is present in many mouse tissues, it is most abundant in testes (Gao et al., 1993). Although this observation originally was explained as reflecting a role for cofactor A in constructing sperm flagellae, instead it may reflect an increased dependence on mouse cofactor A in meiosis itself.

A Comparison with Cofactor A

Cofactor A is a polypeptide that, together with a fraction called cofactor B, is necessary for the release of α- and β-tubulin from the chaperone TCP-1 in a form competent for exchange into exogenous bovine tubulin heterodimer (Gao et al., 1993, 1994). TCP-1 alone can bind both unfolded actin and γ-tubulin polypeptides and release them in a form that migrates normally on a native gel. TCP-1 and TCP-1-like proteins may play an important role in actin and tubulin function in vivo. Complexes containing TCP-1 and actin or tubulin can be isolated from animal cells (Sternlicht et al., 1993). Strains bearing mutant alleles of TCP-1 homologs can exhibit microtubule and actin phenotypes in yeast (Chen et al., 1994; Vinh and Drubin, 1994). In the in vitro assay, proper tubulin folding is assayed not by the measure of folded monomer, but instead by incorporation of the monomer into tubulin dimers. It requires the addition of native tubulin dimers. The presence of cofactor A results in the appearance of β-tubulin dissociated from TCP-1; in contrast, cofactor A does not have the same effect on α-tubulin dissociation from TCP-1. The specificity for β-tubulin strongly implies that cofactor A is not involved in common protein folding pathways. If TCP-1 acts as does GroEL (repetitive binding of unfolded forms until they do fold properly in solution [Weissman et al., 1994]), Rbl2p could act after the release step, to capture and stabilize folded β-tubulin. That model is consistent with our results, with the failure of cofactor A to promote α-tubulin release from the same TCP-1, and with the failure of others to detect any direct interaction between cofactor A and TCP-1 (Gao et al., 1994).

In summary, a screen for overexpressed wild-type genes that restore balance to the components of microtubule assembly identified a stoichiometrically acting component that behaves like a scaffolding element. Changes in the level of Rbl2p exacerbate circumstances that compromise microtubule assembly, suggesting that it may act at a crucial and regulatory step in microtubule morphogenesis.

Experimental Procedures

Strains, Plasmids, and Media

All yeast strains are derivatives of FSY185 (Weinstein and Solomon, 1990) with the exception of the *tub1* mutants (Schatz et al., 1988). We used standard methods (Sherman et al., 1986; Solomon et al., 1992). We used a yeast cDNA (CEN) library from pool 10A provided by H. Liu (Liu et al., 1992). pJA10 was constructed with the PvuII–EagI (pGAL-RBL2) fragment of pA5 into the PvuII–EagI backbone of YEp13 (*LEU2*, 2μ). pJA34 was constructed by isolating mouse cofactor A from a FVB mouse adult testes cDNA library (provided by D. Page) by PCR and cloning the fragment into the SalI–NotI backbone of the pGAL-CEN library plasmid (Liu et al., 1992).

Screen for Suppressors of β-Tubulin Lethality

We transformed 10A into JAY47 and obtained approximately 6.8 × 10^4 original transformants. We grew the transformants in selective glucose media to saturation (expansion of >10^6-fold). We tested 950 galactose survivors for dependence on the plasmid by selecting for loss of the *URA3* plasmid on 5-FOA and then checking for loss of suppression. After isolation of the library plasmid, we identified those that contained either *TUB1* or *TUB3* by a combination of restriction digests, colony hybridization, and DNA sequencing. The isolated plasmids were retransformed into JAY47 and checked for their ability to confer survival on galactose plates.

DNA Sequencing

DNA sequencing on both the cDNA inserts and genomic versions was performed using modified T7 DNA polymerase Sequenase with the dideoxy chain termination method (U. S. Biochemical Corporation). The genomic clones were isolated either from 2μ yeast genomic libraries RB378 and 380 (Carlson and Botstein, 1982) or from a CEN yeast genomic library prepared by C. Thompson and R. Young (Massachusetts Institute of Technology [MIT]). The genomic clones and cDNAs match exactly, indicating that both contain the entire open reading frames.

Immune Techniques

Antibody Production and Purification

A glutathione S-transferase–Rbl2p fusion protein was overexpressed using pGEX-5X (Pharmacia) in Escherichia coli, purified and injected into three rabbits, and boosted at 2, 4, and 6 weeks. Anti-Rbl2p antibodies (248, 249, and 250) were affinity purified against the fusion protein. Rabbit antisera against β-tubulin (206) and α-tubulin (345) are described elsewhere (Weinstein and Solomon, 1990).

Immunofluorescence

We used standard procedures (Solomon et al., 1992). Secondary antibody was fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Cappel). For Rbl2p staining, we also tried extracting fixed cells after attaching to the slides with 0.5% NP-40 in PM2G followed by using

a 0.1% BSA blocking step (M. Magendantz and F. S., unpublished data), by using methanol/acetone fixation (Rout and Kilmartin, 1991), and by varying the time in formaldehyde (10 min to 2 hr) or in first antibody (1–16 hr). DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Boehringer Mannheim) was used to visualize DNA.

Immunoblots

We used standard procedures (Solomon et al., 1992). After gel electrophoresis and transfer to nitrocellulose membranes, blots were blocked (blots 3% BSA, PBSA, sodium azide for 30–120 min. Primary antibodies were incubated for >12 hr at 1/3500 (206 or 345) or at 1/100 (250) and then washed five to seven times (5 min each) in 2% hemoglobin, 0.1% SDS, 0.05% NP-40. Bound antibody was detected by [^{125}I]protein A (New England Nuclear).

Immunoprecipitations

Antibodies were affixed to Affigel-10 beads (Bio-Rad). Yeast strains JAY286 and 381 are FSY185 transformants with two 2μ plasmids, pGAL-RBL2 (pJA10) and either pGAL-TUB2 (pBW54) or pGAL-TUB1 (pQX3). Total protein was harvested by French Press in PME (0.1 M PIPES, 2 mM EGTA, 1 mM magnesium chloride [pH 6.9]) plus protease inhibitors (Solomon et al., 1992) and added to antibody beads for a 1 hr incubation with rotation at 4°C. We washed the beads eight times with PME plus protease inhibitors.

Genetic Analyses

Construction of ΔRBL2

We replaced SnaBI–XhoI of pA21A (genomic *RBL2* plasmid), which completely removes the *RBL2* open reading frame, with SspI–SalI of pNKY51 (Alani et al., 1987), which contains *URA3* flanked by *hisG* repeats for efficient loopout. We used an SspI–MunI disrupting fragment to create a heterozygous knockout in FSY185, confirmed by Southern blot analysis. We sporulated and dissected the heterozygote to produce haploid *ΔRBL2* cells confirmed by Southern and Western blot analysis.

Synthetic Lethality

For *RBL2* overexpression, FSY185 (wild-type diploid) or haploid strains containing *tub1* alleles were transformed with pA5. Transformants were grown overnight in selective raffinose media, and then galactose was added. Viability was assessed at various times by comparing cell number by hemocytometer count to colony-forming units on glucose plates. Alternatively, differences could also be assessed by plating strains to galactose plates and comparing cell number and size.

The *ΔRBL2* JAY422 strain (*ΔRBL2* haploid) was crossed to haploid *tub1* mutants. The diploids were sporulated and dissected. Synthetic interactions were judged by two criteria: percent of dead spores and marker analysis (inability to recover *tub1* allele plus *ΔRBL2* products).

Assays for Meiosis

JAY472 and 474 are transformants of a heterozygous *ADE2/ade2*, homozygous *ΔRBL2/ΔRBL2* strain (JAY428) with A21A (*RBL2* genomic CEN plasmid) or with a control CEN plasmid. JAY472, JAY474, and FSY185 (wild-type) cells were grown to midlog, washed with water, and shifted to 1% potassium acetate (pH 7). Sporulating cells were incubated rotating at room temperature. By visual inspection at 4 days, we scored the presence of asci containing zero to four spores. The criteria for an ascus were rounded shape and thickened cell wall. JAY474 has spore sacs that usually contain fewer than four spores. For quantitation of appearance of *ade2* cells, we allowed strains to sporulate, digested the cell walls with Zymolyase-100, and plated. After 3 days we counted the number of red colonies and the number of total colonies.

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GenBank Accession Number

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Microtubule Function in Morphological Differentiation: Growth Zones and Growth Cones

Minireview

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The mechanistic analysis of cytoskeletal function in cell morphology began with a series of formative drug interference experiments. Several types of animal cells grow in culture with a relatively symmetric morphology but can be induced to take on their characteristic differentiated shape by changes in their growth media. Drugs that depolymerize microtubules inhibit the acquisition of differentiated morphology or, in cells that have established morphology, the drugs cause loss of asymmetry. That phenomenology fits well with the typical geometry of the microtubule network in many cells—a radial pattern extending toward the periphery—since it is the periphery of the cell where the motile events that underlie shape change occur.

A straightforward prediction of these results is the existence of molecules that are responsible for motile specializations at the cell periphery and whose localization depends upon intact microtubules. In fact, very few such molecules have been identified. Instead, the predominant cytoskeletal participant in determinants of cell motility and associations with peripheral molecules is the actin cytoskeleton. For example, bud site selection and growth in S. cerevisiae occur as if they are essentially independent of microtubule functions (Jacobs et al., 1988); rather, they are strongly associated with F-actin structures and functions (Chant and Pringle, 1991). An analysis by Mata and Nurse (Mata and Nurse, 1997 [this issue of *Cell*]) of *tea1*, a gene in Schizosaccharomyces pombe, helps bring microtubules back into the picture. The details of the molecule they are studying present a potentially intriguing parallel with microtubule-associated activities in animal cells.

Control of Growth Morphology in Fission Yeast

The cells of S. pombe elongate at their tips, in a manner that depends upon the stage of the cell cycle (see Nurse, 1994, for a review). At their birth, new daughter cells grow only at the "old end"—the end that existed in the mother cell. After passage through part of the cell cycle, growth initiates at the "new end" as well. This bipolar elongation continues until the onset of mitosis, when it stops at both ends. Thus, the precise temporal and spatial controls on cell growth in S. pombe are integrated with the cell cycle. Both microtubules and microfilaments contribute to cell growth. The position of actin patches during the cell cycle correlates with the positions of cell growth: first at the old end, then at both the old and new ends, and finally in mitosis at the midline where the new septum will form. Further supporting this correlation, the actin inhibitor cytochalasin D blocks cell wall formation. The spatial correlation is less direct with respect to cytoplasmic microtubules. The microtubules extend along the major axis of the cell during interphase, then depolymerize to form the intranuclear spindle. Disruption of microtubules, either by drugs or by mutations in tubulin genes, causes abnormal growth, bending, and even branching.

The phenotypes of mutations that affect growth patterns fall into morphological classes and can be ranked by the extent to which they lose organization (Verde et al., 1995). The *orb* mutant cells are spherical, suggesting total loss of control over the localization of growth. *tea* mutants can initiate a third discrete growth zone, forming T-shaped cells. The *ban* mutants curve like bananas, which could represent disproportionate lengthening of one side, although the cells still elongate at two positions. The microtubule and microfilament cytoskeletons in all three classes are abnormal.

tea1p, Growth Zones, and Microtubules

Cells bearing a disruption of *tea1* (for *t*ip *e*longation *a*berrant) display the curved ban phenotype at 25°C, but some cells show elongation at a third tip at 36°C. As shown by immunofluorescence, tea1p localizes primarily to the ends of cells. However, unlike actin, its distal position persists whether or not there is growth at the tip; tea1p's presence is not sufficient to specify a growth zone.

Over-expression of tea1p substantially reproduces the null morphological phenotypes of bent or three-tipped cells, and the protein is no longer restricted to the growth zones. The morphological defect in over-expressers could be due to this mislocalization of tea1p. However, since localization is not sufficient to specify a growth zone, excess tea1p could disrupt organization by titrating out other relevant components. The cells also apparently regulate tea1p levels to meet physiological requirements. Pheromone response in S. pombe relaxes the restriction of precise antipodal tip growth so that cells can bend toward a mating partner. Treating cells with pheromone causes down-regulation of tea1p.

The authors note two structural features of interest in the 127 kDa tea1p gene product. First, the amino-terminal domain contains 6 internal repeats like those in kelch, a protein associated with the ring canals of Drosophila that mediate transport of nurse cell cytoplasm to the oocyte (Xue and Cooley, 1993). The kelch repeats are believed to be indicative of actin-binding proteins, and the ring canals are themselves associated with actin filaments. Second, the carboxy-terminal domain is predicted to form coiled-coil structures. From sequence comparisons, the authors find two predicted open reading frames in S. cerevisiae containing these same two motifs.

Despite the predicted actin association and the observed colocalization with actin patches, cells bearing mutations in either of two actin-associated proteins, and which have substantially disrupted actin patterns, show normal localization of tea1p. Normal localization of tea1p, however, is dependent upon intact cytoplasmic microtubules. Depolymerization of microtubules induces delocalization of tea1p from growth zones, perhaps with a slight lag. Reassembly of the depolymerized

microtubules to their normal length by removing the drug induces concomitant relocalization of tea1p. The intermediates are intriguing: shortly after repolymerization commences, but before the microtubules return to their original length distribution, tea1p is in bright spots throughout the cell, most of which colocalize with distal ends of microtubules. Indeed, even in untreated cells, many of those dots of tea1p that are not at growth zones co-localize with microtubule ends.

The microtubule cytoskeleton's interactions with tea1p are reciprocal. In a sub-population of null cells, some microtubules grow long enough to curl around the ends of cells. These same extra-long microtubules are seen in the pheromone-treated cells that have reduced levels of tea1p. In contrast, the microtubules are significantly shorter in cells that over-express tea1p.

Models of tea1p Function

Mata and Nurse (1997) conclude that tea1p is required for correct placement of growth zones, although it is neither sufficient nor necessary for growth. But how are the interactions with microtubules to be interpreted?

