Normal Microtubule Function and the Interactions Between the Pathways for Tubulin Folding and Expression in S. cerevisiae

by

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ABSTRACT

Undimerized β-tubulin is toxic to the yeast Saccharomyces cerevisiae. Free β-tubulin can arise if the tubulin heterodimer dissociates or if levels of β-tubulin and α-tubulin are unbalanced. I am using the toxicity of β-tubulin to understand the early steps in microtubule morphogenesis. I have found that a mutation of the gene PLP1 allows cells to survive the toxicity of β-tubulin produced from disparate levels of α- and β-tubulin. The suppression occurs either when α-tubulin is modestly underexpressed relative to β-tubulin, or when β-tubulin is inductively and strongly overexpressed. A significant proportion of the undimerized β-tubulin in plp1Δ cells is less toxic and less functional than in wild type cells. As a result, plp1Δ cells have lower levels of heterodimer. Significantly, plp1Δ cells that also lack Pac10p, a component of the GimC/Pfd complex that helps fold tubulin polypeptides, are even less affected by free β-tubulin. Our results suggest that Plp1p defines a novel step in β-tubulin folding.

My work demonstrates an interaction between the pathways for tubulin folding and the regulation of tubulin expression. Cells that are pac10Δ plp1Δ have much less folded and functional β-tubulin than even plp1Δ cells, and also upregulate β-tubulin through increasing transcription. The upregulation of β-tubulin RNA is dependent on the putative transcription factor Cin5p. In the absence of CIN5, pac10Δ plp1Δ cells have decreased tubulin heterodimer levels, down to approximately 20% that of wild type. The heterodimer levels are also decreased from pac10Δ plp1Δ cells suggesting that the limiting factor in heterodimer formation in pac10Δ plp1Δ cin5Δ is β-tubulin. The pac10Δ plp1Δ cin5Δ cells grow normally, but have mitotic defects such as abnormal nuclear positioning and short anaphase spindles. The Cin5p dependent upregulation of β-tubulin may be a mechanism to maintain tubulin heterodimer levels and so sustain normal microtubule function.

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Chapter 1

Introduction
A BRIEF OVERVIEW

Microtubules are one of the main components of the eukaryotic cytoskeleton and have many essential roles in the cell (Dustin 1984). They are involved in multiple processes including cell division, motility, and intracellular transport and are the major components of structures in the cell such as the flagellar axoneme and the bipolar spindle. Not only are microtubules organized differently in various structures, they also have the capability of dramatically changing their organizational state. In interphase of the cell cycle, microtubules form long arrays and then switch to a bipolar spindle during mitosis that is easily assembled and disassembled for each cell division. The basic subunits of microtubules remain the same in each of the different organizational states, only the associated proteins change.

The regulation of tubulin organization can occur through many aspects. The work cited below describes roles of regulation through microtubule-associated proteins, directional motor proteins, and microtubule dynamics and flux. The work in this thesis involves an entirely different mode of regulation - through the formation of the microtubule. My work focuses on the early steps of microtubule morphogenesis, those involved in the heterodimerization of α- and β-tubulin.

There are many steps involved in the regulation of tubulin heterodimer formation (see Figure 1-1). Tubulin polypeptides are first transcribed and translated. Feedback loops of tubulin RNA regulation through message degradation have been previously described (Ben-Ze'ev et al. 1979; Cleveland and Kirschner 1981). My work also shows a feedback pathway, but through tubulin transcription. Once the tubulin messages are transcribed and translated, the polypeptide
Figure 1-1. The steps involved in tubulin heterodimer formation.

Once tubulin polypeptides are transcribed and translated, they are folded by the chaperonin TriC with the help of the GimC/PFD complex. Newly folded tubulin polypeptides are then able to form heterodimers. Excess β-tubulin is toxic in *S. cerevisiae*.
needs to be properly folded to form tubulin heterodimers. I have identified a protein that is involved in tubulin heterodimer formation, through β-tubulin folding. Furthermore, enough heterodimer must form for the morphogenesis of a microtubule. Our lab has previously shown that a budding yeast cell with 50% of the amount of tubulin heterodimer can survive and grow normally (KATZ et al. 1990). I have now found that cells with 20% the amount of tubulin heterodimer also grows at a normal rate. This sets a new definition for the amount of tubulin needed for survival. This introduction will give a general overview of microtubules and then focus on the topics directly related to my project, mainly tubulin folding, RNA regulation, and the proteins involved in tubulin regulation in *S. cerevisiae*.

**Microtubule Structure and Organization**

A microtubule is a polymer made up of α- and β-tubulin heterodimers. The α- and β-tubulin proteins are very similar, with approximately 50% identity at the level of the amino acid (CLEVELAND 1987). In vertebrates, there are many isotypes of α- and β-tubulin, each with a very specific developmental regulation. These tubulins are highly conserved both within and between species. In the unicellular eukaryotes, *S. cerevisiae* and *S. pombe* there are two α-tubulin encoding genes (HIRAOKA et al. 1984; SCHATZ et al. 1986a; SCHATZ et al. 1986b) and a single β-tubulin encoding gene (NEFF et al. 1983; TODA et al. 1984). The homologies among yeast and vertebrate tubulins are also very striking, showing that these proteins have retained essential structural features throughout eukaryotic evolution.

Microtubules are polar structures. The α- and β-tubulin heterodimers bind each other linearly to form a protofilament that associates laterally with other protofilaments to form a
hollow cylinder (DESAI and MITCHEISON 1997). Since most microtubules are made up of 13 protofilaments, one end of the microtubule will have 13 $\alpha$-tubulin subunits and the other will have 13 $\beta$-tubulin subunits. The ends of a microtubule have been defined as a fast growing plus end and a slow growing minus end (ALLEN and BORISY 1974).

In most cell types, microtubule organizing centers (MTOCs) nucleate microtubules in the cell. MTOCs are also known as centrosomes in vertebrate cells and spindle pole bodies in yeast. The microtubule minus ends are bound to the MTOC. A major and essential component of the MTOC is $\gamma$-tubulin, a member of the tubulin superfamily that is also conserved throughout eukaryotic evolution (JOJ et al. 2003). $\gamma$-tubulin can be found as part of a complex called the $\gamma$-tubulin ring complex ($\gamma$-TuRC) that is thought to help nucleate microtubules at the centrosome by acting as a template for $\alpha$- and $\beta$-tubulin heterodimers to bind.

The structure of the heterodimer has been solved at 3.7 Å resolution (NOGALES et al. 1998) and refined at 3.5 Å (LOWE et al. 2001). This is a remarkable feat because of the difficulties with aggregation and polymerization of purified tubulin. To solve the structure of the tubulin heterodimer, zinc-induced tubulin sheets were stabilized by taxol. The sheet is not a true microtubule, but gives information about the heterodimer contacts that make the protofilament. To complement for lack of a true microtubule structure, the heterodimers are placed onto an 8 Å reconstruction of a microtubule achieved by cryoelectron microscopy to give information about lateral contacts between the protofilaments (LI et al. 2002). This composite image gives more information about where microtubule-binding proteins can interact with tubulin to give specificity and changes in microtubule organization.
Microtubules can serve as highways throughout the cell, allowing movement of different proteins and organelles. The polarity of the microtubules allows for directed transport of cargo through motor proteins. There are two major classes of motor proteins, the dyneins and the kinesins (Thaler and Haimo 1996). Dyneins allow transport towards the minus end of a microtubule, and kinesins drive cargo to the plus ends of microtubules; although there are minus-end directed kinesins. Dyneins and kinesins as well as related proteins have been implicated in many processes in the cell including flagellar movement, bipolar spindle organization, chromosome segregation during mitosis and meiosis, organelle positioning, and vesicle transport. Motor proteins are essential for the diverse roles that microtubules play and have a role in organizing random arrays of microtubules.

**Microtubule Rearrangements**

Microtubules are very dynamic structures, allowing them to have many functions in the cell. Mitchison and Kirschner defined the term dynamic instability while studying microtubules that are nucleated from centrosomes as well as free, finding that a microtubule can switch between a period of growth and a period of shrinkage (1984a; 1984b). Their study shows that at steady state, most microtubules grow slowly with a small number rapidly shrinking. The existence of dynamic instability is further confirmed using real-time microscopy of a single microtubule polymerizing and depolymerizing at varying tubulin concentrations (Walker et al. 1988). The transition of a microtubule from a period of growth to shrinkage is termed catastrophe (McIntosh 1984); the change from shrinkage to growth is called rescue (Walker et
Regulation of the rate and frequency of growth and shrinkage can allow microtubules to abruptly change organizational states.

