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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.

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RBL2        MAPT...QLDIKVKALKRLTKEEGYYQQELKDQEAHVAKLKEDKSVDPYD 47
            ||..  |:.||. .:::|.||  . |:.| |:|| ..:.|:|.:.: : |.
CofactorA   MADPRVRQIKIKTGVVRRLVKERVMYEKEAKQQEEKIEKMKAEDG.ENYA 49

            LKKQEEVLDDTKRLLPTLYEKIREFKEDLEQFL...KTYQGTEDVSDARS 94
            :|||.|:|::.:.::|.   :: .  .||:|:|   |..:::.|: .:||
            IKKQAEILQESRMMIPDCQRRLEAAYTDLQQILESEKDLEEAEEYKEARV 99

            AITSAQELLDSK 106
            .:.|..  |:.
            VLDSVK..LEA. 108
```

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

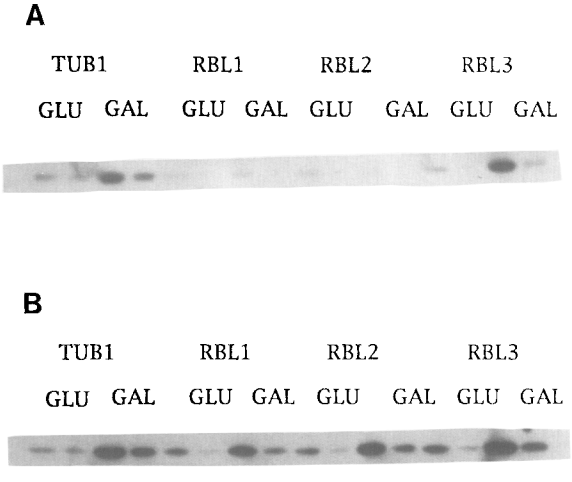


Figure 2. Levels of β- and α-Tubulin in Suppressed JAY47 Cells

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.

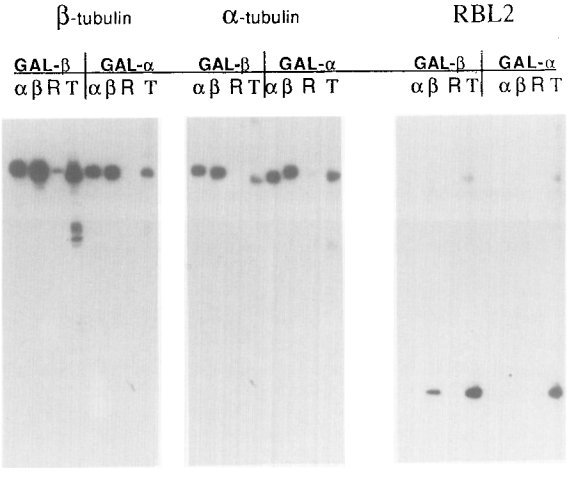
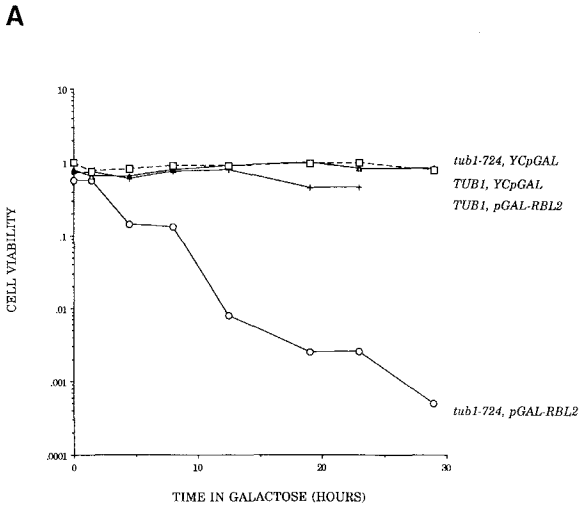
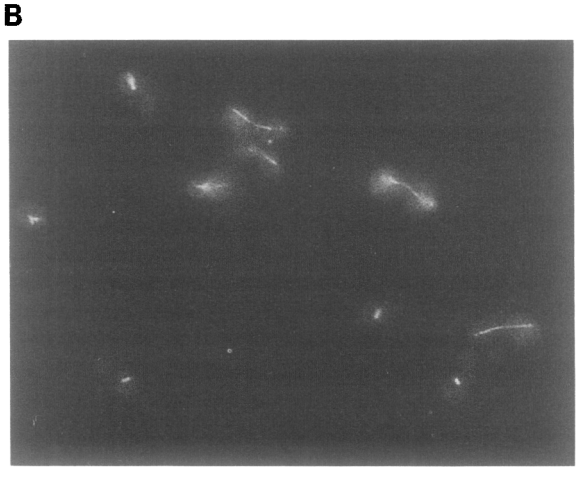


Figure 3. Rbl2p Coimmunoprecipitates with β-Tubulin

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.





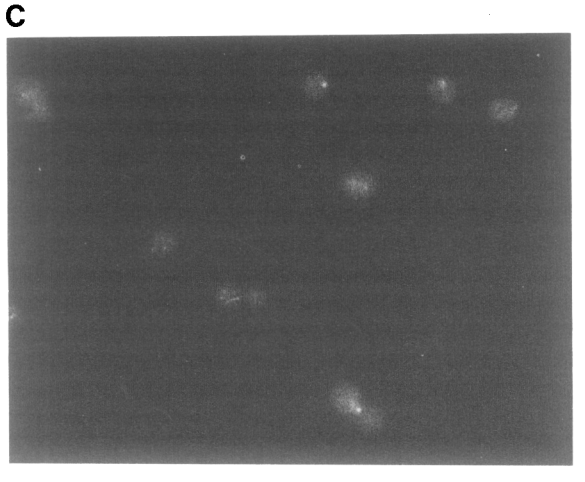