One possibility proposed by the authors is that tea1p is a regulator of microtubule length—a capping activity analogous to those observed for F-actin modulators. The length of microtubules could depend inversely upon the levels of tea1p in the cell, or on the proportion of it that localizes to the cell tips. In this view, the delivery of the growth machinery to the appropriate location in the cell is accomplished by producing microtubules of appropriate length and relying on the constraints of the cell's geometry to point the microtubules in the right direction. In addition to the consequences of altered expression levels on cytoplasmic microtubules, other results also fit such a model. For example, the results show that tea1p can bind to microtubule ends without interacting with the cell surface, but that it does not remain at the cell surface without microtubules. And the authors report that their efforts to clone tea1p by complementation were defeated by plasmid loss; perhaps levels of tea1p affect microtubule organization in the spindle as well. Among the alternative models is the possibility that tea1p localization at the growth zones is important for stabilizing microtubules that reach those positions or for recruiting the growth machinery itself. tea1p could associate with the cell surface in these domains, stabilized there by the dual interactions with microtubules and some cortical element.

The present data do not distinguish among these and other possibilities raised by the reciprocal effects of tea1p levels and microtubule length. However, possible roles for tea1p—or the tea1p complex—should be testable by in vitro assays of microtubule assembly dynamics, identification of proteins that cofractionate with it, and a search for other binding partners. Of particular interest will be testing the prediction from sequence comparisons that tea1p can associate with actin.

As noted above, microtubules have no apparent influence on either bud site selection or growth in the budding yeast, S. cerevisiae; instead, their participation in morphogenesis may be limited to determining nuclear

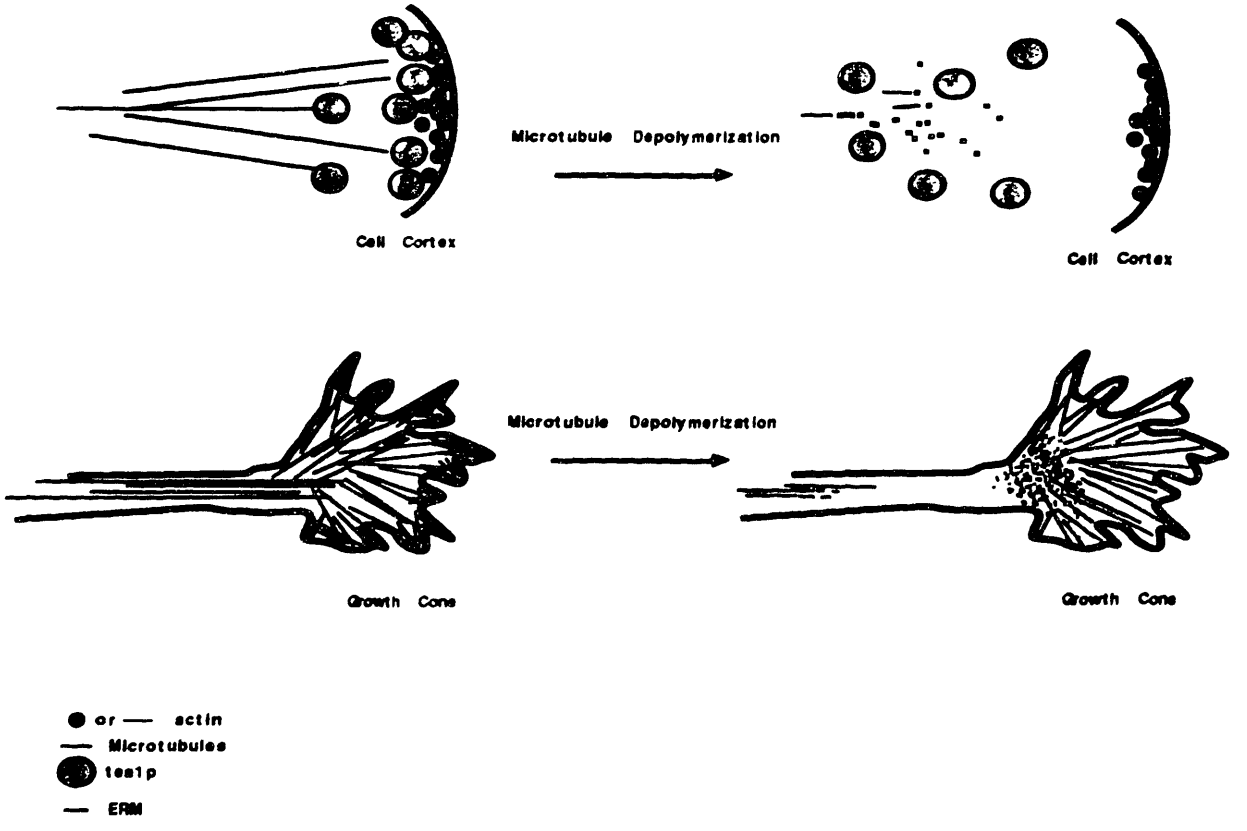

Figure 1. Localization of tea1p and ERM to the Cortical Actin Cytoskeleton Depends upon Intact Microtubules

position and orientation of the spindle. Perhaps the roles of the homologs of tea1p in this organism will modify that view. Interestingly, this distinction between these organisms extends to mechanisms for distribution of mitochondria, which are independent of microtubules in S. cerevisiae but are strongly affected by mutations in both tubulins in S. pombe (Yaffe et al., 1996).

An Animal Cell Parallel

The properties of tea1p are reminiscent of a family of proteins studied in animal cells. Ezrin was first identified as a component of the intestinal microvillar actin cytoskeleton (Bretscher, 1983). It and the other members of the ERM family, radixin and moesin, localize to subdomains of the cell cortex—nonmotile structures such as microvilli and adherens junctions, and motile elements including the leading edges of migrating cells and cleavage furrows. Some in vitro assays suggest that ERM proteins bind to F-actin, but at least in vivo, they colocalize with only a small subset of the cells' actin structures (Tsukita et al., 1997).

Of particular interest is a comparison between the properties of tea1p with those of ERM proteins in cultured hippocampal neurons (Goslin et al., 1989). Anti-ERM antibodies stain these cells at their growth cones almost exclusively. The staining pattern is very similar but not identical to that of the F-actin in growth cones. It is entirely distinct from that of microtubules that are prominent along the length of the process but barely detectable if at all in the growth cones themselves. These neurites can attach along their length to the solid substratum upon which they are grown, so that they do not retract for long periods after addition of microtubule depolymerizing drugs. Those drugs do cause the microtubules along the length of the neurite to depolymerize rapidly, in ordered, distal-to-proximal fashion. During the period when the neurites remain in place but the microtubules are receding, all of the anti-ERM staining leaves the domain of the growth cones. Initially, the delocalized ERM appears in the process, always distal to the receding tubulin staining. Eventually, when no assembled tubulin is detectable, the ERM staining also disappears. Even at this point, staining of two other growth cone markers, GAP43 and F-actin, remain in place. Depolymerization is readily reversible by washing out the drug, and the microtubules regrow along the length of the neurite, in proximal-to-distal fashion. ERM staining reappears, but only in the growth cone and only when microtubule reassembly is complete to the end of the neurite.

Thus, the cortical localizations of tea1p in S. pombe growth zones and of ERM in growth cones occur in patterns similar to that of F-actin. In fact, both may be actin binding proteins. However, their normal localizations are dependent upon intact microtubules. Unlike tea1p, ERM proteins have not yet been detected in close apposition to the ends of the shortened microtubules; and although ERM proteins are not among the tea1p homologs identified by Mata and Nurse (1997) in a search of the database, the two proteins do show significant similarity (Figure 1 and Table 1). That similarity is not in the kelch repeat regions, but rather is dispersed throughout the protein. Perhaps tea1p and ERM depend upon different motifs for their close association with F-actin in situ.

Table 1. Homology among tea1p, Human Ezrin, and Kelch (BESTFIT)

	Identity (%)	Similarity (%)
tea1p and kelch	20.5	40.8
tea1p and ezrin	23.3	47.6
Kelch and ezrin	15.3	35.8

The exact role that ERM proteins play in assumption and maintenance of cellular asymmetry is not known. Depletion experiments using antisense strategies cause significant defects in cell attachment and in morphological differentiations at the cell surface, but these phenotypes may be indirect consequences of disrupting the cortical actin cytoskeleton by removing one of its components (Takeuchi et al., 1994). There is also evidence that ERM proteins interact with Rho in a phospholipid-dependent fashion (Hirao et al., 1996) and with integral membrane proteins (Tsukita et al., 1994), strengthening their possible role as connectors between cytoskeleton and the plasma membrane.

Morphogenesis in Yeast and Animal Cells

There are of course other circumstances in animal cells where microtubules are required to maintain asymmetry at the molecular level. A role for the microtubule cytoskeleton in sorting of proteins to the apical and basolateral domains of epithelial cells is suggested by drug interference experiments, an interpretation that is strengthened by specific depletion of microtubule motors (Lafont et al., 1994). Yet there remains a significant difficulty in comparing the effects of microtubules on morphology in yeasts to their role in animal cells. In animal cells, the loss of microtubules means the loss of asymmetry. Obviously yeasts have other mechanisms that can replace microtubules. Perhaps the yeast cell wall may circumvent a structural role for microtubules in supporting asymmetry as it is formed.

Microtubules apparently participate in fine tuning of the global organization in S. pombe. Their effect is more subtle in S. cerevisiae, but detailed analysis of mutant phenotypes do suggest that microtubules and microfilaments interact, for example to orient the nucleus in mating projections (Read et al., 1992). In fact, the notion that the elements of the cytoskeleton function in concert has arisen from several descriptive experiments. The identification of tea1p and other proteins that act as if they engage more than one of those elements may lead us to mechanisms that will make sense of those observations.

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Formation and Function of the Rbl2p–β-Tubulin Complex

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The yeast protein Rbl2p suppresses the deleterious effects of excess β-tubulin as efficiently as does α-tubulin. Both in vivo and in vitro, Rbl2p forms a complex with β-tubulin that does not contain α-tubulin, thus defining a second pool of β-tubulin in the cell. Formation of the complex depends upon the conformation of β-tubulin. Newly synthesized β-tubulin can bind to Rbl2p before it binds to α-tubulin. Rbl2p can also bind β-tubulin from the α/β-tubulin heterodimer, apparently by competing with α-tubulin. The Rbl2p–β-tubulin complex has a half-life of ~2.5 h and is less stable than the α/β-tubulin heterodimer. The results of our experiments explain both how excess Rbl2p can rescue cells overexpressing β-tubulin and how it can be deleterious in a wild-type background. They also suggest that the Rbl2p–β-tubulin complex is part of a cellular mechanism for regulating the levels and dimerization of tubulin chains.

Much of the work on microtubules has focused on the assembly reaction from α/β-tubulin heterodimer to polymer. This reaction is well characterized in vitro, and genetic and pharmacological studies demonstrate its importance and possible in vivo mechanisms for its regulation. Less well understood are the steps leading to the formation of the heterodimer in the cell. There is now considerable evidence that these steps are themselves subject to cellular controls crucial for microtubule function.

The proper folding of the tubulin chains in vivo (22, 23) and in vitro (6, 12, 26) apparently requires the action of chaperone complexes (variously abbreviated as TriC, CCT, TCP/c-cpn). Unlike other proteins that are TriC substrates, however, α- and β-tubulin require other proteins in vitro to exchange into exogenous heterodimers, as assayed by native gel electrophoresis (2, 7, 8). The extent to which this in vitro reaction is applicable to the in vivo situation is unknown, beginning as it does with fully denatured protein rather than newly synthesized protein (5). Comparison of elements of the in vitro reaction with cellular activities reveals both similarities and differences. For example, yeast strains with altered forms of TCP-1 genes do exhibit cytoskeleton defects (3, 15, 22–24). On the other hand, a protein that is required for the in vitro reaction is the homolog of a yeast protein, Cin1p, that is not essential in vivo but which may be involved in microtubule functions (10, 20, 21).

A recent study of the in vitro folding reaction identified cofactor A, which promotes the recovery of β-tubulin, as a monomer from the chaperonin (7). However, in this assay, the form of β-tubulin released by cofactor A does not exchange into exogenous dimer. A genetic analysis of cellular responses to β-tubulin levels identified Rbl2p as a yeast structural homolog of cofactor A; Rbl2p is a nonessential protein that suppresses the lethality associated with overexpression of β-tubulin (1). The murine cofactor A was shown to partially replace Rbl2p in this in vivo assay (1). Although results of the in vitro assay first suggested that cofactor A was a co-chaperonin, the yeast experiments demonstrated that Rbl2p interacts with β-tubulin directly, rather than with TCP-1. Results of a revised version of the in vitro assay agree with the observation that Rbl2p/cofactor A interacts with β-tubulin rather than with TriC and that Rbl2p is not essential for β-tubulin folding (21). More recently, Melki and colleagues (14) have shown that cofactor A, like Rbl2p, binds noncovalently to β-tubulin. This cofactor A–β-tubulin complex elutes from a gel filtration column in a position consistent with it being a 1:1 heterodimer.

To analyze the function of Rbl2p in the cell, we have isolated and characterized a stable complex of Rbl2p and β-tubulin, formed both in vivo and in vitro, that lacks α-tubulin. The data suggest that Rbl2p binds to a folded form of β-tubulin and predict possible roles for Rbl2p in the regulation of tubulin assembly.

MATERIALS AND METHODS

Plasmids, strains, and media. pQE-60/RBL2 was used to produce recombinant His_6-Rbl2p in *Escherichia coli*. This plasmid was constructed by PCR to add *Nco*I and *Bgl*II sites to the *RBL2* gene just before the start codon and just after the penultimate codon, respectively. The PCR primers were 5'TAGGACACC ATGGCACCCACACAATTG3' and 5'AATCTGAGATCTTTTAGAATCGA GTAATTC3'. The PCR product was cloned into the *Nco*I and *Bgl*II sites of Qiagen vector pQE-60.

pGRH allows inducible expression of His_6-Rbl2p in *Saccharomyces cerevisiae*. pQE-60/RBL2 was digested with *Hin*dIII, blunted, and then digested with *Mfe*I. This fragment was cloned into pA5 (*URA3 CEN* GAL1-10 promoter [1]) that had been digested with *Not*I, blunted, and then digested with *Mfe*I. pMM11 is a 2-μm plasmid encoding His_6-Tub2p under control of the GAL1-10 promoter. Starting with the vector pBW47 containing the 3' half of *TUB2* (25), we used PCR to generate a fragment containing an *Nco*I site 5' of codon 291 and a *Bgl*II site just after the penultimate codon. The 5' primer was 5'CCGGACACCATGGCAGC AAATGTTTGAT3'. The 3' primer was 5'CAATCTTAGATCTTTCAAAATT CTCAGTGAT3'. This fragment was cloned into the *Nco*I and *Bgl*II sites of pQE-60, placing six histidine codons at the carboxy terminus followed by a stop codon. A *Sal*I-*Hin*dIII fragment was cut from this construct and cloned into pBW54 (25) from which the *Sal*I-*Sac*I fragment had been removed.