A model to explain how dynamic instability occurs is based on the binding and hydrolysis of GTP. Both \(\alpha\)- and \(\beta\)-tubulin bind GTP (NOGALES 2001). The GTP bound to \(\beta\)-tubulin is exchangeable to GDP, unlike the GTP bound in \(\alpha\)-tubulin that is nonexchangeable. For microtubule polymerization, both \(\alpha\)- and \(\beta\)-tubulin must be GTP bound; however, the \(\beta\)-tubulin GTP can be hydrolyzed. Most of the lattice of the microtubule is made up of GDP bound \(\beta\)-tubulin (CARLIER and PANTALONI 1981). When a nonhydrolyzable analog of GTP is used in a microtubule polymerization assay, the microtubule can still polymerize, but depolymerizes very slowly and suppresses microtubule dynamics (HYMAN et al. 1992; MEJILLANO et al. 1990). These results suggest that GTP hydrolysis is not needed for microtubule polymerization, but is needed for microtubule dynamics.

The prevailing model to explain dynamic instability is the GTP cap model (MITCHISON and KIRSCHNER 1984a; MITCHISON and KIRSCHNER 1984b). In this model, most of the lattice making up the microtubule contains GDP bound \(\beta\)-tubulin that is stabilized by a layer, or cap, of GTP bound \(\beta\)-tubulin. If this layer is lost, for example by hydrolysis of GTP, the microtubule rapidly depolymerizes (Figure 1-2). In support of this model are several microtubule severing assays. If a microtubule is severed, the newly created plus-end rapidly depolymerizes, suggesting that the severing exposes the unstable GDP-bound tubulin (TRAN et al. 1997; WALKER et al. 1989). If a microtubule assembled from a slowly hydrolyzable GTP analog is severed, the plus end does not undergo catastrophe (TRAN et al. 1997). In this case, the "cap" encompasses the entire length of the microtubule and is not released by the shearing.
Figure 1-2. A model for dynamic instability.
Polymerization of microtubules occurs with the addition of heterodimer with GTP bound β-tubulin. The GTP is hydrolyzed to GDP soon after addition to the microtubule lattice. Most of the lattice is made up of GDP bound β-tubulin. A cap of GTP containing β-tubulin can act as a stabilizing structure. Depolymerization of microtubules occurs with loss of the stabilizing GTP cap. The transition from polymerization to depolymerization is termed catastrophe. The transition from depolymerization to polymerization is termed rescue. Dynamic instability is the existence of populations of microtubules that are polymerizing and depolymerizing. Adapted from Desai and Mitchison 1997.
The regulation of dynamic instability allows microtubules to make drastic rearrangements throughout the cell cycle (ANDERSEN 1999). Altering one of the parameters of dynamic instability—the rate of growth, the rate of shrinkage, or the frequencies of catastrophe and rescue—significantly contributes to the change from the radial, seemingly stable array of interphase microtubules into a highly organized and dynamic bipolar spindle. In fact, a study using Xenopus cell extracts from different stages of the cell cycle and monitoring microtubule dynamics through video-microscopy shows that the polymerization and depolymerization rates are very similar in interphase and mitosis, but that the frequency of catastrophe is much greater in mitosis (BELMONT et al. 1990).

Stabilizing and destabilizing factors present in the cell can regulate microtubule dynamics. Microtubule-associated proteins (MAPs) are known to bind the microtubule lattice and stabilize microtubules (ANDERSEN 1999). Many MAPs are regulated during the cell cycle by phosphorylation, which inhibit their stabilizing ability. MAPs can act differently to stabilize microtubules. For example, MAP4 promotes the frequency of rescue without changing the frequency of catastrophe (OOKATA et al. 1995). XMAP215 promotes elongation and microtubule turnover (GARD and KIRSCHNER 1987; VASQUEZ et al. 1994). In contrast, microtubule destabilizers can also act through different mechanisms. First, there are proteins that sever microtubules such as katanin, a protein that is active only during mitosis in Xenopus cell extracts (MCNALLY and THOMAS 1998; MCNALLY and VALE 1993). Second, proteins such as XKCM1 promote catastrophe of microtubules and their absence leads to abnormally long microtubules (WALCZAK et al. 1996). An example of a third type of microtubule destabilizer is
Stathmin/Op18. *In vitro*, this protein regulates microtubule dynamics by sequestering free tubulin heterodimers (Curmi et al. 1997; Jourdain et al. 1997). The regulation of microtubule stabilizing and destabilizing factors can greatly change as the cell progresses through the cell cycle allowing spindle formation and break down.

Besides dynamic instability, microtubules can also turn over through treadmilling. Tubulin subunits can flow unidirectionally by adding to one end and being lost at the other. Treadmilling was first described for actin filaments (Wegner 1976) and then for tubulin *in vitro* (Margolis and Wilson 1978). Changes in the microtubule length are very slow by treadmilling, at a rate of 1 um/min, where dynamic instability can allow a change in length at up to 20 um/min (Andersen 1999). Thus, treadmilling will not allow the drastic change of microtubule organization that occurs between interphase and mitosis, but does allow microtubules to slowly turnover.

Studies show that *in vivo*, microtubule flux does occur, even in mitosis with microtubules attached at the plus end to a kinetochore and to the minus end at the microtubule organizing center. The term “flux” is used in these studies because it is unclear whether the tubulin is moving poleward through the microtubule by treadmilling or through the activities of microtubule motors (Margolis and Wilson 1998). Using fluorescence microscopy, marked tubulin located at the kinetochore slowly moves towards the pole at a rate of 0.5 um/min (Mitchison 1989). In a further study, Mitchison and Salmon show that in newt lung cells, during Anaphase-A, when chromosomes move toward the spindle poles, 63% of kinetochore fiber shortening is due to depolymerization, and 37% is due to flux (Mitchison and Salmon 1992). There is an even greater role for flux in Anaphase-A of Xenopus meiotic extracts (Desai
et al. 1998) as well as mitotic divisions in Drosophila embryos (MADDOX et al. 2002), showing that microtubule flux has a role \textit{in vivo} and may be needed for proper microtubule dynamics.
TUBULIN FOLDING AND HETERODIMER FORMATION

A newly translated polypeptide must be properly folded to undertake its appropriate function in the cell. Folding into a native form can be quite a challenge in the cytoplasm, a crowded molecular environment containing many macromolecules (ELLIS 1997). Furthermore, since translation in a eukaryotic cell can take several minutes, the N-terminal amino acids must be stabilized until the entire folding domain of the protein is translated (FELDMAN and FRYDMAN 2000). Although Anfinsen’s early studies led to the finding that the amino acid sequence of a ribonuclease contains all of the information needed to achieve the native form of a protein, (ANFINSEN 1973) in vivo, most proteins need the help of a molecular chaperone to prevent improper hydrophobic interactions, often resulting in aggregation.

The ability to properly fold starts during the translation process at the ribosome. The ribosome itself can protect 20 to 30 amino acids while inside the structure (FEDOROV and BALDWIN 1997). During translation, the nascent polypeptide can be bound by a chaperone that is present at the polypeptide exit site of the ribosome (HARTL and HAYER-HARTL 2002). This interaction prevents aggregation of a protein, allowing it to fold once the entire domain is translated. Most small proteins only need the ribosome-binding chaperones such as NAC (nascent chain-associated complex) for proper folding. Longer polypeptides can be bound by a group of chaperones that are not directly associated with the ribosome. These are mainly the Hsp70 class of chaperones. Even larger, aggregation prone polypeptides are bound by the chaperonins, or passed by the other chaperones to the chaperonin.
The chaperonins are a subset of the Hsp60 family of chaperones and are large toroidal complexes that have a central cavity for protected folding. There are two groups of chaperonins. Group I chaperonins are present only in eubacteria and in organelles originating from endosymbiosis. Members of this group include the \textit{E. Coli} chaperonin GroEL, and Hsp60 of the mitochondria and chloroplasts. Group II chaperonins are found in archea and eukaryotes. Members of this group include the thermosome (also called TF55) in archae and TriC (TCP-1 containing Ring Complex), also called CCT (Chaperonin Containing TCP-1), and c-cpn (cytosolic chaperonin).

The Group I and II chaperonins have many similarities. Crystal structures of both GroEL from \textit{E. coli} and the thermosome from \textit{T. acidophilum} have been analyzed, showing comparable domains between the two groups and a similar double toroid structure (BRAIG et al. 1994; DITZEL et al. 1998; XU et al. 1997). Both chaperonins contain a middle hinge region separating two distinct subunits, each with an ATP binding equatorial domain and a substrate binding apical domain. As well as having similar structures, both chaperonins require ATP for folding (FRYDMAN et al. 1992; GAO et al. 1992; GAO et al. 1993). They also act through multiple rounds of binding and release (FENTON and HORWICH 1997). Only about 10\% of actin monomers reach a native form after being bound to TRiC once. GroEL also acts through a similar mechanism, using multiple rounds of binding and release to create native protein.