Figure 4. Synthetic Interaction of *RBL2* Overexpression with *tub1-724*

(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 *ΔRBL2* 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*	–	–
tub1-738 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).

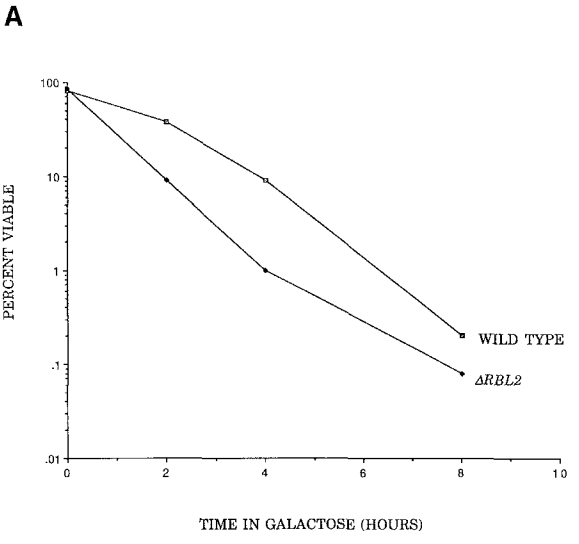


Figure 5. Phenotypic Consequences of Altered Stoichiometry between Rbl2p and Tubulin

(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. *ΔTUB3* 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 10^8 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.

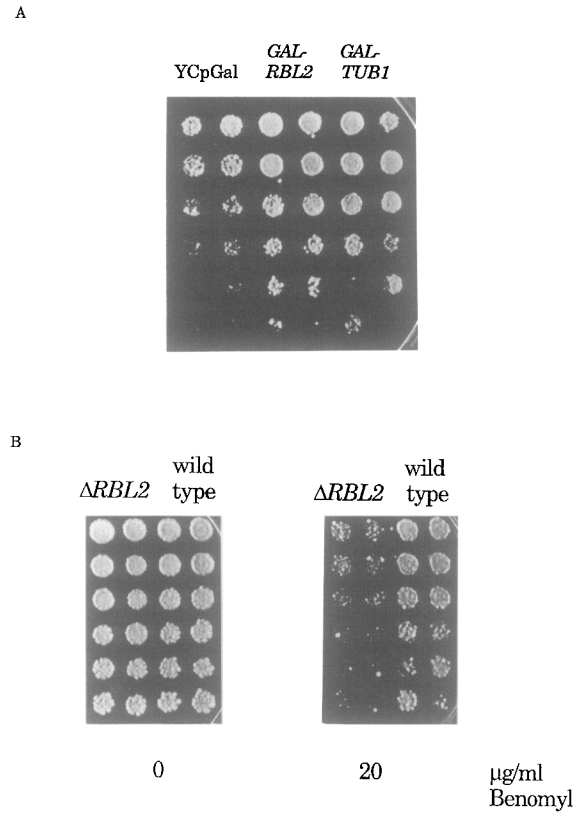


Figure 6. Levels of Rbl2p Affect Growth on Benomyl, a Microtubule-Depolymerizing Drug

(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.

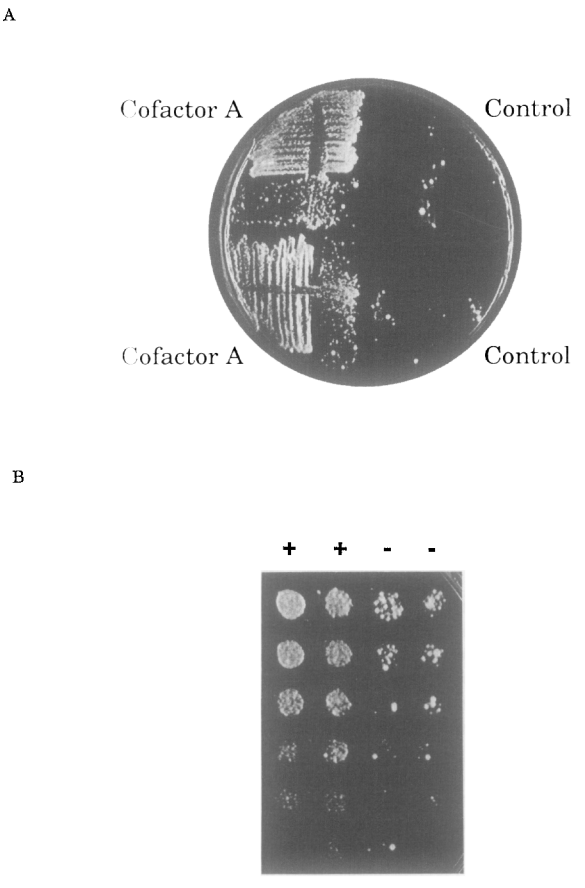


Figure 7. Overexpression of Murine Testes Cofactor A Has Phenotypes Reminiscent of Rbl2p in Yeast

(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 10^7 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	<^	=	<^
β-OVEREXPRESSION	<^	=	>*
Δtub3	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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