All yeast strains used in this study are derivatives of FSY182, -183, or -185 (25). JAY47 is a diploid containing a third copy of the *TUB2* gene integrated at the *TUB2* locus and under control of the *GAL* promoter (1). JAY614 is FSY185 plus pGRH. JAY570 is JAY47 plus pGRH. FSY820 is a derivative of FSY182 containing a deletion of the chromosomal *RBL2* locus (1). This strain was transformed with a *CEN* plasmid (marked with *URA3*) bearing *tub2-590* under control of the GAL promoter (25) and with a CEN plasmid (*HIS3*) encoding His_6-Rbl2p under control of the *RBL2* promoter. The latter plasmid was created by cutting pGRH with *Mfe*I and *Pvu*II and subcloning the fragment into pJA33, a plasmid containing the entire *RBL2* gene (1) from which the *Mfe*I-*Eco*RV fragment had been removed. FSY821 is similar to FSY820 except that it constitutively expresses *tub2-590* and contains *TUB2* under control of the *GAL* promoter. We

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crossed FSY127 (*tub2-590* [11]) with FSY 611 (*rbl2 URA3 hisG*). We identified sporulated segregants bearing the marker for the *Δrbl2* allele (by the *URA3* gene) and the *tub2-590* allele by Western blotting. These segregants were plated on 5-fluoro-orotic acid, and cells that had looped out the *URA3* sequence were recovered. Finally, these cells were transformed with plasmids pBW54, containing *GAL-TUB2* (25), and pJA33, a *CEN* plasmid encoding His_6-Rbl2p under control of the *RBL2* promoter. LTY333 contains the pMM11 plasmid in FSY182 (*Δtub1 Δtub3* PRB539) (16). LTY292 is FSY182 plus pGRH (16).

We used standard methods and media (18, 19).

Purification of His_6-tagged proteins. The Ni-nitrilotriacetic acid (NTA) slurry and column materials were from Qiagen. We used protocols that are slight modifications of both those described earlier (13) and those recommended by the manufacturer. Immunoblot signals were quantified from multiple burns within the linear range by using the IS-1000 Digital Imaging System (Alpha Innotech Corporation).

In vivo association experiments. We grew LTY292 overnight in selective raffinose medium and then induced expression with galactose for 10 to 16 h. We harvested protein by glass bead smash (19), using approximately 4×10^8 cells per experiment. We used a volume of PME buffer plus protease inhibitors (19) equal to the volume of the cell pellet. After centrifugation (13,000 × g, 30 min), 850 μl of extract was mixed with 130 μl of Ni-NTA slurry that had been preincubated with buffer I (20 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate buffer [pH 8.0]). After a 1-h incubation at 4°C, we washed the Ni-NTA beads three times with 10 ml of buffer I plus 10% glycerol. Bound proteins were eluted by incubation with an equal volume of buffer I containing 400 mM imidazole or with 2× gel sample buffer (4% sodium dodecyl sulfate [SDS], 0.2 M dithiothreitol, 20% glycerol).

In vitro association experiments. We harvested protein from FSY185 (wild-type) cells. After breaking the cells in PME buffer with a French press, we immediately added 300 to 500 μl of bacterial lysate containing recombinant His_6-Rbl2p and then proceeded as described above. The lysate was prepared from *E. coli* cells containing pQE-60 RBL2 which had been induced with isopropyl-β-D-thiogalactopyranoside for 6 h. Approximately 2.5 ml of packed cells were opened by sonication in 20 ml of buffer I, followed by centrifugation at 31,000 × g for 20 min. To assay denatured proteins, we brought 1 ml of extract to a final concentration of 6 M guanidine hydrochloride for 5 min at 0°C. We diluted the sample (or untreated control) 100-fold into PME buffer plus protease inhibitors plus 500 μl of recombinant His_6-Rbl2p (~0.1 mg/ml), incubated the mixture for 1 h at 4°C, and then isolated the complex as described for the in vivo association experiments.

Dissociation experiments. We prepared His$_6$-Rbl2p–β-tubulin complex from JAY570 protein extracts, and His_6–α β-tubulin heterodimer from LTY333 protein extracts. Cells were opened in PME buffer by French press as described above, and the extracts were centrifuged at 13,000 × g and then mixed with Ni-NTA slurries also as described above. Unbound proteins were washed away by three washes (15 ml per 130 μl of resin), and we resuspended the samples in PME buffer plus protease inhibitors (25-fold dilution). At various times, we centrifuged aliquots of the samples, removed the supernatant, and eluted bound proteins with buffer I containing 400 mM imidazole or with 2× gel sample buffer.

RESULTS

Characterization of a His_6-Rbl2p–β-tubulin complex formed in vivo. Previous work demonstrated that Rbl2p can form a complex with β-tubulin but not α-tubulin in vivo, demonstrating the existence of a second pool of β-tubulin in the cell in addition to that of the α/β-tubulin heterodimer (1). We originally isolated this complex by immunoprecipitation with anti-β-tubulin or anti-Rbl2p antibodies. However, this isolation method has drawbacks. First, the monoclonal anti-α-tubulin antibody has only a modest level of affinity, so the immunoprecipitates are somewhat unstable. Second, on SDS-polyacrylamide gels, the sizes of the tubulin polypeptides are similar to those of the immunoglobulin G heavy chains, which are abundant in the precipitate and interfere with analyses.

To avoid these problems in analyses of the Rbl2p–β-tubulin complex, we constructed a version of the *RBL2* gene encoding a form of the protein with six histidines at its carboxy terminus. By several criteria, this modified form of Rbl2p has the same activities as does the unmodified form.

First, overexpressed His_6-Rbl2p suppresses the lethality associated with overexpression of β-tubulin with an efficiency of 49%, under conditions where only 0.01% of cells containing the YCpGAL control plasmid survive. This value is only slightly less than the 70% efficiency achieved with unmodified Rbl2p (1).

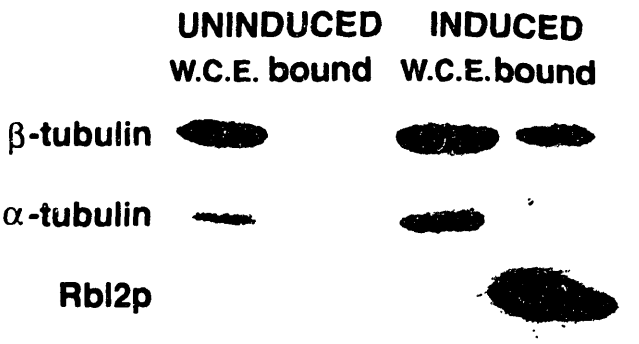

FIG. 1. The His_6-Rbl2p–β-tubulin complex formed in vivo is devoid of α-tubulin. LTY292 cells carrying a plasmid-borne gene specifying His_6-Rbl2p under control of the *GAL* promoter were grown for 2 h in medium containing raffinose (uninduced) or galactose (induced). The His_6-Rbl2p was separated from extracts by using Ni-NTA beads. The whole-cell extracts (W.C.E.) and bound proteins were analyzed by immunoblotting for β-tubulin, α-tubulin, and Rbl2p as shown. The bottom film was exposed six times longer than the top two. The presence of β-tubulin in the bound proteins required expression of His_6-Rbl2p, but no α-tubulin was detected in the bound proteins under either condition.

Second, like Rbl2p, His_6-Rbl2p overexpression confers resistance to the microtubule-depolymerizing drug benomyl.

Third, protein binding to His_6-Rbl2p is similar to that of unmodified Rbl2p. We mixed extracts of cells that inducibly overexpress His_6-Rbl2p with Ni-NTA beads (see Materials and Methods). β-Tubulin binding to the beads strictly depended upon His_6-Rbl2p expression (Fig. 1). This complex, like the one previously characterized by immunoprecipitation, contains no detectable α-tubulin.

The level of Rbl2p–β-tubulin complex increases when both of its components are co-overexpressed (data not shown), although we detected no increase in Rbl2p levels when β-tubulin alone was overexpressed. Analysis of extracts from the co-overexpressing strains by gel filtration identified a peak containing β-tubulin and Rbl2p which eluted at an apparent molecular mass of ~60 kDa (3a), consistent with the finding by Melki and colleagues that cofactor A and β-tubulin form a 1:1 heterodimer (14). As shown in Fig. 2 below, we also detected this complex in extracts of wild-type cells, but the level of the complex was extremely low. To estimate the relative sizes of the two pools, we used immunoprecipitation to measure the proportion of the cellular β-tubulin not associated with α-tubulin. The anti-α-tubulin antibody was covalently attached to beads and was incubated with wild-type extract. Under conditions where that antibody leaves ~1% of the total α-tubulin in the supernatant, we found that <2% of the β-tubulin also remained. We can therefore place a limit on the proportion of β-tubulin associated with Rbl2p as being no greater than 2% of the total β-tubulin.

Ordering the formation of Rbl2p–β-tubulin complex and α/β-tubulin heterodimer in vivo. To order the formation of these two β-tubulin complexes with respect to one another, we constructed yeast strains that would allow us to monitor the compartmentalization of newly synthesized β-tubulin relative to that of the steady-state pool. In FSY820 cells, the only source of Rbl2p is a low-copy-number plasmid that constitutively expresses His_6-Rbl2p under the control of the *RBL2* promoter. On the chromosome the constitutively expressed β-tubulin gene is wild-type *TUB2*. An inducibly expressed β-tubulin gene, *tub2-590*, is on a second plasmid under control of the *GAL* promoter. The product of that gene, Tub2-590p, is a fully functional β-tubulin protein. Because it lacks the carboxy-terminal 12 amino acids, we could distinguish between the

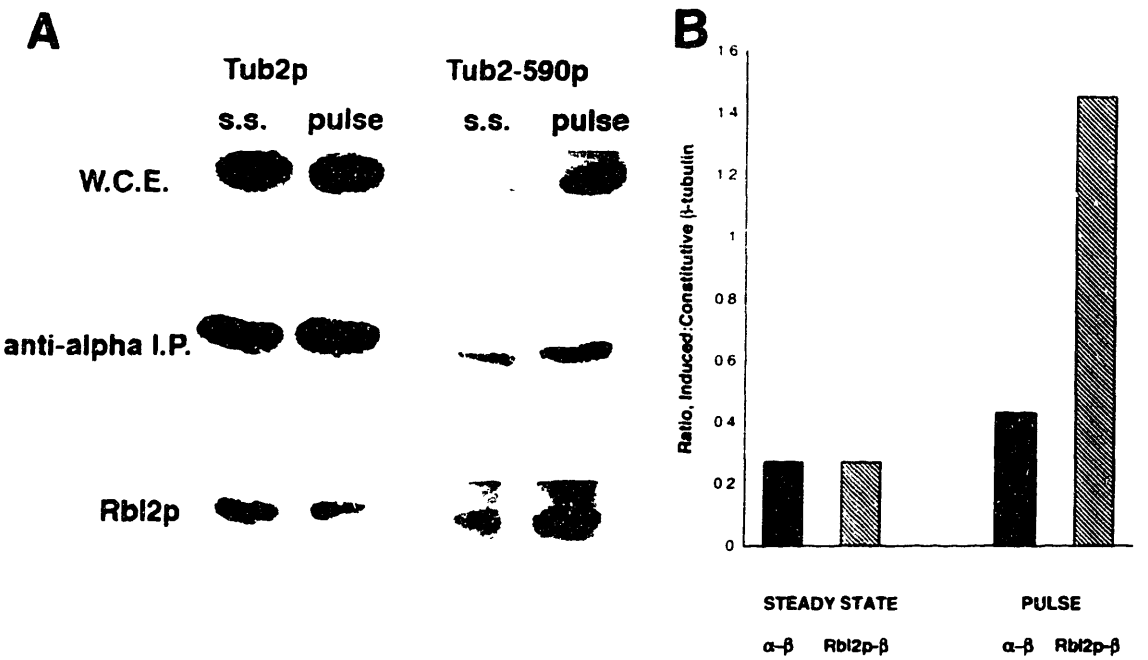

FIG. 2. Newly synthesized β-tubulin can bind to Rbl2p in vivo. Distribution of β-tubulin between the Rbl2p and α-tubulin pools in FSY 820 cells is shown. (A) Immunoblots of whole-cell extracts (W.C.E.), anti-α-tubulin immunoprecipitates (I.P.), and proteins bound to His_6-Rbl2p either at steady state (s.s.) or after a brief (galactose for 10 min followed by glucose for 10 min) induction of Tub2-590p (pulse) are shown. The blots were probed with antibodies specific for either wild-type Tub2p (206) or the faster-migrating Tub2-590p (590). Sample volumes and exposure times were adjusted to give detectable signals from all fractions. (B) Analysis of the data shown in panel A. The ratios represent the proportions of the induced β-tubulin (Tub2-590p) relative to the constitutive β-tubulin (Tub2p) present as the α/β-tubulin heterodimer or associated with Rbl2p in the uninduced cells (steady state) and after a brief induction of Tub2-590p expression (pulse).

β-tubulin proteins by using two antibodies (206 and 339) that bind specifically to the wild-type and truncated forms, respectively (11). In addition, Tub2-590p migrates faster on SDS-polyacrylamide gels than the wild-type Tub2p.

To examine the partitioning of newly synthesized β-tubulin, a culture of FSY820 cells grown in raffinose was exposed to galactose for 10 min and then to glucose for an additional 10 min. We fractionated extracts of these cells with anti-α-tubulin antibodies to isolate the α/β-tubulin heterodimers and with Ni-NTA beads to bind the His_6-Rbl2p–β-tubulin complexes. As a steady-state control, an identical culture of raffinose-grown FSY820 cells was shifted to glucose for 20 min. The distributions of the two β-tubulin proteins in whole-cell extracts and in the two fractions were assayed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting.