Although group I and group II chaperonins share similar structures, they also have substantial differences. For example, group I chaperonins require a cochaperonin for function, GroES for GroEL and Hsp10 for Hsp60 (FELDMAN and FRYDMAN 2000). Group II chaperonins do not have an essential cochaperonin. The structure of the thermosome revealed that an integral
flexible protrusion allows the cavity to be closed upon substrate binding and open for substrate release. This allows the similar cage-like structure to create a secluded folding environment (DITZEL et al. 1998) without the necessity of two chaperones acting cooperatively.

Another difference between the two groups of chaperonins is the complexity of the subunits that make up the structures. Unlike GroEL, the thermosome and TRiC are hetero-oligomeric complexes. GroEL is a homo-oligomeric complex of two rings with seven subunits each (WEISSMAN et al. 1995). In archaea, the chaperonin contains eight or nine membered rings with two or three distinct subunits in each of the rings. TRiC is made up of 8-10 subunits in each of the two rings. Although the subunits are different proteins, they are approximately 30% identical (WILLISON and HORWICH 1996). The evolution of multiple subunits in the ring may have been to allow the differences in specificity of substrate binding.

**TRiC the cytosolic chaperonin**

TRiC was initially thought to be a chaperonin specific for cytoskeletal proteins, tubulin and actin, but now we know that it has other substrates. Cold and heat sensitive mutations in the Tcp1p subunit of TRiC in *S. cerevisiae*, gave rise to cytoskeletal defects such as nuclear missegregation, spindle abnormalities, and disorganized actin cables (URSIC and CULBERTSON 1991; URSIC et al. 1994) *In vitro*, TRiC helps fold tubulin and actin polypeptides (FRYDMAN et al. 1992; GAO et al. 1992; YAFFE et al. 1992) and binds tubulin and actin polypeptides *in vivo* (STERNLICHET et al. 1993; THULASIRAMAN et al. 1999) More recently, the data suggest that TRiC has other substrates including the VHL-Elongin BC tumor suppressor complex in mammalian cells, cyclin E, and myosin II (FELDMAN et al. 1999; SRIKAKULAM and WINKELMANN 1999).
Mass spectroscopy identified many more proteins that copurify with TRiC (Hynes et al. 1996). Furthermore, approximately 9-15% of newly synthesized polypeptides in the cell can co-immunoprecipitate with TRiC, suggesting that there are many unknown substrates (Thulasiram et al. 1999). Further analyses will likely identify these substrates.

Though TRiC has many substrates, the chaperonin is specialized for folding tubulin polypeptides. When tubulin and actin intermediates are presented to TRiC, they bind with much higher affinity than noncytoskeletal protein intermediates and through specific interactions. This is not true for the mitochondrial chaperonin, which binds all of the intermediates with equivalent affinities (Melki and Cowan 1994). A study using cryoelectron microscopy shows that tubulin interacts with a larger region of the chaperonin than actin (Llorca et al. 2000). The area of substrate binding for tubulin includes a region beneath the helical protrusions as well as at the base of the apical domain. Actin only binds below the helical protrusions. Specificity for substrates also lies in the subunits in the rings. For example, tubulin is bound to five specific subunits in the eight-membered ring (Llorca et al. 2000). Actin interacts with only three subunits (Llorca et al. 1999). The hetero-oligomeric ring may allow a more specialized folding machine.

The GimC/Prefoldin Complex

Although the TRiC does not have a cochaperonin that is part of the integral structure of the chaperonin, there is a folding complex that acts in conjunction with TRiC. This complex is termed GimC/PFD (prefoldin) and is also found throughout the archael and eukaryotic cytoplasm (Hartl and Hayer-Hartl 2002). The GimC/PFD complex is a 90kDa six-membered structure
that is made up of α- and β- subunit types and acts independently of ATP. GimC/PFD is proposed to bind and stabilize nonnative cytoskeletal polypeptides to prevent aggregation (Geissler et al. 1998; Vainberg et al. 1998). Furthermore, a study found that GimC/PFD helps accelerate folding of TRiC and prevents release of nonnative polypeptides (Siegers et al. 1999). The substrates of GimC/PFD include the tubulins and actin in eukaryotic cells.

GimC/PFD is a helical complex made up of six subunits. The crystal structure of GimC in the archaeum M. thermoautotrophicum reveals that this complex has a jellyfish appearance with a large central cavity and six long rods of coiled-coils (Figure 1-3) (Siebert et al. 2000). The rods have hydrophobic ends and act to bind substrates. The central cavity acts to protect and isolate a nonnative polypeptide. Electron microscopy of the eukaryotic GimC/PFD complex shows a similar structure (Martin-Benito et al. 2002).

The GimC/PFD complex in eukaryotic cells, but not in archae, is highly specialized for binding cytoskeletal proteins as substrates (Leroux et al. 1999; Vainberg et al. 1998). Tubulin and actin are not present in the archaeal cytosol (Doolittle 1999). GimC in archae acts more like an Hsp70 chaperone, binding many types of nonnative polypeptides and may make up for the lack of Hsp70 chaperones in archae (Leroux et al. 1999). However, archaebel subunits can integrate into the yeast complex, and can partially rescue the phenotypes of two of the yeast gim mutations (Leroux et al. 1999). The difference in substrate specificity may lie in the complexity of the two complexes. In eukaryotes the six-membered complex has six independent proteins. In archae there are only two different proteins in the six-membered complex.
Figure 1-3. Structural components of the GimC/PFD complex.
Ribbon models of side and top views of the MtGimC structure from Siegert et al. 2000. α–subunits are shown in gold and β-subunits are shown in blue. Gene names of yeast, human, and archaean homologues of the members of the GimC/PFD.
The GimC/PFD complex was originally isolated as a complex that promotes formation of native tubulin and actin in both yeast and mammalian cells (Geissler et al. 1998; Vainberg et al. 1998). In yeast, a screen that looked for synthetic lethality with a γ-tubulin mutant isolated the six GIMs (Genes Involved in Microtubule biogenesis), GIM1-6 (Geissler et al. 1998). One of the GIMs that my thesis will focus on is PAC10/ GIM2. The Gim proteins coimmunoprecipitate to form a complex. GimC is not essential, and deletion of any of the genes results in similar phenotypes. These phenotypes include cold sensitivity with depolymerized microtubules at 14 degrees, and sensitivity to the microtubule depolymerizing drug benomyl.

The homologous mammalian structure was purified from rabbit reticulocyte lysate extracts as a complex bound to newly translated actin polypeptides (Vainberg et al. 1998). They termed this complex as prefoldin because it can bind unfolded actin, not native actin and transfer it to TRiC. However, TRiC itself can be associated with the ribosome (McCallum et al. 2000), so GimC/PFD may also act to retrieve unfolded polypeptides from the cytoplasm and bring them back to TRiC. The six proteins that they named PFD1-6 are homologous to the members of the yeast GIMs (Figure 1-3) (Geissler et al. 1998).

**Tubulin Cofactors**

In an in vitro folding experiment, actin and γ-tubulin, but not α- and β-tubulin, can fold to a functional form with the presence of the cytosolic chaperonin, and ATP (Gao et al. 1992; Melki et al. 1993). In this assay, tubulin polypeptides are labeled then denatured using urea and guanidine-HCl. The denatured polypeptide is then diluted into rabbit reticulocyte lysate
containing purified TRiC as well as ATP. The tubulin folded by TRiC in the lysate cannot incorporate into heterodimer without the help of additional protein cofactors. These cofactors are thought to further fold and heterodimerize α- and β-tubulin released from TRiC. From the reticulocyte cell lysate, the Cowan lab has purified and cloned five proteins they named cofactors A, B, C, D, and E (GAO et al. 1994; GAO et al. 1993; ROMMELAERE et al. 1993; TIAN et al. 1996; TIAN et al. 1997). Cofactors C, D, and E are essential for production of native tubulin polypeptides in this *in vitro* reaction. Cofactors A and B are not required, but their absence leads to a decreased efficiency of heterodimer production (LEWIS et al. 1996).

A folding pathway of α- and β-tubulin was determined using these assays (reviewed in (LEWIS et al. 1996) (see Figure 1-4). Cofactor A is involved only in β-tubulin folding, and cofactor B is unique to α-tubulin. Cofactors D, C, and E are necessary for folding both subunits. However, cofactor D binds to β-tubulin and cofactor E binds to α-tubulin. Cofactor A will pass the β-tubulin it binds to cofactor D, and cofactor B will pass α-tubulin to cofactor E. Once cofactors D and E have bound to tubulin, they can form a hetero-tetramer, allowing the tubulin subunits to be close enough to interact and form a heterodimer catalyzed by GTP hydrolysis in the presence of cofactor C. Thus, these cofactors are believed to interact with a quasi-native form of tubulin polypeptides released from the chaperonin to further fold and heterodimerize them.

Four of the cofactors described in the *in vitro* folding assay also have *S. cerevisiae* homologs and are thought to have tubulin specific functions. Cofactor A, B, D and E are 20-32% identical to Rbl2p (ARCHER et al. 1995), Alf1p (TIAN et al. 1996), Cin1p (HOYT et al. 1997) and Pac2 respectively. Both Alf1p and Pac2p have a CLIP-170 domain that is found in several
### Figure 1-4. Model of tubulin heterodimerization *in vitro*.

When released from the chaperonin, α-tubulin is bound by cofactor B (F_B) or cofactor E (F_E). If bound by cofactor B, it is transferred to cofactor E. β-tubulin released from the chaperonin is bound to either cofactor A (F_A) or cofactor D (F_D). If bound by cofactor A, it is transferred to cofactor D. Cofactor D/β-tubulin and cofactor E/α-tubulin then bind each other. Cofactor C binds and exchanges the β-tubulin GTP for GDP, an essential step in this pathway to allow tubulin monomers to form a heterodimer. Adapted from Tian *et al.* 1997.

<table>
<thead>
<tr>
<th>Mammalian Cofactor</th>
<th>S. cerevisiae homologue</th>
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<tr>
<td>F_A</td>
<td>Rbl2p</td>
</tr>
<tr>
<td>F_B</td>
<td>Alf1p</td>
</tr>
<tr>
<td>F_C</td>
<td>unidentified</td>
</tr>
<tr>
<td>F_D</td>
<td>Cin1p</td>
</tr>
<tr>
<td>F_E</td>
<td>Pac2p</td>
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*Chaperonin*
microtubule associated proteins (Feierbach et al. 1999; Hoyt et al. 1997; Pierre et al. 1992; Tian et al. 1997). Furthermore, deletions of any of the cofactor homologs have microtubule phenotypes such as sensitivity to the microtubule depolymerizing drug benomyl and cold sensitivity (Archer et al. 1995; Hoyt et al. 1997; Tian et al. 1996). As in the in vitro folding assay, Rbl2p and Cin1p can bind β-tubulin and Pac2p and Alf1p can bind α-tubulin (Archer et al. 1995; Feierbach et al. 1999; Fleming et al. 2000). Cin1p and Pac2p can also bind each other, as is the case with cofactors D and E (Fleming et al. 2000; Lewis et al. 1996).

Although the cofactor homologues in yeast are involved in a pathway leading to normal microtubule stability, they may not be acting the same way in vivo as described in vitro. In the in vitro folding assay, cofactors D, C, and E are absolutely required for tubulin heterodimerization. Cofactors A and B are not required but inefficient folding resulted in their absence. In S. cerevisiae, none of the cofactors are essential, even when deleted in pairwise combinations (Hoyt et al. 1997) (Feierbach et al. 1999; Fleming et al. 2000). Thus, these cofactors cannot have the essential function of making tubulin heterodimers. They could act redundantly with another undefined class of folding proteins, or they could have a different function.

Data reported from our lab suggest that the cofactors may actually be involved in a pathway to reclaim dissociated tubulin heterodimers instead of de novo formation of heterodimers (Fleming et al. 2000). In S. cerevisiae, excess β-tubulin is lethal and has toxic consequences when dissociated from α-tubulin (Burke et al. 1989; Weinstein and Solomon 1990) and the cofactors may be needed to prevent the accumulation of toxic β-tubulin. For example, the cofactors are essential in benomyl and in the cold when microtubules depolymerize (Hoyt et al. 1997) (Feierbach et al. 1999; Fleming et al. 2000). Also, both Cin1p and Pac2p
are required in an α-tubulin mutant, *tubl-724* that causes a weak heterodimer. In this case, the mutant α-tubulin destabilizes the heterodimer favoring dissociation (VEGA et al. 1998). The cofactors may be needed to promote heterodimerization of released free tubulin to prevent the toxic consequences of β-tubulin or to maintain appropriate levels of heterodimer in the cell.

Rbl2p, the Cofactor A homologue also acts differently than that described in the *in vitro* folding reaction. *RBL2* (Rescues β-tubulin lethality), was isolated from our lab in a screen for genes that when overexpressed rescues the toxicity of excess β-tubulin (ARCHER et al. 1995). Like Cofactor A, Rbl2p can bind β-tubulin in a complex that excludes α-tubulin (ARCHER et al. 1995; LEWIS et al. 1996). However, in the *in vitro* reaction, Cofactor A can not bind a form of β-tubulin that is capable of exchanging into heterodimer, but has to pass the β-tubulin to Cofactor D for further folding (GAO et al. 1994). In contrast, Rbl2p is capable of binding β-tubulin both prior to heterodimerization and after release from heterodimer- thus in the same form, or readily converted to the form that binds to α-tubulin (ARCHER et al. 1998). In a further study, Rbl2p is found to rescue toxic β-tubulin at substoichiometric concentrations. This result suggests that Rbl2p does not rescue by sequestering the population of free β-tubulin, but possibly by shuttling it into an aggregate (ABRUZZI et al. 2002). Thus, Rbl2p may have an entirely different role in the cell than described *in vitro*.

Thus, the cofactor homologs found in *S. cerevisiae* have different roles *in vivo* than in the *in vitro* folding assay. There could be many reasons for these differences. First of all, the *in vitro* folding assay may not represent how the cofactors are working in the cell. The folding assay uses radiolabeled tubulin that is denatured by guanidine HCl and then asked what factors are required for exchange into exogenous heterodimer. The tubulin present in the cell may not be that
denatured and may not actually require these cofactors. Second, the cofactors in yeast have redundent functions with other proteins that have not been identified. A third possibility is that the cofactors in yeast have different functions from that in animal cells. Our lab favors the first hypothesis, however we cannot rule out the other two.
TUBULIN REGULATION

Previous experiments using animal cells show that tubulin heterodimers are involved in a feedback response. Addition of the microtubule depolymerizing drug colchicine, or injection of free tubulin polypeptides decreases the amount of both α- and β-tubulin messages (BEN-ZE'EV et al. 1979; CLEVELAND 1983; CLEVELAND et al. 1981). Addition of a drug vinblastine that causes aggregation of tubulin into crystals, or the microtubule stabilizing drug taxol, does not cause a decrease in tubulin RNA levels, but increases tubulin synthesis. Similar experiments were performed in sea urchin embryos (GONG and BRANDHORST 1988). In the presence of microtubule depolymerizing drugs, tubulin RNA levels decrease, but microtubule stabilizing drugs cause increases in tubulin RNA. Similarly, our lab has found that the addition of the microtubule depolymerizing drug nocodazole also causes a decrease in tubulin RNA levels in the yeast S. cerevisiae (Magendantz and Solomon, unpublished observation). These results suggest that there is autoregulation of the tubulin messages resulting from a change in polymerized tubulin levels.

The Cleveland lab demonstrated that the downregulation of β-tubulin messages in animal cells is posttranscriptional and cotranslational (PACHTER et al. 1987). There are 4 amino acids at the N-terminus of the polypeptide that are required for autoregulation (YEN et al. 1988). They hypothesize that a ribonuclease may recognize the four amino acids and degrade the message as ribosomes are translocating. However, the ribonuclease that specifies β-tubulin has not been identified, and there is no direct evidence that the increased rate of degradation is due to an enhanced ribonuclease.

The mechanism of regulation of α-tubulin RNA levels is even less clear. Addition of microtubule depolymerizing drugs to enucleated cells gave a decrease of β-tubulin message.
levels (CARON et al. 1985; PITTENGER and CLEVELAND 1985). The decrease of α-tubulin RNA was weak, suggesting that for α-tubulin, autoregulation can be an event in the nucleus as well as the cytoplasm (PITTENGER and CLEVELAND 1985). Furthermore, addition of the first four N-terminal amino acids of β-tubulin onto the α-tubulin message does not increase the degradation of α-tubulin messages (BACHURSKI et al. 1994). These experiments demonstrate that α-tubulin downregulation clearly acts through a different regulatory mechanism than β-tubulin.

Our lab has shown that a downregulation of tubulin RNA levels occurs in S. cerevisiae. Deletion of the GimC/PFD member Pac10p/Gim2p causes a decrease in both α- and β-tubulin RNA levels (SMITH 2000). Since Pac10p is part of a complex involved in folding of tubulin polypeptides, this could be a feedback response to an increase in unfolded α-tubulin. The feedback must be in response to α-tubulin because in this thesis I show that unfolded β-tubulin does not trigger this response, but does trigger an entirely different response. Further data show that high-level overexpression of one of the α-tubulin genes causes a decreased level of the other α-tubulin RNA and β-tubulin RNA as well (see Appendix I). In this experiment, the folding pathway may be saturated by the excess α-tubulin, leaving unfolded α-tubulin, triggering a feedback response to decrease tubulin levels.