Results from a representative experiment are shown in Fig. 2A. The different fractions are represented by very different exposures because they are so different in abundance. Some Tub2-590p, the induced β-tubulin protein, is detectable in the steady-state cell extracts because the *GAL* promoter is weakly expressed in raffinose medium.

To monitor the newly synthesized β-tubulin, we recovered the fractions associated with α-tubulin and with Rbl2p and normalized the recoveries using the constitutively expressed Tub2p. Figure 2B presents an analysis of the results shown in Fig. 2A. The short exposure to galactose increased the level of Tub2-590p approximately fourfold, while the levels of wild-type β-tubulin were unaffected. The ratio of Tub2-590p to Tub2p associated with Rbl2p increased about 5.4-fold after the induction. In contrast, the ratio for the two β-tubulin proteins present as α/β-tubulin heterodimer increased only about 1.6-fold.

This difference in partitioning of newly synthesized β-tubulin is not the consequence of a subtle difference in the properties of the two proteins. We repeated this experiment using FSY821 cells, in which the constitutive and inducible β-tubulin genes are switched. In this experiment, the levels of the inducible Tub2p increased sixfold after the same induction protocol. After the induction, the relative proportion of this newly synthesized β-tubulin protein to the constitutive Tub2-590p was 10-fold higher in the Rbl2p pool but only about 1.5-fold higher in the α/β-tubulin heterodimer (data not shown).

These data demonstrate that newly synthesized β-tubulin can bind to Rbl2p before it incorporates into α/β-tubulin. If the opposite were true, i.e., if β-tubulin could bind to Rbl2p only after it had been in heterodimer, we would not detect any enrichment for the induced protein in the Rbl2p pool, since the heterodimer pool is at least 50-fold larger than the Rbl2p pool. It is important to note, however, that this result does not demonstrate that this order is obligatory (see Discussion).

Formation of Rbl2p–β-tubulin in vitro. To determine if Rbl2p could bind only to newly synthesized β-tubulin or if instead it could bind to β-tubulin that had previously been in α/β-tubulin heterodimers, we used an in vitro assay. We expressed His_6-Rbl2p in *E. coli* and incubated it with extracts of wild-type yeast cells and then assayed for bound proteins by using Ni-NTA beads. We found that Rbl2p bound β-tubulin in a time-dependent fashion (Fig. 3). Like the complex formed in vivo, this in vitro complex contained only a trace amount of α-tubulin, which amount did not increase with time. Therefore, it is likely that the α-tubulin detected represents adventitious binding.

The time course demonstrates a linear rate of association between Rbl2p and β-tubulin for at least 4 h. Extrapolated back to zero time, the kinetics give evidence for a small but reproducible initial burst of complex formation. One interpre-

tation of this biphasic time course is that it represents reaction with two distinct in vitro pools of β-tubulin. The low rate at which the majority of the complex forms may represent a rate-determining release of free β-tubulin from the heterodimer, by far the predominant population of tubulin in the extract. The initial burst could represent the diffusion-controlled reaction of a small equilibrium population of undimerized β-tubulin in the yeast extracts, which should bind to Rbl2p at the diffusion-controlled limit. The level of β-tubulin that reacts at this high rate fits well with our estimate of the level of β-tubulin not associated with α-tubulin in extracts (see above).

These results suggest that Rbl2p can interact with β-tubulin molecules that have previously been dimerized and hence completely folded. Conversely, the ability of β-tubulin to bind to Rbl2p in vitro is abolished by denaturation. We treated wild-type yeast protein extracts with 6 M guanidine hydrochloride and then diluted the protein into solutions containing His_6-Rbl2p. Conventional chaperone binding and folding assays often make use of substrates that are denatured by treatment with 6 M guanidine hydrochloride. Relative to the control reaction mixtures that were diluted but not exposed to denaturing agent, the amount of β-tubulin bound to Rbl2p was only barely detectable (Fig. 4). In contrast, virtually no bound α-tubulin was detected in either the denatured or untreated samples. Immunoblots of these samples with anti-Rbl2p demonstrated that the amounts of Rbl2p bound to beads were the same in the treated and untreated samples. This result is consistent with the failure of β-tubulin denatured in this fashion to bind the murine Rbl2p homolog, cofactor A, in the in vitro system (7, 8).

Stability of the Rbl2p–β-tubulin complex. The in vitro experiment described above suggests that β-tubulin can transfer from α-tubulin to Rbl2p. The in vivo experiment suggests that β-tubulin can interact with Rbl2p before it interacts with α-tubulin. A crucial issue for understanding Rbl2p function is how these two complexes of β-tubulin compare to one another. Accordingly, we measured their stabilities in vitro.

We isolated the His_6-Rbl2p–β-tubulin complex from extracts of yeast cells that overproduce both proteins and then measured the dissociation of the complex by monitoring the loss of β-tubulin from the Ni-NTA beads (see Materials and Methods). Under the conditions of this experiment, the His_6-Rbl2p protein does not dissociate from the beads. As shown in Fig. 5, the Rbl2p–β-tubulin complex dissociates exponentially through about two half-lives. These results are consistent with a simple dissociation reaction with a half-life of about 2.5 h, corresponding to a dissociation rate constant, k_{off}, of 8×10^{-5} s^{-1}.

The stability of this complex should be compared to that of the α/β-tubulin heterodimer with which it can interact. The equilibrium dissociation constant for that heterodimer is reported to be $\sim 8 \times 10^{-7}$ M (4). We cannot measure the comparable constant for the Rbl2p–β-tubulin complex, either directly or indirectly (by measuring its association rate constant), since we do not have a source of native monomeric β-tubulin. However, the bimolecular association constants for molecules of similar sizes are on the order of 10^7 to 10^8 M^{-1} s^{-1} (9). If we choose the conservative value of 10^6 M^{-1} s^{-1}, the Rbl2p–β-tubulin complex would have a K_d of $\sim 10^{-10}$. That value would make the Rbl2p–β-tubulin complex significantly more stable than the α β-tubulin heterodimer. To compare the stabilities of the complexes more directly by similar assays, we prepared α/β-tubulin heterodimer from cells expressing His_6-Tub2p (see Materials and Methods). We isolated this complex on Ni-NTA beads and then monitored its dissociation by assaying for loss of the α-tubulin polypeptide. The rate of α-tubulin loss from the beads is low, consistent with a half-life for the heterodimer of about 10 h (Fig. 5). Therefore, by using essentially the same method to assay the stabilities of the two complexes, it can be concluded that the α/β-tubulin heterodimer dissociates much more slowly than does the Rbl2p–β-tubulin complex.

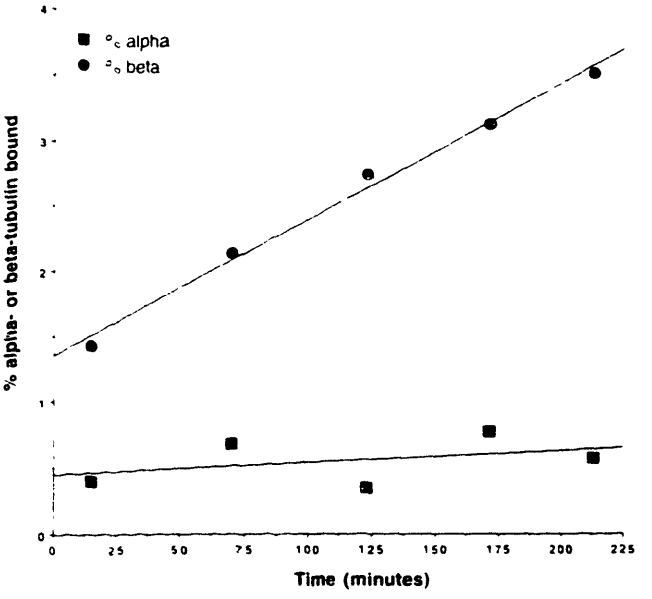

FIG. 3. Formation of His_6-Rbl2p–β-tubulin complex in vitro. Bacterial extract containing His_6-Rbl2p was incubated with wild-type yeast cell extracts at 4°C. At various times, an aliquot was removed and added to Ni-NTA beads for 15 min. The beads were washed, and the bound proteins were assayed by elution followed by immunoblotting with anti-tubulin antibodies. The data are reported as percent β-tubulin and α-tubulin bound as a function of time.

DISCUSSION

That β-tubulin can interact specifically with a protein other than α-tubulin suggests several possible functions for such a complex. The results presented above characterize the formation and properties of the Rbl2p–β-tubulin complex.

The results demonstrate that the formation in vitro of the Rbl2p–β-tubulin complex is dependent upon the conformation of β-tubulin. Although there may be conformational alterations of β-tubulin prior or subsequent to binding Rbl2p, the form of β-tubulin that binds Rbl2p is at least in equilibrium

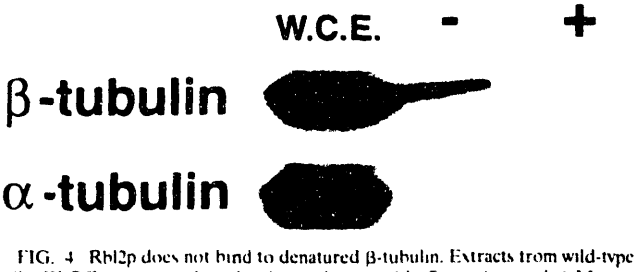

FIG. 4. Rbl2p does not bind to denatured β-tubulin. Extracts from wild-type cells (W.C.E.) were incubated either with control buffer (−) or with 6 M guanidine hydrochloride (+) for 5 min, diluted 100-fold, and then incubated with His_6-Rbl2p plus Ni-NTA beads. The specifically bound proteins were eluted from the beads and assayed for the presence of both β-tubulin and α-tubulin by immunoblotting. The binding of β-tubulin to Rbl2p was essentially abolished by the preincubation with denaturing agent. No bound α-tubulin was detected under either condition.

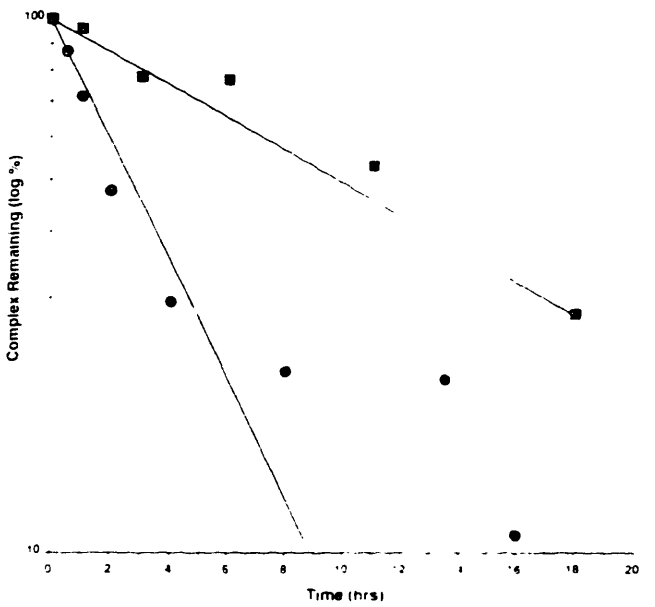

FIG. 5. Dissociation of His$_6$-Rbl2p–β-tubulin and Tub2p–His$_6$–β-tubulin in vitro. The dissociation rates of these two complexes were measured by incubating cell extracts with Ni-NTA beads, resuspending the beads in EMB buffer at 4°C, and then measuring the levels of the complexes remaining at various times by immunoblotting. The data are reported as semilog plots of β-tubulin (circles) and α-tubulin (squares) dissociation. Extracts from cells expressing either Tub2p and His$_6$-Rbl2p or His$_6$-Tub2p alone were used to measure the dissociation of β-tubulin or α-tubulin, respectively.

with the form that binds α-tubulin. That both the in vivo and in vitro complexes are essentially devoid of α-tubulin argues that Rbl2p competes with α-tubulin for binding to β-tubulin, perhaps because both ligands bind to similar sites on β-tubulin. These characteristics of the Rbl2p–β-tubulin complex are consistent with the comparable efficiencies of Rbl2p and α-tubulin in rescuing cells from β-tubulin lethality. In cell extracts, it is clear that much more β-tubulin is associated with α-tubulin than with Rbl2p, reflecting not only relative stabilities but also the likelihood that there is much less Rbl2p than α-tubulin in wild-type cells. We note that the overproduction of Rbl2p is modestly toxic (1), a phenotype that may be explained by the ability of high levels to compete successfully with α-tubulin and so to sequester β-tubulin.

Two results are consistent with a role for Rbl2p in the pathway leading to heterodimer formation. First, the in vitro data suggest that the α/β-tubulin heterodimer is more stable than the Rbl2p–β-tubulin complex. Of course this comparison is of dissociation rates that need not reflect conditions in vivo. For example, that the tubulin heterodimer can have an alternative fate to dissociation, i.e., polymerization, may affect its apparent stability in the cytoplasm. In addition, there may be effectors that modify the stability of either β-tubulin complex. Second, the pulse induction experiment shows that β-tubulin can interact with Rbl2p before it interacts with α-tubulin. This result is consistent with the interactions in vitro reported for the refolding of completely denatured β tubulin (21), which suggest that the murine homolog of Rbl2p, cofactor A, binds β-tubulin shortly after its release from the Tcp-1 complex. However, at steady state the amount of α-tubulin available for dimerization with the newly synthesized material may be limiting. Under that circumstance, the induced β-tubulin may be forced into association with uncomplexed Rbl2p. Therefore, this experiment does not permit us to conclude that this sequence of formation of the two β-tubulin complexes is obligatory or that β-tubulin ordinarily passes through the Rbl2p complex as part of dimer formation. The experiment does establish that Rbl2p may be on the pathway of heterodimer formation for newly synthesized β-tubulin.