The mechanism of upregulation in animal cells by addition of microtubule stabilizing drugs has mostly been unexplored. In the original experiments, Ben-Ze’ev et al. propose that the regulatory system exists to maintain a constant pool of free tubulin (1979). In response to this model, our lab perturbed the tubulin genes in S. cerevisiae to ask if additional or decreased levels of tubulin genes can influence the amount of tubulin present in the cell (KATZ et al. 1990). In a diploid containing only one copy of the single β-tubulin gene, the level of β-tubulin RNA or
protein is not upregulated, resulting in 50% of the amount of β-tubulin present in the cell. An upregulation of β-tubulin is not necessary because the cells grew normally with normal microtubules. In this strain, α-tubulin protein levels are decreased, likely because of the inability to bind a partner. The finding that excess copies of the α-tubulin gene do not result in increased levels of α-tubulin protein supports this hypothesis. From these experiments, Katz et al conclude that the cell does not upregulate tubulin levels in response to lower intracellular levels. In this thesis, I will present evidence that there is an upregulatory mechanism controlling β-tubulin transcription. The major differences between my strain and the strain used in this experiment are that there is a tubulin folding defect, the levels of tubulin are down to 20-30% that of wild type, and these cells do not have a phenotypically normal spindle. Thus, the trigger to upregulate tubulin RNA levels may require a decrease of tubulin polypeptides beyond a certain threshold level.

**Quantitative Regulation**

Tubulin levels are tightly controlled to maintain equivalent levels of α- and β-tubulin. Since excess β-tubulin is toxic in *S. cerevisiae* (Burke et al. 1989; Weinstein and Solomon 1990), perturbations that cause an excess of β-tubulin compared to α-tubulin can have harmful consequences. For example, in budding yeast there are two α-tubulin genes, *TUB1* and *TUB3*. The minor α-tubulin gene, *TUB3* only encodes approximately 15% of the amount of α-tubulin in the cell (Schatz et al. 1986b). A deletion of *TUB3* leads to free β-tubulin, causing phenotypes such as an enhancement of chromosome missegregation and sensitivity to the microtubule depolymerizing drug benomyl. High-level overexpression of β-tubulin from a galactose
inducible promoter results in microtubule depolymerization and subsequent cell death (Burke et al. 1989; Weinstein and Solomon 1990). The cell must avoid such excesses by strictly regulating tubulin levels.

Deletion of the GimC/PFD component Pac10p also causes an imbalance of tubulin levels leading to microtubule phenotypes such as cold and benomyl sensitivity. As mentioned above, tubulin RNA levels are slightly decreased in the cell. Polypeptide levels are decreased as well, however not to the same extent (Smith 2000). α-tubulin protein levels are decreased more than β-tubulin. The GimC/PFD complex seems to have a greater role in folding α-tubulin than β-tubulin. The benomyl phenotypes of pac10Δ cells can be rescued by excess α-tubulin (Alvarez et al. 1998; Geissler et al. 1998), suggesting that there is a pool of functional folded β-tubulin that is toxic because it lacks a partner. Also, pac10Δ cells are much more sensitive than wild type cells to overexpression of β-tubulin presumably because they already have excess β-tubulin, have saturated the Rbl2p protection mechanism, and are compromised in α-tubulin folding. The imbalance of tubulin levels can have detrimental phenotypes for yeast survival.

I have found a mutant that can overcome the toxic consequences of excess β-tubulin, even from high-level overexpression. Interestingly, this mutation enhances survival of toxic β-tubulin in the presence of a mutation in the GimC/PFD complex. In this thesis I will present data showing that these cells can survive excess β-tubulin because it is in a form that is nonfunctional and not toxic. Furthermore, these cells have increased β-tubulin RNA levels that may be a response to too little heterodimer present in this cell. If the transcription factor that causes an upregulation is knocked out, the cells have very low tubulin levels, causing spindle defects.
Literature Cited


Chapter 2

A novel step in β-tubulin folding is important for heterodimer formation in *S. cerevisiae*
ABSTRACT

Undimerized β-tubulin is toxic in the yeast S. cerevisiae. It can arise if levels of β-tubulin and α-tubulin are unbalanced or if the tubulin heterodimer dissociates. We are using the toxicity of β-tubulin to understand the early steps in microtubule morphogenesis. We find that deletion of PLP1 suppresses toxic β-tubulin formed by disparate levels of α- and β-tubulin. That suppression occurs either when α-tubulin is modestly underexpressed relative to β-tubulin, or when β-tubulin is inducibly and strongly overexpressed. Plp1p does not rescue by changing tubulin expression. Instead, a significant proportion of the undimerized β-tubulin in plp1Δ cells is less toxic than in wild type cells. It is also less able to combine with α-tubulin to form heterodimer. As a result, plp1Δ cells have lower levels of heterodimer. Importantly, plp1Δ cells that also lack Pac10, a component of the GimC/PFD complex, are even less affected by free β-tubulin. Our results suggest that Plp1p defines a novel early step in β-tubulin folding.
INTRODUCTION

Microtubule function requires the participation of genes with a wide range of molecular roles. Biochemical experiments first identified proteins that modulate the assembly and dynamics of the microtubule polymer. These proteins contribute to the formation and function of the many diverse microtubule structures (DESAI and MITCHISON 1997). Other experiments, both biochemical and genetic analyses, demonstrate activities that affect earlier steps in microtubule morphogenesis, in particular the folding of the tubulin polypeptides to allow formation of \( \alpha-\beta \) tubulin heterodimers, the subunits of the microtubule polymer.

The efficient formation of heterodimer is crucial for the cell. First, cell viability requires some minimal level of tubulin to support essential cell functions. Second, undimerized \( \beta \)-tubulin, arising either because it is in excess with respect to \( \alpha \)-tubulin or because the heterodimer dissociates or does not form properly, is extremely toxic in yeast (BURKE et al. 1989; JAVERZAT et al. 1996; VEGA et al. 1998; WEINSTEIN and SOLOMON 1990). Even at low levels, undimerized \( \beta \)-tubulin can disrupt microtubule assembly (SCHATZ et al. 1986); at higher levels, it is lethal. In contrast, a very large excess of \( \alpha \)-tubulin has only modest consequences for the cell (WEINSTEIN and SOLOMON 1990). Among cytoskeletal proteins, the problem of maintaining balance between major components – an issue encountered in other morphogenetic pathways (FLOOR 1970; STERNBERG 1976) - affects only tubulin; but not of course actin, intermediate filament subunits or even the prokaryotic predecessor of tubulin, FtsZ (ERICKSON 1995), all of which are monomeric proteins acting as primary subunits. Why the microtubule subunit
contains two rather similar polypeptides, one of which is toxic on its own, is not understood.

Several gene products are involved in forming heterodimer. They include the cytosolic chaperonin (TRiC), an essential structure that participates in the folding of many proteins (DUNN et al. 2001; URSIC and CULBERTSON 1991). In addition, the non-essential GimC or prefoldin complex (GimC/PFD) (GEISSLER et al. 1998; VAINBERG et al. 1998) cooperates with TRiC in folding a subset of those proteins, including actin and the tubulin family. Third, five proteins act as cofactors in an in vitro assay for incorporation of tubulin polypeptides into heterodimer (TIAN et al. 1997). These cofactors are not essential for heterodimer formation in budding yeast (ARCHER et al. 1995; GEISER et al. 1997; HOYT et al. 1997; STEARNS et al. 1990), and may act in a pathway to reclaim dissociated heterodimers (FLEMING et al. 2000).

The specific role of GimC/PFD in the formation of functional cytoskeletal proteins is not known. It may interact with nascent polypeptides and transfer them to the TRiC (HANSEN et al. 1999; VAINBERG et al. 1998), although the TRiC itself may also interact with nascent chains bound to the ribosome (MCCALLUM et al. 2000). GimC/PFD may bind incompletely folded cytoskeletal proteins released from the TRiC and help return them to the chaperonin for another round of sequestered folding (VAINBERG et al. 1998). Alternatively, GimC/PFD may prevent release of an incorrectly folded polypeptide from TRiC (SIEGERS et al. 1999). The GimC/PFD complex interacts with both α- and β-tubulin ((SIEGERS et al. 1999; VAINBERG et al. 1998), and our unpublished results), and is important but not required for folding of both proteins.
A conspicuous phenotype of deleting PAC10, which encodes Pac10p/Gim2p, one of the yeast GimC/ PFD components, is supersensitivity to microtubule depolymerizing drugs, a common microtubule phenotype (Alvarez et al. 1998; Geissler et al. 1998). In pac10Δ cells, this phenotype can be explained by changes in tubulin expression; the levels of both α- and β-tubulin are reduced to approximately 55% and 85% of wild type, respectively (Alvarez et al. 1998). The resulting modest excess of β-tubulin can account for the benomyl supersensitivity of pac10Δ cells, since overexpressed α-tubulin or Rbl2p (Rescues β-tubulin Lethality), a β-tubulin binding protein that protects cells against excess β-tubulin (Archer et al. 1995), completely suppresses the drug phenotype (Alvarez et al. 1998; Geissler et al. 1998). In fact, the level of undimerized β-tubulin in pac10Δ cells makes them inviable either in the absence of RBL2; or when the minor α-tubulin gene TUB3, is deleted, producing an additional ~15% undimerized β-tubulin (Alvarez et al. 1998).

I am using S. cerevisiae to study tubulin heterodimer formation as an early step in microtubule morphogenesis, using the toxicity of free β-tubulin as a probe of its state in vivo. I screened for loss-of-function mutations that would rescue a strain which contains three non-essential mutations affecting tubulin expression, and that is inviable in the absence of a low-copy plasmid expressing α-tubulin. This screen identifies deletion of the PLP1 gene as a suppressor of this lethality. I show that plp1Δ rescues cells from excess β-tubulin in several circumstances, including high-level overexpression. Suppression by plp1Δ does not occur through differential expression of Rbl2p or tubulin proteins. Instead, it affects the properties of undimerized β-tubulin. The data suggest that
Pp1p may affect the state of β-tubulin in the cell by facilitating the efficient transfer of nascent β-tubulin polypeptides to the folding apparatus.
MATERIALS AND METHODS

Strains and media

All yeast strains are derivatives of FSY183 (WEINSTEIN and SOLOMON 1990). I used standard yeast manipulation methods and media (GUTHRIE and FINK 1991; SHERMAN et al. 1986; SOLOMON et al. 1992). I used the pNK51 vector containing hisG-URA3-hisG sequences to disrupt the entire PLP1 and GRR1 open reading frames (ALANI et al. 1987). STE4 and STE18 were deleted using a PCR based method (LONGTINE et al. 1998). Deletion of PAC10, TUB3, and RBL2 was previously described (ABRUZZI et al. 2002; ALVAREZ et al. 1998; ARCHER et al. 1995). I HA-tagged the chromosomal PLP1 gene using the pFA6a-3HA-kanMX6 module (LONGTINE et al. 1998).

Mutagenesis

I mutagenized SSY14 (pac10::HIS3 grr1::hisG tub3::hisG + pAIA510b/TUB1) with the mTn-lacZ/LEU2 insertion library (BURNS et al. 1994) according to the Yale Genome Analysis Center protocol (http://ygac.med.yale.edu/). I plated library transformants to synthetic complete media lacking leucine, uracil, and histidine. Colonies grew 4 days and were then replica plated to 5-FOA. I used the vectorette PCR rescue method described by the Botstein lab http://genome-www.stanford.edu/group/botlab/protocols/vectorette.html to identify the position of the inserts.

DNA sequencing

Sequencing of the transposon inserted allele of PLP1 was performed by the MIT Biopolymers Facility.
Immunoblotting

I followed standard procedures for immunoblotting (SOLOMON et al. 1992), using anti-α–tubulin antibody #345 (SCHATZ et al. 1987) and anti-β-tubulin antibody #206 (BOND et al. 1986). Tubulin protein levels were normalized to CPY. Immunoblot detection was performed as previously described (ABRUZZI et al. 2002).

GroE-trap experiments

Strains with integrated TUB2-LEU2-GAL-TUB2 were transformed with a plasmid containing the gene encoding GroEL D87K trap under a copper inducible promoter. The strains were grown in raffinose to log phase. At 0 hr, CuSO₄ to 100uM and galactose to 2% were added, and Cells plated to non-inducing media at different time points.

Gel filtration chromatography

To determine the state of tubulin polypeptides in each strain, I used gel filtration chromatography as described previously (ABRUZZI et al. 2002).

Polysome Gradients

Polysomes were isolated using a modification of the protocol previously published (ROTENERG et al. 1988).
RESULTS

A deletion of *PLPI* rescues the synthetic lethality of *pac10Δ grr1Δ tub3Δ* triple mutants

To find genes involved in the regulation of tubulin expression or toxicity, I screened for the rescue of *pac10Δ grr1Δ tub3Δ* triple mutants using a transposon based insertion library. As noted above, both *pac10Δ* and *tub3Δ* contain undimerized β-tubulin, and both those strains and *grr1Δ* cells (A. Smith, M. Magendantz and F. S., unpublished results) have lower levels of tubulin. Our lab has found that *grr1Δ* is synthetically lethal with both *pac10Δ* and *tub3Δ*. Grr1p is a member of the SCF complex and targets proteins for ubiquitination and subsequent degradation (SKOWYRA et al. 1997). Unfortunately, Grr1p is involved in many processes and we do not currently understand why tubulin levels are decreased in this strain. The deletion of *GRR1* was added to decrease the level of tubulin polypeptides to a greater extent and to reduce background cells that are able to survive at a very low frequency that are *pac10Δ tub3Δ*. Further characterization of *grr1Δ* with a mutation isolated from this screen will be presented in Appendix II.

The screen is based on the observation that the *pac10Δ grr1Δ tub3Δ* triple mutant can survive with a plasmid containing *TUB1*, the major α-tubulin gene. Therefore, the inviability of the triple mutant is most likely due to the presence of toxic, undimerized β-tubulin. Rescue of the triple mutant must suppress at least two of the three mutations since each of the pair-wise combinations is synthetically lethal, suppressible by either excess α-tubulin or excess Rbl2p (ALVAREZ et al. 1998, my unpublished results). Thus, mutations that allow these cells to live could be involved in regulating expression levels of α-tubulin, β-tubulin or Rbl2p; or in regulating the toxicity of undimerized β-tubulin.
SSY14 cells, \(pac10\Delta\ grr1\Delta\ tub3\Delta\) covered with \(TUB1\) on a \(URA3\) marked \(CEN\) plasmid, grow on synthetic complete medium but not on medium containing 5-FOA, which forces loss of the covering plasmid. Using the \(mTn-lacZ/LEU2\) insertion library (BURNS et al. 1994), I recovered 3 independent insertions that could survive in the absence of the plasmid containing \(TUB1\). All three insertions were within the gene \(PLP1\) (FLANARY et al. 2000). To verify this interaction, I recreated the quadruple mutant by deleting the entire open reading frame of \(PLP1\) in SSY14 cells and showed that it can grow without the extra \(\alpha\)-tubulin expressed from the covering plasmid (Figure 2-1A).

**The \(pac10\Delta\ grr1\Delta\ tub3\Delta\ plp1\Delta\) quadruple mutant survives with very low tubulin levels**

I used immunoblotting to measure the levels of Tub1p and Tub2p in the \(pac10\Delta\ grr1\Delta\ tub3\Delta\ plp1\Delta\) quadruple mutant. A deletion of either \(PAC10\) or \(GRR1\) causes a decrease in the levels of both Tub1p and Tub2p by 20%-45% (ALVAREZ et al 1998; A. Smith, M. Magendantz and F. S., unpublished). In addition, deletion of \(TUB3\) decreases the total amount of \(\alpha\)-tubulin by 15% (SCHATZ et al. 1986). Normalizing to a control protein (carboxypeptidase \(Y\)), I found that tubulin levels are dramatically reduced in the quadruple mutant: \(\alpha\)-tubulin levels to 27 \(\pm\) 5%, and \(\beta\)-tubulin levels to 32 \(\pm\) 6%, of wild type (Figure 2-1B). The quadruple mutant cells grow even more slowly than \(grr1\Delta\) cells, which have an elongated G1 phase (BARRAL et al. 1995). The quadruple mutant has the lowest level of tubulin shown to be sufficient for growth in yeast. Cells with 50% of the wild type tubulin complement grow at normal rates (KATZ et al. 1990).
Figure 2-1. *pac10Δ grr1Δ tub3Δ plp1Δ* cells are viable and express very low tubulin levels.
A) *pac10Δ grr1Δ tub3Δ plp1Δ* cells can survive in the absence of a plasmid containing *TUB1*. Serial dilutions of these cells grow on standard media ("YPD"), although more slowly than *pac10Δ* and *grr1Δ* mutants. *pac10Δ grr1Δ tub3Δ* mutants cannot survive on 5-FOA plates in the absence of the plasmid containing *TUB1*. (B) α- and β-tubulin levels of wild type and *pac10Δ grr1Δ tub3Δ plp1Δ* cell extracts were determined by immunoblotting. Values reported (average of 3 experiments) are the levels of tubulin polypeptides normalized to carboxypeptidase Y (CPY); the wild type values are defined as 1.0.
*plp1Δ suppresses the toxicity of free β-tubulin*

Deletion of *PLP1* partially rescues the benomyl supersensitivity of *pac10Δ* and *tub3Δ* cells (Figure 2-2). This suppression is apparent at relatively low benomyl concentrations – 10μg/ml for *pac10Δ plp1Δ* cells and 4μg/ml for *tub3Δ plp1Δ* cells. At higher drug concentrations, the suppression is not detectable. Thus, *plp1Δ* suppresses the phenotypes of undimerized β-tubulin arising from altered expression levels. The *plp1Δ* mutation in an otherwise wild type background does not confer either benomyl resistance or sensitivity at any concentration of drug.