These results do encourage further comparisons with the in vitro assay for Rbl2p cofactor A in heterodimer formation. We show here that Rbl2p can bind to β-tubulin that has been in the α/β-tubulin heterodimer. In contrast, Gao et al. originally reported that β-tubulin bound to cofactor A fails to exchange into exogenous heterodimer in the in vitro reaction (7). That result could mean that the formation of Rbl2p–β-tubulin from tubulin heterodimer is not reversible. Alternatively, the inability to detect this exchange reaction may reflect the slow dissociation of β-tubulin from the Rbl2p complex relative to the length of the in vitro assay. It may also reflect the fact that the level of α-tubulin available to bind the released β-tubulin in that assay may be very low, limited by the rate of dissociation from the heterodimer.

What might Rbl2p do in cells? We can consider here two possible roles. First, the demonstration that Rbl2p–β-tubulin can form from newly synthesized protein, before that β-tubulin is incorporated into heterodimer, demonstrates that Rbl2p may participate as a scaffolding protein for β-tubulin in the assembly of the tubulin heterodimer. If so, it obviously does not define the sole pathway for formation of this essential protein, since *RBL2* is itself not essential in wild-type cells. Alternatively, Rbl2p could serve as a buffer to sequester free β-tubulin. Even modest excesses of β-tubulin are deleterious to the cell. For example, strains deleted for the *TUB3* gene, and so lacking about 15% of their normal α-tubulin complement, show distinct microtubule phenotypes (17), which are completely suppressed by an extra copy of *RBL2* under control of its own promoter (1). Experimentally, the extreme toxicity of β-tubulin is best remedied by two proteins that bind to it specifically, α-tubulin and Rbl2p. The cell could find an advantage in using Rbl2p rather than excess α-tubulin in this role. Increased levels of α-tubulin would have the consequence of changing the level of heterodimer, which in turn could affect the balanced dynamics likely to be an important part of successful microtubule function.

In some genetic backgrounds, including those carrying mutations in α-tubulin genes, *RBL2* function is essential (1). Detailed analysis of these situations may provide more insight both into Rbl2p function and into cellular mechanisms for regulating tubulin assembly.

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An α-Tubulin Mutant Destabilizes the Heterodimer: Phenotypic Consequences and Interactions with Tubulin-binding Proteins

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Many effectors of microtubule assembly in vitro enhance the polymerization of subunits. However, several *Saccharomyces cerevisiae* genes that affect cellular microtubule-dependent processes appear to act at other steps in assembly and to affect polymerization only indirectly. Here we use a mutant α-tubulin to probe cellular regulation of microtubule assembly. *tub1-724* mutant cells arrest at low temperature with no assembled microtubules. The results of several assays reported here demonstrate that the heterodimer formed between Tub1-724p and β-tubulin is less stable than wild-type heterodimer. The unstable heterodimer explains several conditional phenotypes conferred by the mutation. These include the lethality of *tub1-724* haploid cells when the β-tubulin–binding protein Rbl2p is either overexpressed or absent. It also explains why the *TUB1/tub1-724* heterozygotes are cold sensitive for growth and why overexpression of Rbl2p rescues that conditional lethality. Both haploid and heterozygous *tub1-724* cells are inviable when another microtubule effector, *PAC2*, is overexpressed. These effects are explained by the ability of Pac2p to bind α-tubulin, a complex we demonstrate directly. The results suggest that tubulin-binding proteins can participate in equilibria between the heterodimer and its components.

INTRODUCTION

Microtubules participate in a variety of specific functions crucial for morphological differentiation, cell growth, and cell movement. The diversity of these functions requires that microtubules assemble into quite different structures even within the same cell. Many of those structures are dynamic, allowing them to disassemble rapidly and thus provide the components necessary to form another microtubule organelle. Possible mechanisms for regulation of these processes can be envisioned at several levels: primary sequences of tubulin genes (Joshi and Cleveland, 1989; Hoyle and Raff, 1990), message stability (Pachter *et al.*, 1987), folding and dimerization of the protein subunits (Ursic and Culbertson, 1991; Chen *et al.*, 1994), properties of the polymer (Mitchison and Kirschner, 1984; Saxton *et al.*, 1984), and the interaction of the polymer with non-tubulin proteins (Caceres and Kosik, 1990; Dinsmore and Solomon, 1991).

Recently, several diverse experimental approaches have identified proteins that may participate in tubulin heterodimer formation. In vitro assays for proper folding of denatured α- and β-tubulins require several protein cofactors that transiently interact with the individual polypeptide chains (Melki *et al.*, 1996; Tian *et al.*, 1996, 1997). These complexes of tubulin polypeptides with cofactors may be intermediates that form between release of tubulin polypeptide from the TCP1-containing ring complex and its incorporation into preexisting heterodimers by exchange. In at least some cases, those polypeptides form binary or higher-order complexes with the tubulins that are stable enough to be isolated but are still reactive (Tian *et al.*, 1997).

Homologues of these cofactors (except cofactor C) are identified by diverse screens for mutations that affect microtubule processes in budding yeast. The

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processes affected include sensitivity to microtubule-depolymerizing drugs (Stearns *et al.*, 1990), fidelity of mitotic chromosome transmission (Hoyt *et al.*, 1990), response to overexpression of β-tubulin (Archer *et al.*, 1995), and interactions with mitotic motors (Geiser *et al.*, 1997). Although most of these cofactors are essential for the in vitro assay, none of their *Saccharomyces cerevisiae* homologues are essential for viability. Therefore, they may participate in the folding and heterodimerization of tubulin polypeptides, but there must be pathways that do not depend on them.

The genetic data alluded to above suggest that there may be multiple steps in tubulin assembly subject to cellular control. Analysis of tubulin mutants can provide access to those steps. A panel of α-tubulin mutants cold sensitive for growth arrest at their restrictive temperature with diverse microtubule phenotypes (Schatz *et al.*, 1988). Some of the mutants arrest with no microtubules (class 1), some with a normal complement of microtubules (class 2), and the rest with an apparent excess of microtubules (class 3). This variability suggests that the conditional defects in these mutant α-tubulin proteins can affect different aspects of microtubule assembly and function. Certain of these mutations are suppressed by specific mutations in β-tubulin (Schatz *et al.*, 1988) and others by extra copies of the mitotic check point *BUB* genes (Guénette *et al.*, 1995) or by yeast homologues of the mammalian checkpoint gene *RCC1* (Kirkpatrick and Solomon, 1994). However, there is too little structure–function information for tubulin to permit an understanding of the phenotype in terms of the tubulin mutation itself.

Another distinction among the *tub1* mutants is uncovered when they are assayed in the presence of varying Rbl2p levels. Rbl2p binds β-tubulin to form a 1:1 complex (Melki *et al.*, 1996; Archer *et al.*, 1998). Rbl2p binding to β-tubulin excludes α-tubulin binding to β-tubulin. Four class 1 α-tubulin mutants are synthetically lethal with deletion of *rbl2*. Two of those are also synthetically lethal with overexpression of *RBL2*, but several other class 1, 2, or 3 mutants show no such interactions (Archer *et al.*, 1995).

The present study analyzes and exploits the properties of one of those two mutants. The *tub1-724* mutation fails to support growth at 18°C and only partially supports growth at 25°C but grows as well as wild-type cells at 30°C (Schatz *et al.*, 1988; see Figure 5). The lethality and loss of microtubules at the nonpermissive temperature is not a consequence of degradation of α-tubulin; the steady-state α-tubulin levels in these cells is the same as that in an isogenic wild-type control (our unpublished results). Upon induction of *GAL-RBL2*, *tub1-724* cells at permissive temperature rapidly lose assembled microtubule structures, and within 20 h <0.1% of the cells are viable (Archer *et al.*, 1995).

The data presented here demonstrate that tubulin heterodimer containing this mutant α-tubulin protein is less stable than the wild-type heterodimer. We use this property to analyze interactions between *tub1-724* and altered levels of two of the cofactor homologues mentioned above. The results provide a structure–function correlation for tubulin as well as insight into the cellular activities of the β-tubulin–binding protein Rbl2p and the putative α-tubulin–binding protein Pac2p.

MATERIALS AND METHODS

Strains, Plasmids, and Media

All yeast strains are derivatives of FSY185 (Weinstein and Solomon, 1990) with the exception of the *tub1* mutants (Schatz *et al.*, 1988) (Table 1). We used standard methods for yeast manipulations (Sherman *et al*, 1986; Solomon *et al.*, 1992).

Viability Measurements and Immunofluoresence

LTY374, LTY8, LTY376, and LTY11 were grown overnight in SC-Ura-Leu raffinose media. Log phase cells were then induced with 2% galactose, and at various time points aliquots of cells were taken and counted using a haemocytometer. Known numbers of cells were then plated to SC-Ura glucose plates. Cell viability was measured as the percent of counted cells able to form colonies on the SC-Ura glucose plates. At various time points cells were fixed for immunofluorescence in 3.7% formaldehyde. Anti-β-tubulin staining was done with #206 (Bond *et al.*, 1986) at 1.2000 in PBS containing 0.1% BSA.

Phenotypes of TUB1 or tub1-724 Heterozygous Diploids

Δ*tub1*, Δ*tub3* strains containing *tub1-724* or *TUB1* gene on *LEU2:CEN* plasmids were crossed to FSY183 (wild type) containing YCpGAL, pPA45, or pA5. The diploid strains were grown to saturation overnight in SC-Ura-Leu-His glucose liquid media. The saturated cultures were serially diluted in 96-well dishes and spotted onto SC-Ura glucose and SC-Ura galactose plates.

Rescue of JAY47

JAY47 (Archer *et al.*, 1995) was transformed with genomic *CEN. URA3* plasmids containing *TUB1*, *tub1* alleles or with *CEN:URA3: RBL2*. Cells were plated to SC-Leu-Ura glucose plates at 30°C and to SC-Leu-Ura galactose plates 30, 18, and 15°C. The number of colonies on galactose relative to glucose was measured.

DNA Sequencing

DNA sequencing was performed using modified T7 DNA polymerase Sequenase with the dideoxy chain termination method (United States Biochemical, Cleveland, OH).

Immune Techniques

Immunoblots. Standard procedures were used (Solomon *et al.*, 1992). After gel electrophoresis and transfer to nitrocellulose membranes, we blocked blots with TNT (0.025 M Tris, 0.17 M NaCl, 0.05% Tween 20, pH 7.5) for 30–120 min. Primary antibodies were incubated for >12 h at 1:3500 (#206 or #345; Weinstein and Solomon, 1990) or at 1:100 (#250; Archer *et al.*, 1995) and then washed five times (5 min each) in TNT. Bound antibody was detected by ^{125}I-protein A (New England Nuclear, Boston, MA) or (for 12CA5)

^{125}I-sheep anti-mouse immunoglobulin G (New England Nuclear). Commercial preparations of anti-HA were used (Boehringer Mannheim, Indianapolis, IN).

Immunoprecipitations. The procedure described previously (Archer *et al.* 1995) was used with slight modifications. The monoclonal antibodies A1BG7 (anti-α) and B1BE2 (anti-β), raised against the carboxyl-terminal 12 amino acids of Tub1p and Tub2p, respectively, were affixed to Affigel-10 beads (Bio-Rad, Hercules, CA). Yeast strains FSY157 and FSY182 were grown up at 30 C. Total protein was harvested by glass bead lysis in PME (0.1 M piperazine-*N,N'*-bis[2-ethanesulfonic acid], 2 mM EGTA, 1 mM magnesium chloride, pH 6.9) plus protease inhibitors and was added to antibody beads for a 1-h incubation with rotation at 4 C. We washed the beads eight times with PME plus protease inhibitors. Bound proteins were eluted by boiling in SDS sample buffer and resolved by SDS-PAGE analysis.

Purification of His_6-tagged Proteins

The Ni-NTA nickel slurry and column materials were from Qiagen (Chatsworth, CA). We used protocols from the Qiagen handbook and modifications of this protocol that have been previously described (Magendantz *et al.*, 1995).

In Vivo His_6-Rbl2p-β-Tubulin Association Experiments

Yeast strains LTY291 and LTY292 are FSY157 and FSY182 transformants with a CEN pGAL-*RBL2-HIS*$_6$ (pGHR). We grew LTY291 and LTY292 overnight at 30°C in selective media containing raffinose to about 2×10^9 cells per experiment. Galactose (2%) was added to induce His_6-RBL2 expression. After 0, 1, and 2 h, protein was harvested by glass bead lysis in 1 ml of PME buffer plus protease inhibitors. We applied 0.85 ml of protein extract to 130 μl of Ni-NTA beads. We washed and eluted the bound proteins as previously described (Magendantz *et al.*, 1995). Eluted proteins were subjected to SDS-PAGE analysis and probed for α-tubulin, β-tubulin, and Rbl2p and quantitated by densitometry.

In Vivo HIS_6-(HA)-Pac2p-α-Tubulin Association Experiments

We grew yeast strains LTY539, LTY541, LTY439, and LTY440 overnight in selective raffinose media at 30 C. Galactose (2%) was added to induce Pac2p-(HA)-His_6 and α-tubulin or β-tubulin expression. Cells (6.0×10^9) were harvested by glass bead lysis per experiment in 1.1 ml of PME buffer plus protease inhibitors. We applied 1 ml of protein extract to 25 μl of Ni-NTA beads. We washed and eluted the bound proteins as previously described (Magendantz *et al.*, 1995). Eluted proteins were subjected to SDS-PAGE analysis and probed for α-tubulin, β-tubulin, and HA(12CA5). For Pac2p, the bead eluants represent 120 times the load of whole-cell extract. For α- and β-tubulin, the bead eluants represent 500 times the load of whole-cell extract.

RESULTS

Characterization of Cold-sensitive tub1 Mutants

The conditional loss of assembled microtubules in class 1 α-tubulin mutants could arise from cold sensitivity of any of several steps in microtubule morphogenesis. However, the synthetic lethality of Tub1-724p with both Rbl2p deletion and overexpression suggests that the mutant defect arises from a weaker heterodimer (Figure 1). If the heterodimer formed by the Tub1-724p dissociates more readily than does wild-type heterodimer, the increase in free, undimerized β-tubulin could be toxic in the absence of the β-tubulin binding capacity provided by Rbl2p. Conversely, an excess of Rbl2p, which has only minor phenotypes in a wild-type cell, could compete effectively with the mutant α-tubulin protein for β-tubulin and so diminish the level of tubulin subunits to cause loss of microtubules and cell death. The experiments described below present tests of this model.