*Plp1p’s microtubule function is independent of phosducin-like homology*

The gene encoding the yeast Plp1p (for Phosducin-Like Protein) was identified and named based on its sequence similarity to mammalian phosducin (FLANARY et al. 2000). In retinal cells, phosducin binds the βγ subunits of G proteins, and so inhibits their re-binding to the α subunit to reconstitute the trimeric protein. In yeast, the only known Gβγ protein function is in the mating response pathway. Mating pheromone binding is signaled through the G protein βγ subunits Ste4p and Ste18p. Consistent with the sequence homology, Plp1p can bind Ste4p and Ste18p released from the mating pheromone receptor when alpha factor is present (FLANARY et al. 2000). However, neither deletion nor overexpression of *PLP1* has a significant effect on response to the mating pheromone (FLANARY et al. 2000).

I determined that neither Ste4p nor Ste18p are required for the rescue of microtubule mutants by *plp1Δ*. First, *plp1Δ* rescue of *pac10Δ grr1Δ* cells is unaffected by deletion of *STE4* and *STE18* (data not shown).
Figure 2-2. \textit{plp1\Delta} suppresses the benomyl phenotypes of \textit{pac10\Delta} and \textit{tub3\Delta} at low benomyl concentrations.

Wild type, \textit{plp1\Delta}, \textit{pac10\Delta}, \textit{pac10\Delta plp1\Delta}, \textit{tub3\Delta}, and \textit{tub3\Delta plp1\Delta} cells were grown overnight in rich media. Serial dilutions of a saturated culture were spotted onto plates containing benomyl as indicated. Cells grew equivalently on rich plates without benomyl (not shown).
Also, plp1Δ still reduces the benomyl sensitivity of pac10Δ ste4Δ ste18Δ. Thus, the role of Plp1p in microtubule regulation is independent of any functional homology to phosducin it may have.

Plp1p is also distinct from the recently described rat protein PhLP (McLaughlin et al. 2002). PhLP is related to phosducin but is only about 15% identical to yeast Plp1p and Plp2p. Overexpression of PhLP severely affects protein folding, while overexpressed Plp1p has no phenotype (data not shown). Thus, there appears to be no significant structural or functional relationship between these proteins.

plp1Δ does not suppress undimerized β-tubulin by modifying tubulin expression levels

The benomyl sensitivity of pac10Δ cells can be attributed at least in part to excess β-tubulin, since it is rescued by overexpression of Rbl2p (Alvarez et al. 1998; Geissler et al. 1998). Therefore, the suppression by plp1Δ could be achieved by increasing the amount of α–tubulin or decreasing the amount of β–tubulin in the cell. However, the ratio of β– to α–tubulin actually increases in pac10Δ plp1Δ relative to pac10Δ (Figure 2-3). In agreement with previous reports, (Alvarez et al. 1998, Geissler, 1998 #1836), in pac10Δ cells α– and β-tubulin levels are reduced to 54 ± 10% and 85 ± 10% of wild type, respectively. The ratio of β– to α–tubulin is approximately 1.6, and there is an excess of approximately 30% of β-tubulin compared to α-tubulin. In a pac10Δ plp1Δ double mutant, α–tubulin (48± 5% of wild type levels) and β-tubulin (92 ± 3% of wild type levels) are comparable to those in pac10Δ cells. Thus, both the ratio of β– to α-tubulin, about 1.9, and the amount of excess β-tubulin, about 44%, are even higher in the pac10Δ plp1Δ cells than in pac10Δ cells. There is no effect of plp1Δ on tubulin levels in
Figure 2-3. *plp1Δ* does not rescue by decreasing β–tubulin levels or increasing α-tubulin levels.
Extracts from wild type, *plp1Δ*, *pac10Δ*, and *pac10Δ plp1Δ* were analyzed for levels of α– tubulin, β–tubulin and CPY by immunoblotting, Values reported are the average of 5 experiments.
an otherwise wild type background: α–tubulin levels are 95± 10% and β-tubulin levels are 96 ± 10%. Therefore, despite the fact that the pac10Δ plp1Δ double mutant actually has more excess β–tubulin than do pac10Δ cells, it grows normally and has lower sensitivity to benomyl.

**plp1Δ does not suppress undimerized β-tubulin through RBL2**

The toxicity of overexpressing β-tubulin can be suppressed by overexpression of either α-tubulin or Rbl2p (ARCHER et al. 1995; WEINSTEIN and SOLOMON 1990). Therefore, it is possible that the plp1Δ mutation upregulates Rbl2p expression to suppress strains carrying excess β-tubulin. However, plp1Δ rescues the synthetic lethality of rbl2Δ tub3Δ double mutants and rbl2Δ pac10Δ (Figure 2-4). Therefore, suppression by plp1Δ is not mediated by Rbl2 function.

**plp1Δ protects cells against high-level overexpression of β-tubulin**

The toxicity of overexpressed β–tubulin can be demonstrated in two ways. Both assays demonstrate that plp1Δ suppresses the effects of even high levels of undimerized β-tubulin. First, haploid cells carrying an additional chromosomal copy of β–tubulin under the control of the galactose inducible promoter show dramatically reduced plating efficiency on galactose plates; only about 0.06% of cells form colonies on medium containing galactose, compared to medium containing glucose (Figure 2-5A). Deletion of PAC10 makes the cells even more sensitive – about .03% live on galactose. In both of these backgrounds, the plp1Δ mutation increases plating efficiency – about 4-fold to 0.27% in PAC10 cells, and about 300-fold to 18% in pac10Δ cells.
Figure 2-4. *plp1Δ* does not rescue through Rbl2p expression. A deletion of *PLP1* rescues *rbl2Δ tub3Δ* and *rbl2Δ pac10Δ*. Cells deleted for both *RBL2* and *TUB3* carried a low-copy plasmid expressing *TUB3* and *URA3*. These cells grow normally on standard media ("glucose") but do not grow on media containing 5-FOA, which selects for loss of the plasmid expressing *URA3* ("5-FOA"). If those cells are deleted for *PLP1*, they grow normally on both media. Cells deleted for both *RBL2* and *PAC10* carried a low-copy plasmid expressing *RBL2* and *URA3*. These cells grow normally on standard media ("glucose") but do not grow on media containing 5-FOA, which selects for loss of the plasmid expressing *URA3* ("5-FOA"). If those cells are deleted for *PLP1*, they grow normally on both media.
Second, the \( plp1\Delta \) mutation affects the kinetics of \( \beta \)-tubulin toxicity. Cells were grown to log phase in raffinose, and then at zero time, galactose was added to induce overexpression of \( \beta \)-tubulin. At various times, cells were plated to glucose to repress the galactose inducible promoter and to enable counts of viable cells. Compared to otherwise wild type cells, \( pac10\Delta \) cells lose viability more rapidly (ALVAREZ et al. 1998). In both backgrounds, \( plp1\Delta \) substantially slowed the rate of cell death (Figure 2-5B). In cells lacking the prefoldin component Pac10p, \( plp1\Delta \) has an even more dramatic phenotype.

Both the plating efficiency and kinetic experiments show that deletion of \( PLP1 \) reduces the toxicity of excess \( \beta \)-tubulin to a greater extent in \( pac10\Delta \) than in \( PAC10 \) cells (Figure 2-5), even though the \( pac10\Delta \) mutation makes cells substantially more sensitive to overexpressed \( \beta \)-tubulin. Indeed, the \( pac10\Delta plp1\Delta \) double mutants are much more resistant to excess \( \beta \)-tubulin than wild type cells. Control experiments show that the extent of \( \beta \)-tubulin overexpression was comparable in all 4 strains (data not shown).