The only difference between the primary sequences of *TUB1* and *tub1-724* genes predicts substitution of threonine for arginine at codon 106 (AGA becomes ACA). Arginine-106 is a highly conserved residue among α-tubulins. The possible significance of this mutation for heterodimer stability is presented in DISCUSSION.

Coimmunoprecipitation of α- and β-Tubulin from Wild-Type and tub1-724 Mutant Cells

We assessed the stability of the wild-type and mutant α-β heterodimers by coimmunoprecipitation. Extracts from *tub1-724* mutant cells (FSY157) and wild-type cells (FSY182) grown at 30°C were incubated with antibodies to either α-tubulin or β-tubulin coupled to Affigel beads. The beads were washed extensively to remove adventitiously adhering proteins, and specifically bound proteins were released by SDS. The tubulin chains in the immunoprecipitates were analyzed by immunoblotting (Figure 2). From extracts of wild-type cells, antibodies against each of the tubulin polypeptides coprecipitate the other chain with high efficiency; the ratio of the tubulins in the coprecipitates is comparable to the original extracts. This result suggests that under the conditions of tubulin immunoprecipitation, normal heterodimer largely remains intact. From extracts of *tub1-724* cells, however, the anti-tubulin antibodies complex efficiently with the specific tubulin chain against which they are directed but precipitate the other tubulin chain only poorly.

Because we recover only a small fraction of Tub1-724p heterodimer by immunoprecipitation, we cannot directly compare the stability of the mutant and wild-type heterodimers. We previously established that at least 98% of the β-tubulin in wild-type cells is in the form of α-β heterodimer (Archer *et al.*, 1998). Because *tub1-724* cells grow normally at 30°C, presumably most of the tubulin in those cells is in heterodimer in vivo. Thus, the dissociation of the heterodimer likely occurs in the course of the immunoprecipitation itself, which exposes the heterodimer to large dilutions at low temperature (4°C). Under similar conditions, the wild-type heterodimer has a half-life of ~10 h (Archer *et al.*, 1998).

Table 1. Strains and plasmids

Strain/plasmid	Genotype	Reference
Strains		
FSY185	*a/α; ura3-52/ura3-52, leu2-3,112/leu2-3,112, his3Δ200/his3Δ200, lys2-801/lys2-801, ade2/ADE2*	Weinstein and Solomon, 1990
FSY183	*a; ura3-52; leu2-3,112, his3Δ200, lys2-801*	Weinstein and Solomon, 1990
FSY157	*a; ura3-52; leu2-3,112; his3Δ200; lys2-801, Δtub1::HIS3, Δtub3::TRP1* [pRB624]	Schatz *et al.*, 1988
FSY182	*a; ura3-52; leu2-3,112, his3Δ200, lys2-801; Δtub1::HIS3, Δtub3::TRP1* [pRB539]	Schatz *et al.*, 1988
LTY8	FSY157 plus YCpGAL	Archer *et al.*, 1995
LTY11	FSY182 plus YCpGAL	This study
LTY291	FSY157 plus pGHR	This study
LTY292	FSY182 plus pGHR	This study
LTY374	FSY157 plus pPA45	This study
LTY376	FSY182 plus pPA45	This study
JAY47	*a/α, ura3-52/ura3-52, leu2-3,112/leu2-3,112, his3Δ200/his3Δ200, lys2-801/lys2-801, ade2/ADE2, TUB2/TUB2-LEU2-GAL-TUB2*	Archer *et al.*, 1995
LTY319	JAY47 plus YCp50	This study
LTY321	JAY47 plus A21A	This study
LTY323	JAY47 plus pLV32	This study
LTY325	JAY47 plus pA1A5106	This study
LTY338	JAY47 plus pLV30	This study
LTY340	JAY47 plus pLV38	This study
LTY343	JAY47 plus pLV36	This study
LTY345	JAY47 plus pLV37	This study
LTY392	*a/α; ura3-52/ura3-52; leu2-3,112/leu2-3,112, his3Δ200/his3Δ200; lys2-801/lys2-801; TUB1/Δtub1::HIS3, TUB3/Δtub3 TRP1.* [pRB539, pA5]	This study
LTY393	like LTY392 but with pPA45 rather than pA5	This study
LTY395	like LTY392 but with YCpGAL rather than pA5	This study
LTY396	*a/α; ura3-52/ura3-52, leu2-3,112/leu2-3,112, his3Δ200/his3Δ200; lys2-801/lys2-801; TUB1/Δtub1::HIS3,TUB3/Δtub3 TRP1.* [pRB624, pPA45]	This study
LTY397	like LTY396 but with pA5 rather than pA45	This study
LTY440	JAY47 plus YCpGAL	This study
LTY439	JAY47 plus pLV56	This study
LTY399	like LTY396 but with YCpGAL rather than pA45	This study
LTY539	FSY183 plus pJA3 and pLV62	This study
LTY541	FSY183 plus pJA3 and pRS317	This study
LTY540	FSY183 plus YCpGAL and pLV62	This study
Plasmids		
YCp50	*CEN-URA3*	Kirkpatrick and Solomon, 1994
pA1A5106	*TUB1-CEN-URA3*	Kirkpatrick and Solomon, 1994
pA21A	*RBL2-CEN-URA3*	Archer *et al.*, 1995
pA5	*GAL 1-10-RBL2-URA3*	Archer *et al.*, 1995
pGHR	*GAL 1-10-HIS$_6$-RBL2 URA3*	Archer *et al.*, 1998
pRB624	*tub1-724-CEN-LEU2*	Schatz *et al.*, 1988
pRB539	*TUB1-CEN-LEU2*	Schatz *et al.*, 1988
pPA45	*GAL 1-10-PAC2-CEN-URA3*	Alvarez *et al.*, 1998
YCpGAL	*GAL1-10-CEN-URA3*	Archer *et al.*, 1995
pLV30	*tub1-704* in YCp50	This study
pLV32	*tub1-724* in YCp50	This study
pLV36	*tub1-737* in YCp50	This study
pLV37	*tub1-747* in YCp50	This study
pLV38	*tub1-714* in YCp50	This study
pLV56	*GAL 1-10-PAC2-HA-HIS$_6$-CEN-URA3*	This study
pLV62	*GAL1-10-PAC2-HA-HIS$_6$-CEN-LYS2*	This study
pRS317	*CEN-LYS2*	Sikorski and Hieter, 1989

Formation of Rbl2p-β-Tubulin Complex in Wild-Type and tub1-724 Mutant Cells

Rbl2p is complexed with β-tubulin in vivo, and the level of that complex increases as the cellular level of Rbl2p increases (Archer *et al.*, 1995, 1998). The model presented in Figure 1 predicts that overexpressed Rbl2p will form a complex with β-tubulin more readily in *tub1-724* cells than in wild-type cells. To test that possibility, we introduced a plasmid encoding His$_6$-Rbl2p under the control of the galactose promoter into wild-type *TUB1* cells or *tub1-724* mutant cells. The transformants were grown at the permissive temperature for the mutant in noninducing medium and then were shifted to inducing medium containing galactose for 1 or 2 h. We used nickel-agarose beads to purify the His$_6$-Rbl2p-β-tubulin complex. The bound

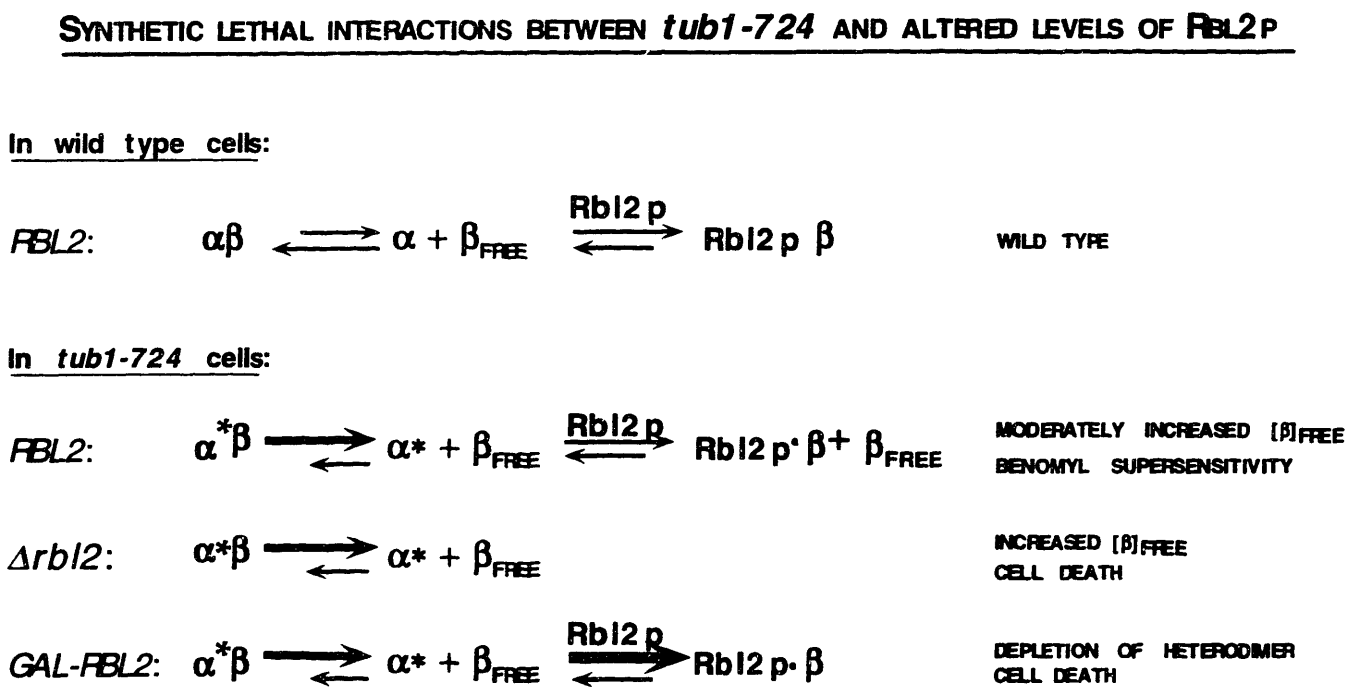

Figure 1. Synthetic lethal interactions between *tub1-724* and altered levels of Rbl2p: a model. Cells expressing *tub1-724* as their sole source of α-tubulin die when Rbl2p is either absent or overexpressed. Those relationships are explicable if the heterodimer formed by the Tub1-724p (α*β) dissociates more readily than that formed by the wild-type Tub1p (αβ). In the presence of a normal complement of *RBL2*, the mutant cells would have a high concentration of free β-tubulin (β_{free}), which may be responsible for the conditional phenotypes of the mutant (e.g., benomyl supersensitivity). In the absence of Rbl2p, the activity of β_{free} would increase to toxic levels. In contrast, an excess of Rbl2p could bind to β-tubulin and so enhance dissociation of the mutant heterodimer, promoting dissociation to levels below those necessary for viability.

proteins were eluted and analyzed by immunoblotting with antibodies against α-tubulin, β-tubulin, or Rbl2p. As expected, the levels of His_6-Rbl2p-β-tubulin complex increase upon induction in both control and mutant cells, but three- to fivefold more complex forms in *tub1-724* cells relative to wild type (Figure 3). In these experiments, we detect only a trace of α-tubulin bound to the nickel columns, and its level does not increase with time in galactose (Archer *et al.*, 1998; our unpublished results). This result suggests either that Rbl2p competes more efficiently with Tub1-724p than with wild-type α-tubulin for binding to β-tubulin in vivo, or that there is a greater pool of free β-tubulin available for binding to Rbl2p in the *tub1-724* mutant (see DISCUSSION). Either possibility is consistent with Tub1-724p forming a less stable heterodimer with β-tubulin than wild-type α-tubulin.

Rescue of β-Tubulin Lethality by Wild-Type and Mutant α-Tubulins

An excess of either α-tubulin or Rbl2p rescues cells from β-tubulin lethality (Archer *et al.*, 1995; Alvarez *et al.*, 1998); the rescue likely depends on the ability of these two proteins to bind β-tubulin. Even a modest excess of α-tubulin, expressed under the control of its own promoter from a low-copy plasmid, increases the survival of cells overproducing β-tubulin by two to three orders of magnitude. If Tub1-724p binds β-tubulin with low affinity, we would expect it to rescue β-tubulin lethality poorly. To test this hypothesis, wild-type or mutant alleles of α-tubulin were introduced into JAY47, a diploid strain with a normal complement of tubulin genes plus a third, integrated copy of the β-tubulin gene *TUB2* under the control of the galactose promoter. We measured the percent survivors on galactose relative to glucose at both the permissive (30°C) and the nonpermissive (18°C) temperatures (Table 2). Rescue of β-tubulin lethality by *tub1-724* is substantially less efficient (0.84%) than by wild-type *TUB 1* (15.4%). The efficiency of rescue is further diminished at the nonpermissive temperature for the mutant: at 18°C, *tub1-724* rescues β-tubulin lethality (0.06%) to essentially the same extent as the negative control (0.03%). In contrast, four other mutant α-tubulins rescue at levels comparable to that of the wild type, and their efficiency is unaffected by the temperature of growth. In fact, the activity of those alleles persists even at 15°C (our unpublished results). These results are consistent with the conclusion that

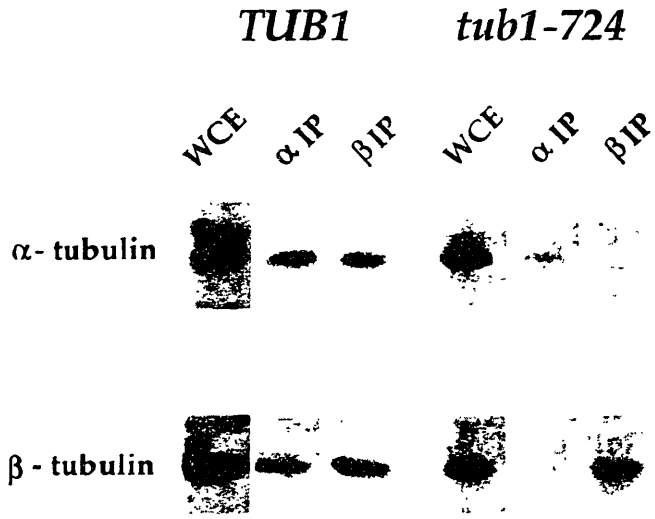

Figure 2. α- and β-Tubulin coimmunoprecipitate with low efficiency from *tub1-724* cells. Immunoblots with anti-α-tubulin (top row) and anti-β-tubulin (bottom row) of whole-cell extracts (WCE) and the precipitates with the two antibodies (αIP and βIP) from wild-type *TUB1* or mutant *tub1-724* cells

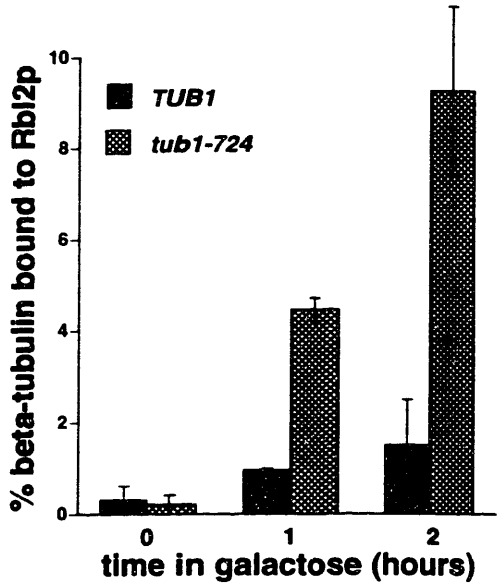

Figure 3. The Rbl2p-β-tubulin complex in vivo is enhanced in *tub1-724* cells. Cells growing in raffinose (0 h) were induced with galactose to express His_6-Rbl2p for 1 or 2 h. His_6-Rbl2p was isolated by affinity chromatography of the whole-cell extracts, and the levels of β-tubulin in the original extract and bound to Rbl2p were assayed by immunoblotting. The results are the averages of two independent experiments for each strain and time point with the ranges indicated by error bars. In both of these experiments, the wild-type strain produced slightly more His_6-Rbl2p upon induction (our unpublished results). Solid bars, *TUB1* cells; cross-hatched bars, *tub1-724* cells.

Tub1-724p binds β-tubulin with lower affinity than does wild type α-tubulin.

Cold Sensitivity of TUB1/tub1-724 Heterozygotes and Their Suppression by Excess Rbl2p

The *tub1-724* phenotype is not completely suppressed in a heterozygote with *TUB1*. A diploid strain containing only single chromosomal copies of *TUB1* and *TUB3* plus a low-copy plasmid expressing *tub1-724* is cold sensitive for growth at 18°C. In contrast, heterozygotes containing *TUB1* and other *tub1* mutants show the same temperature sensitivity as do wild-type cells (our unpublished results). The conditional growth of *TUB1/tub1-724* heterozygotes must reflect a property of the mutant heterodimer, rather than a deficiency in tubulin levels, because diploid cells with only 50% of their wild-type complement of tubulin are wild type for growth at low temperatures (Katz *et al.*, 1990).

We hypothesized that the cold sensitivity of these *TUB1/tub1-724* heterozygous cells is due to the free β-tubulin produced by dissociation of the mutant heterodimer. Consistent with that explanation, the cold sensitivity of the heterozygotes is substantially suppressed by overexpression of *RBL2* from the galactose promoter (Figure 4). The presence of excess Rbl2p can bind the free β-tubulin and so protect the cell from its deleterious consequences. This result is in striking contrast to the lethal effect of *GAL-RBL2* in cells expressing *tub1-724* as their sole source of α-tubulin (see above).

Overexpression of PAC2 in tub1-724 Cells

Pac2p is a candidate for an α-tubulin–binding protein in yeast. It is the homologue of cofactor E in the in vitro system described above. Cofactor E plays an essential role in this assay: it is believed to bind to α-tubulin after its release from the TCP1-containing

Table 2. Rescue of excess β-tubulin lethality by α-tubulin alleles

Plasmid	30°C	18°C
	.04	.03
RBL2	6.8	7.2
TUB1	12.0	15.4
tub1-724	.84	.06
tub1-704	10.9	18.9
-714	14.0	20.0
-737	4.1	8.5
-747	11.8	21.0

JAY47 cells, which contain an integrated *GAL-TUB2* gene, carrying the indicated α-tubulin alleles on plasmids were plated to media containing either galactose or glucose and incubated at either 30 or 18°C. Rescue is reported as the percentage of cells that form colonies on galactose plates compared with glucose plates.

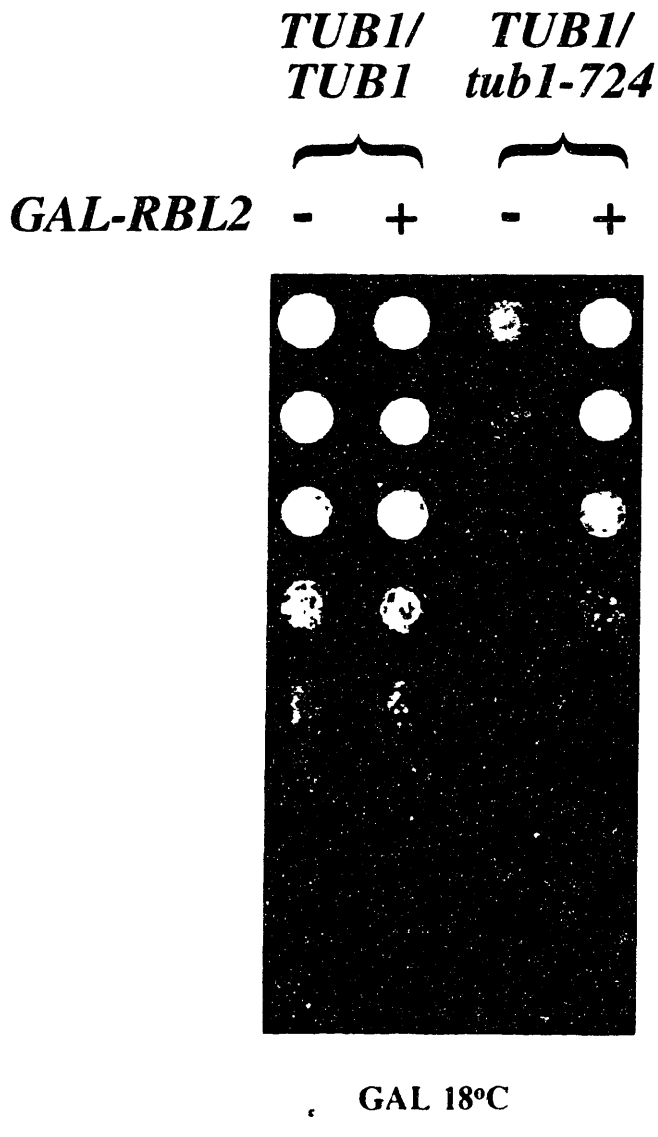

Figure 4. Overexpression of *RBL2* suppresses *TUB1/tub1-724* heterozygous cells. Serial (fourfold) dilutions of saturated cultures were plated to galactose-containing media and allowed to grow at 18°C. The cells were either wild-type diploids or *TUB1/tub1-724* cells, carrying either YCpGAL or CEN GAL *RBL2*

ring complex (Tian *et al.* 1997). This binary complex is then thought to form a quaternary complex with cofactor D and β-tubulin. The cofactor E-α-tubulin complex is rather unstable and is detectable on native gels only after it is stabilized by glutaraldehyde fixation.

The *Schizosaccharomyces pombe* homologue of cofactor E is essential in vivo (Hirata *et al.*, 1998). In budding yeast *PAC2* is not essential, but mutations in *pac2* affect microtubule functions. *pac2* mutations are supersensitive to benomyl (Hoyt *et al.* 1997). It is required in cells deleted for *cin8*, which encodes a kinesin-related protein that participates in anaphase (Geiser *et al.*, 1997), or deleted for *pac10* (Alvarez *et al.*, 1998), which affects ratios of α-tubulin to β-tubulin (Alvarez *et al.*, 1998; Geissler *et al.*, 1998).

If Pac2p is an α-tubulin–binding protein, we would predict that at elevated levels it would be deleterious to cells containing the unstable *tub1-724* heterodimer. As shown in Figure 5, induction of *GAL-PAC2* in haploid *tub1-724* cells grown at permissive temperature (30°C) causes rapid loss of viability, down 10-fold in ~3 h. In contrast, *GAL-PAC2* has only a modest effect on the viability of wild-type cells (Figure 5). In that time, the induction of *GAL-PAC2* causes microtubule disassembly in the mutant but not in wild-type cells; representative micrographs are shown in Figure 6. From such fields, we find that overexpression of *PAC2* increases the proportion of *tub1-724* cells that have no microtubules by 10-fold (53.2 vs. 5.4%) but has no effect on wild-type cells (10.1% for both strains).

Both phenotypes of elevated Pac2p levels on *tub1-724* haploid cells are the same as produced by elevated levels of Rbl2p (Archer *et al.*, 1995). Therefore, these results could represent Pac2p binding to either β-tubulin or α-tubulin. However, the effect of *GAL-PAC2* expression in *TUB1/tub1-724* heterozygotes does distinguish between these two possibilities. As shown in Figure 7, overexpression of *PAC2* in the heterozygotes causes a significant loss of cell viability at the permissive temperature. This result contrasts with that shown in Figure 4 above, showing that overexpression of *RBL2* actually suppresses the phenotype of the *TUB1/tub1-724* heterozygotes.

These results are explicable if the Tub1-724p-β-tubulin heterodimer is relatively unstable (Figure 1). The increased levels of an α-tubulin–binding protein might be expected to increase free β-tubulin to toxic levels in both *tub1-724* haploids and *TUB1/tub1-724* heterozygotes. This outcome is in contrast to the effect noted for excess Rbl2p in the heterozygotes, where the increased capacity to bind β-tubulin would be expected to reduce its levels and so suppress the *TUB1/tub1-724* phenotypes. Taken together, these results suggest that Pac2p can bind to α-tubulin in vivo and so are consistent with the conclusion of the in vitro experiments (Tian *et al.*, 1997).

Isolation of a Pac2p-α-Tubulin Complex

To demonstrate directly a Pac2p-α-tubulin complex, we used a form of Pac2p that contains the HA tag followed by 6 histidines at its carboxyl terminus. This modified allele is functionally indistinguishable from wild type Pac2p in both Δ*pac2* and *tub1-724* cells (our unpublished results). We can isolate a complex containing α-tubulin and Pac2p-(HA)-His_6 from extracts of cells overexpressing both proteins (Figure 8, lane c); no β-tubulin is detected in this complex. We cannot

SYNTHETIC EFFECTS OF PAC2p OVERPRODUCTION IN *tub1-724* CELLS

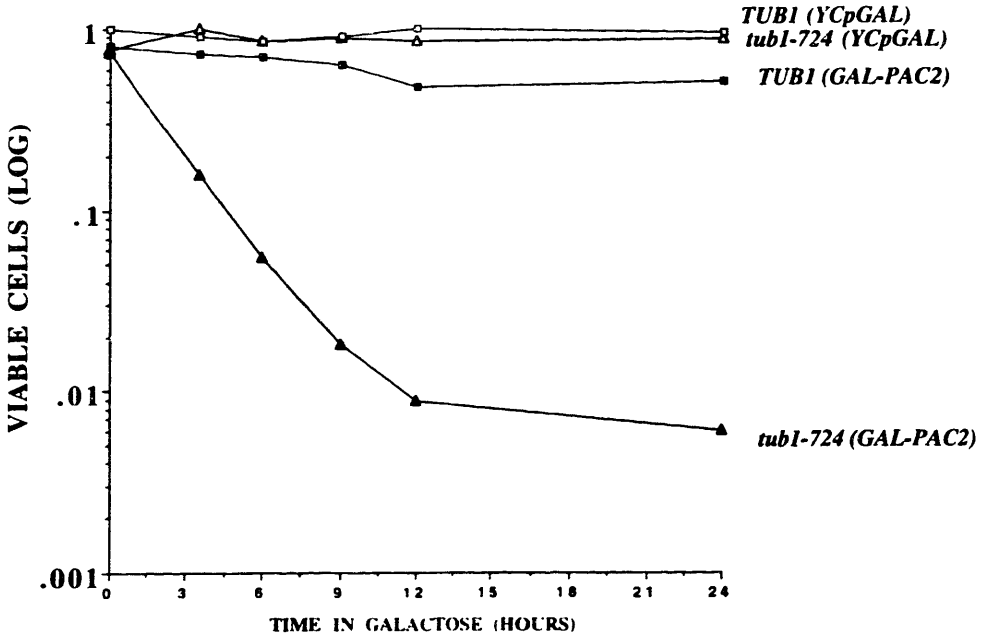

Figure 5. Overexpressing *PAC2* is lethal in *tub1-724* cells. *tub1-724* (triangles) and wild type (squares) containing either control plasmid (open symbols) or *GAL-PAC2* (filled symbols) cells growing at 30°C were shifted to galactose-containing media at zero time, and aliquots were taken at intervals and scored for total cells and viable cells.

detect this complex unless both Pac2p and α-tubulin are overexpressed. In contrast, overexpression of both Pac2p and β-tubulin does not produce a complex between those two proteins (Figure 8, lane g). These results support the conclusion that Pac2p can bind α-tubulin in vivo. Overexpression of Pac2p-(HA)-His_6 alone in *tub1-724* cells does not produce measurable levels of the Pac2p-α-tubulin complex (our unpublished results).

DISCUSSION

A Tubulin Mutation That Affects Heterodimer Stability

tub1-724 is one of a set of α-tubulin mutants generated by chemical mutagenesis and selected on the basis of their conditional growth at low temperature. Because of the familiar cold lability of microtubules evident both in vivo and in vitro, a reasonable prediction might have been that mutants so selected would arrest because their microtubules were especially cold labile at temperatures permissive for wild-type cells. Instead, only a subset of the mutants arrest with no microtubules; the others have at least normal complements of assembled tubulin.

Here we have characterized the properties of the protein encoded by one of the mutants that arrest with no microtubules, *tub1-724*. We previously showed that cells expressing only this α-tubulin allele are dead when Rbl2p is either overexpressed or absent. Because Rbl2p is a β-tubulin–binding protein, we hypothesized that these lethal interactions could reflect an unstable heterodimer formed by Tub1-724p (Figure 1). Several of the experiments presented above demonstrate that the mutant heterodimer does act as if it were unstable relative to wild type. The mutant heterodimer does not remain intact in vitro during immunoprecipitation. Similarly, in vivo the mutant heterodimer reacts more readily with excess Rbl2p to produce Rbl2p-β-tubulin. An alternative measure of Tub1-724p binding to β-tubulin is manifest in its inability to rescue cells from β-tubulin overexpression even at permissive temperature for the mutant (Table 2); success in that assay most likely depends on the ability of the α-tubulin protein to bind β-tubulin. These results indicate that Tub1-724p has a reduced affinity for β-tubulin. However, the normal growth of the mutant cells requires that most of its tubulin be in heterodimers, rather than as free α- and β-tubulin. We previously showed that the microtubules in 50% of cells overproducing β-tubulin are completely depolymerized when β-tubulin levels are 1.4-fold greater than wild type (Weinstein and Solomon, 1990).

A weaker heterodimer could readily explain the arrest phenotype of *tub1-724* cells. At the restrictive temperature, increased dissociation of the mutant het-

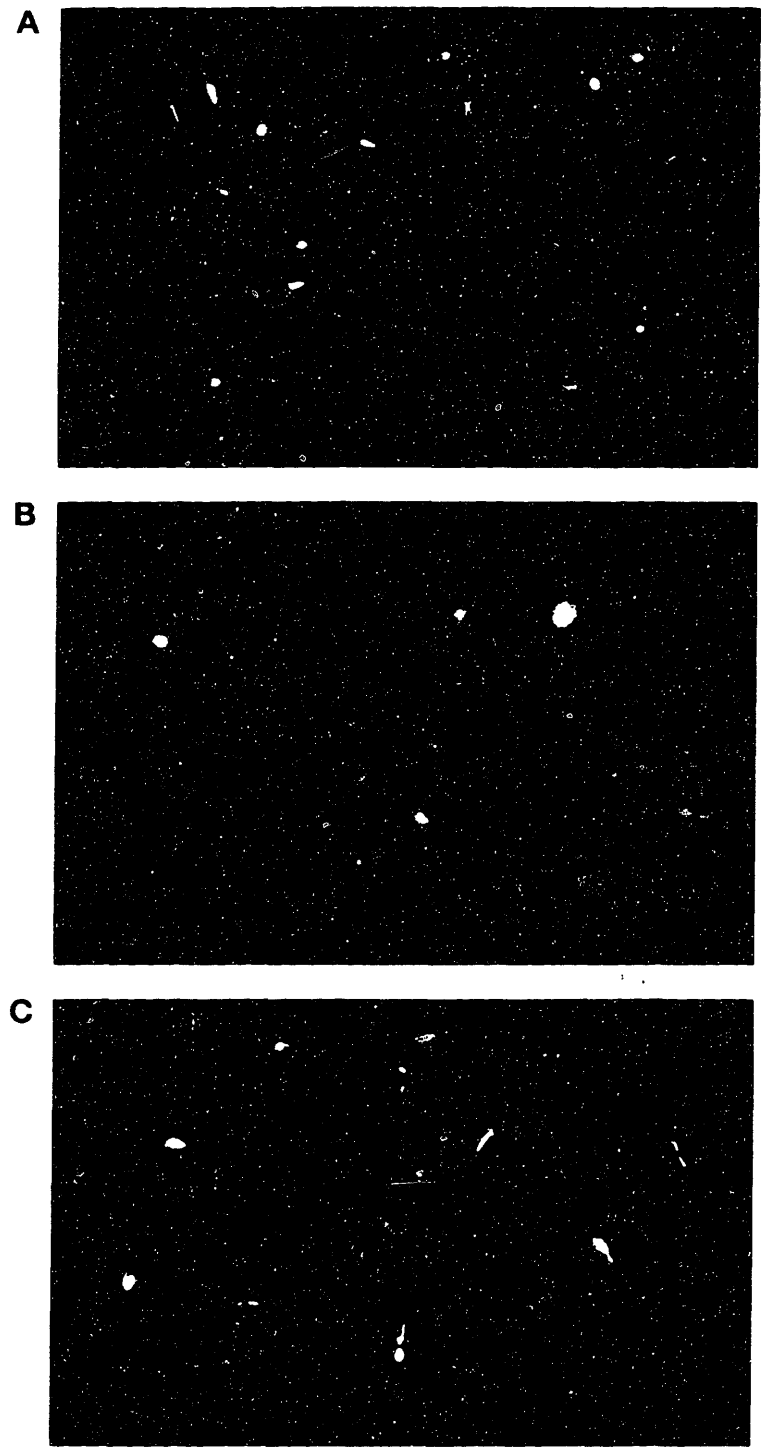

Figure 6. Microtubule disassembly in *tub1-724* cells overexpressing *PAC2*. Anti-tubulin immunofluorescence of *tub1-724* cells containing the control plasmid YCpGAL (A) or a *CEN-GAL-PAC2* (B) and wild-type cells containing a *CEN-GAL-PAC2* plasmid (C). Cultures were grown in galactose for 3.5 h before fixation for immunofluorescence.

erodimer could be lethal either by decreasing the level of heterodimer below that necessary to maintain microtubules or by increasing the level of undimerized β-tubulin, which in turn causes microtubule disassembly and cell death even at modest excess (Katz *et al.*, 1990; Weinstein and Solomon, 1990).

The single mutation in Tub1-724p predicted from the DNA sequence is loss of a positive charge at po-

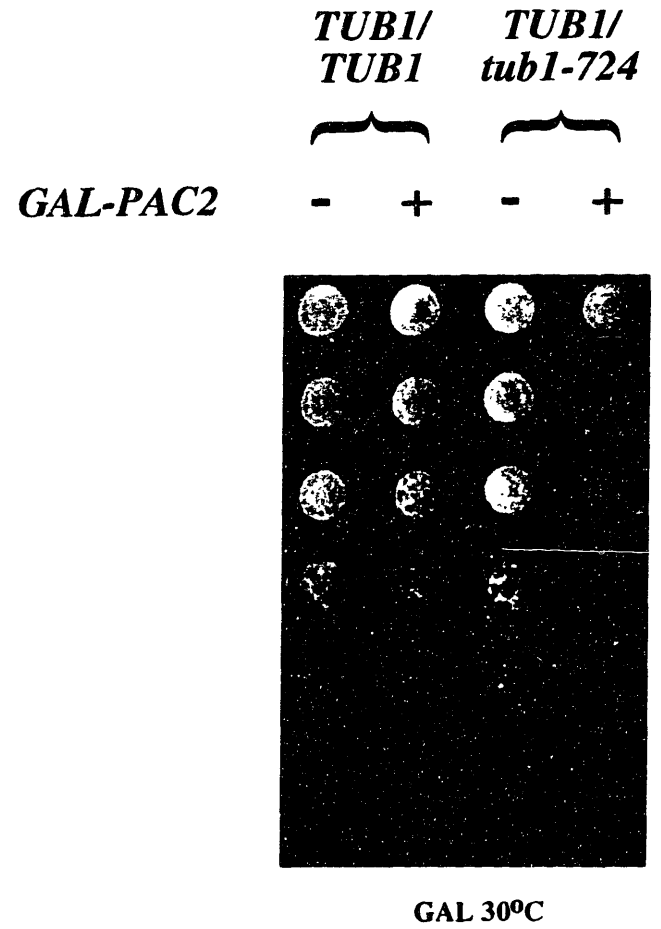

Figure 7. Overexpression of *PAC2* is lethal in *TUB1/tub1-724* heterozygous cells. Serial (fourfold) dilutions of saturated cultures were plated to galactose-containing media and allowed to grow at 30°C. Strains were wild-type diploids or *TUB1/tub1-724* cells containing either YCpGAL or *CEN-GAL-PAC2*.

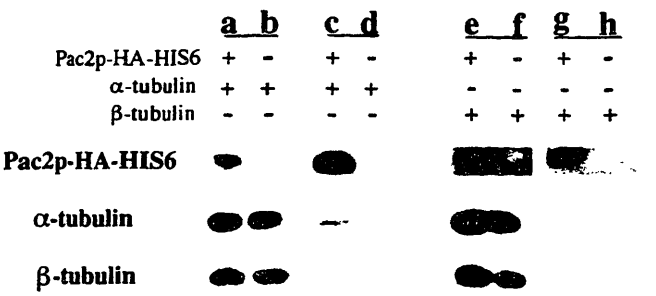

Figure 8. Binding of α-tubulin to Pac2p-(HA)-His_6 in double-overexpressing cells. Whole-cell extracts (lanes a, b, e, and f) and eluants from nickel-agarose beads (lanes c, d, g, and h) were analyzed by SDS-PAGE and immunoblotting for HA-tagged Pac2p, α-tubulin, and β-tubulin. The fractions were from cells overexpressing Pac2p-(HA)-His_6 and α-tubulin (a and c), Pac2p-(HA)-His_6 and β-tubulin (e and g), α-tubulin alone (b and d), and β-tubulin alone (d and h). For Pac2p, the bead eluants represent 120 times the load of whole-cell extract. For α- and β-tubulin, the bead eluants represent 500 times the load of whole-cell extract.

sition 106. Based on the structure of tubulins reported by Nogales *et al.* (1998), this residue occurs in the region between the B3 and H3 loops that contact the phosphates of the nonexchangeable GTP. That site is at the postulated interface between α- and β-tubulin in the heterodimer. The wild-type arginine at this position probably contributes to phosphate binding and so may indirectly participate in α-β interactions. Further analysis to understand the physical properties of mutations in this region are under way.

This analysis of Tub1-724p provides insight into the primary molecular defect that explains the mutant phenotypes. In general, the defects of mutant tubulins are largely understood in terms of the arrest phenotype rather than their execution point. For example, mutations in yeast β-tubulin can selectively affect a subset of microtubules (Sullivan and Huffaker, 1992) or cause cells to become benomyl dependent (Huffaker *et al.*, 1988). Similarly selective tubulin mutations have been identified in other organisms as well (Oakley and Morris, 1980). However, the precise molecular basis for the defective arrest phenotype is not yet understood. A possible exception is the disruption produced by substitution of lysine for the highly conserved glutamate at position 288 in the *Drosophila* β2 protein; this mutation causes an apparent packing defect, so that the protofilaments do not close to form a tubule (Fuller *et al.*, 1987). However, the same substitution in yeast β-tubulin has no apparent effect (Praitis *et al.*, 1991). The generalizability of the mutation found in Tub1-724p also requires further testing.

Genetic Interactions between tub1-724 and PAC2

Instability of the Tub1-724p-β-tubulin heterodimer predicts that overexpression of an α-tubulin–binding protein should be deleterious to *tub1-724* cells, perhaps by producing more toxic free β-tubulin in the mutant cells. The work of Tian *et al.* (1997) suggests that the vertebrate homologue of the yeast protein Pac2p binds α-tubulin. As predicted, overexpression of *PAC2* is lethal in *tub1-724* cells and causes loss of all assembled microtubules. Consistent with this result, we can recover a complex containing Pac2p and α-tubulin from double-overexpressing cells. These results demonstrate for the first time that Pac2p can bind α-tubulin in vivo. This result does not distinguish among many possible functions for *PAC2*. It may act as does cofactor E in the in vitro assay, facilitating the incorporation of α-tubulin into heterodimers (Tian *et al.*, 1997), but it is not essential for that reaction, because *PAC2* is not an essential gene in vivo (Hoyt *et al.* 1997). Δ*pac2* is synthetically lethal with other microtubule mutants: Δ*cin8* (Geiser *et al.*, 1997), Δ*pac10* (Alvarez *et al.*, 1998), and *tub1-724* (Vega, unpublished results).

Regulating Microtubule Function

The first analyses of microtubules at a molecular level focused on protein factors that could be responsible for assembly in an in vitro reaction. It is striking that so many of the genes that appear to affect microtubules in vivo almost certainly do not participate in the polymerization reaction itself. In this sense, the *CIN* genes (Hoyt *et al.*, 1990; Stearns *et al.*, 1990), the *PAC* genes (Geiser *et al.*, 1997), the *GIM* genes (Geissler *et al.*, 1998), and the *RBL* genes (Archer *et al.*, 1995), although identified—in some cases more than once—by a wide variety of approaches, have fundamental properties in common. They are not essential for cell viability in budding yeast, and their deletion does not confer a quantitative defect in microtubule assembly. Conversely, their overexpression does not increase the level of assembly, as could be expected for a modulator of microtubule assembly. For only one of these proteins, alp1, a *CIN1* homologue in fission yeast, is there evidence suggesting that it binds along the length of the microtubule (Hirata *et al.*, 1998).

A role for these proteins arises from the in vitro system for incorporating separated tubulin chains into heterodimer. Alone among proteins that have been analyzed in such assays, the tubulin polypeptides appear to require factors that act after release from the chaperonin. Without those factors, there is no exchange of newly folded polypeptide with the exogenously added heterodimer. Some of the protein factors are homologous to gene products in *S. cerevisiae* and *S. pombe* that affect microtubule functions, and in *S. pombe* some of them are essential (Hirata *et al.*, 1998). That they are not essential in *S. cerevisiae*, however, suggests that there must be other mechanisms for folding tubulin and forming heterodimer in those cells.

These proteins may also have alternative functions. Rbl2p levels affect how cells survive alterations in the ratios of α- to β-tubulin (Archer *et al.*, 1995). Levels of Pac10p and the *GIM* genes affect those ratios (Alvarez *et al.*, 1998; Geissler *et al.*, 1998). It is clear that yeast cells are sensitive to those ratios. These proteins may participate in maintaining proper balance of the tubulin components, which may become an important step, especially under times of stress. Such a role could help explain why expression of *RBL2* mRNA increases when cells are incubated with a microtubule-depolymerizing drug (Velculescu *et al.*, 1997), although there is no evidence that the tubulin chains themselves are expressed in greater amounts. The results from these several approaches suggest that the early steps of microtubule morphogenesis may be crucial for cell function.

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