The results described above demonstrate that undimerized \( \beta \)-tubulin produced by differential levels of expression relative to \( \alpha \)-tubulin shows substantially lower than expected toxicity in \( plp1\Delta \) cells. This conclusion applies both to \( pac10\Delta \) cells, which contain a modest excess of \( \beta \)-tubulin, and to cells overexpressing \( \beta \)-tubulin under the control of the strong \( GAL \) promoter.

**Additional \( \alpha \)-tubulin does not affect the benomyl phenotype of \( pac10\Delta plp1\Delta \)**

Our findings raise the possibility that \( plp1\Delta \) affects the properties of the excess \( \beta \)-tubulin. To assay the state of \( \beta \)-tubulin in the absence of Plp1p, I first asked if the \(~40\%\) excess undimerized \( \beta \)-tubulin in \( pac10\Delta plp1\Delta \) cells could interact with \( \alpha \)-tubulin. The supersensitivity of \( pac10\Delta \) cells to benomyl can be rescued by \( \alpha \)-tubulin on a low copy
A

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<th>Strain</th>
<th>% Survival</th>
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<tr>
<td>GALβ–tubulin</td>
<td>.06</td>
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<td><strong>pac10Δ</strong> GALβ–tubulin</td>
<td>&lt;.03</td>
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<tr>
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<td>.27</td>
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<tr>
<td><strong>pac10Δ plp1Δ</strong> GALβ–tubulin</td>
<td>18</td>
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B

- **pac10Δ plp1Δ**
- **plp1Δ**
- **pac10Δ**
- **wildtype**

% Viability (Log) vs. Hours in Galactose
Figure 2-5. Deletion of PLP1 rescues β–tubulin lethality.

(A) Strains containing an integrated galactose inducible β–tubulin expressing gene were grown overnight in raffinose and then plated on glucose and galactose media. The percent survival was determined by comparing the number of colonies on the galactose and glucose media. (B) Strains containing an integrated galactose inducible β–tubulin expressing gene were grown in raffinose to early log phase. At time zero, galactose was added to induce overexpression of TUB2. At the given time points, the cells were plated to glucose (to repress the galactose promoter) and the plating efficiency determined.
plasmid (ALVAREZ et al. 1998; GEISSLER et al. 1998), because the additional α-tubulin binds the excess β-tubulin to form more heterodimer. Significantly, the residual benomyl sensitivity of pac10Δ plp1Δ is not rescued by additional α-tubulin, even with high-level overexpression from a galactose-inducible promoter (Figure 2-6). This result suggests that, in the double mutant, the excess β–tubulin is in a form that is unable to heterodimerize with α-tubulin.

**Cells deleted for PLP1 have normal levels of tubulin expression but reduced levels of heterodimer**

I next tested whether the loss of PLP1 affects the form of β-tubulin in a wild type background. As shown in Figure 2-3, the levels of α- and β-tubulin in plp1Δ cells are equivalent to wild type levels. I used gel-filtration chromatography (ABRUZZI et al. 2002) to show that in plp1Δ cells, about half of both the α– and β-tubulin proteins are present in a heterodimer peak as well as in a much larger form that elutes in the void volume of the column (Figure 2-7). In wild type cells, >90% of the α– and β-tubulin is found in a single heterodimer peak. The tubulin in the void volume of the column, found in plp1Δ, is most likely aggregated (ABRUZZI et al. 2002). This could be a form of nontoxic β-tubulin present in the form of an aggregate in plp1Δ cells.

**The GroE trap for unfolded proteins suppresses β-tubulin toxicity**

The results described above suggest that PLP1 function helps convert β-tubulin to a form that either can become heterodimer, or – in the absence of sufficient α-tubulin – can become toxic. I asked if ectopic expression of a chaperonin trap could reduce loss of viability in cells overexpressing β-tubulin. The GroEL D87K mutant binds unfolded
Figure 2-6. The excess β-tubulin in pac10Δ plp1Δ cells is in a nontoxic and nonfunctional form. Excess α-tubulin does not suppress the benomyl phenotypes of pac10Δ plp1Δ. Wild type, pac10Δ, and pac10Δ plp1Δ cells containing a plasmid carrying a galactose inducible TUB1 gene or an empty plasmid were grown in selective media overnight. Serial dilutions of a saturated culture were spotted onto rich plates ("YPD") to ensure equivalent growth (not shown) and plates containing galactose and 10ug/ml of benomyl.
**Figure 2-7.** *plp1Δ* cells contain aggregated tubulin. Wild type and *plp1Δ* cell extracts were resolved on a Sephacryl 300S column, and fractions analyzed for α- and β-tubulin by immunoblot. The graph represents the percent of the total amount of tubulin in each fraction. α-tubulin: solid line; β-tubulin: dashed line.
polypeptides but does not release them (Farr et al. 1997; Siegers et al. 1999; Weissman et al. 1994), and so should reduce the pool of free β-tubulin. If overexpressed β-tubulin has the same potential to become toxic in wild type and plp1Δ cells, the trap should rescue to the same extent in the two strains.

To test this idea, genes encoding β-tubulin and GroEL trap were induced for 5 hours, and the cells then plated to medium permissive for growth but that repressed inducible promoters. Under these conditions, overexpression of β-tubulin kills ~80% of the cells, but when the GroE trap is co-overexpressed only ~35% of the cells are killed (Figure 2-8). Thus, the presence of GroE-trap is sufficient to substantially suppress β-tubulin lethality. These results suggest that the exogenous chaperonin can bind a form of β-tubulin with the potential to be toxic. In contrast, the presence of the GroE trap only modestly increases the survival of plp1Δ cells overexpressing β-tubulin, from 45% to 65% (Figure 2-8). The amount of β-tubulin bound by the GroE trap in this experiment, as determined by co-immunoprecipitation experiments with anti-GroE, is the same in both wild type and plp1Δ backgrounds (data not shown). The results suggest that the form of β-tubulin that can be toxic is less abundant in plp1Δ cells than in wild type cells.

**Plp1p co-fractionates with polysomes**

The differential effects of a plp1Δ mutation on wild type compared to pac10Δ cells suggest that Plp1p acts upstream of Pac10p. By gel filtration, I found that Plp1p behaves as if it were associated with a very large complex (data not shown). I used sucrose gradients to determine that a portion of Plp1p was present in fractions containing polysomes (Figure 2-9).
Figure 2-8. GroE-Trap suppresses β-tubulin toxicity.

Wild type and plp1Δ cells containing an integrated galactose inducible β-tubulin gene, with and without a plasmid containing a copper inducible GroE-trap, were grown in raffinose media to saturation. Five hours after galactose and copper were added to induce expression of β-tubulin and GroE-trap, cells were plated to glucose. The graph represents per cent viable cells.
In buffers that preserve polysomes, a substantial fraction of the Plp₁p co-fractionates with the integral ribosomal component Rpl₃p (Vilardell et al. 1997) in a domain of the gradient that contains polysomes (determined by UV absorption). Treatment of parallel samples with EDTA disrupts the polysomes, and both the Plp₁p and the Rpl₃p shift in the gradient to lighter fractions. This behavior is typical of that observed for other proteins associated with ribosomes (Mangus and Jacobson 1999). The results suggest that a pool of Plp₁p may be associated with the ribosomes in intact polysomes.
Figure 2-9. Plp1p Is Associated with Polysomes.

(A) Extracts from cells with an endogenous HA-tagged PLPI were applied to a 15-45% sucrose gradient, and fractions analyzed for HA-Plp1p and the ribosomal subunit Rpl3p.

(B) As above, with the addition of EDTA to cell extracts to dissociate polysomes. The Rpl3p and Plp1p peaks both shift to lighter fractions.
DISCUSSION

The experiments described above use β–tubulin toxicity as a probe to understand the properties and regulation of this essential protein. Previously discovered suppressors of excess β-tubulin lethality - α-tubulin and Rbl2p - act by binding directly to the toxic protein. The screen used here, for loss-of-function mutations as suppressors of excess β–tubulin, led to the identification of a protein – Plp1p - important in forming toxic β–tubulin. In cells lacking Plp1p, the consequences of excess β-tubulin are substantially diminished, especially in the absence of the GimC/PFD complex.

**Plp1p mediates most but not all β-tubulin toxicity**

The suppression of undimerized β-tubulin toxicity by plp1Δ is manifest in those strains where the excess is caused by higher levels of β-tubulin than α-tubulin. That condition occurs in strains deleted for the minor α-tubulin gene TUB3; in strains deleted for the prefoldin component PAC10; and in strains that inducibly overexpress the β-tubulin gene TUB2.

The ability of plp1Δ to rescue phenotypes caused by excess β-tubulin could be explained if Plp1p had a role in tubulin expression, so that it's deletion reduced expression of β-tubulin or increased expression of α-tubulin. However, plp1Δ in otherwise wild type cells has no effect on expression of either tubulin. Similarly, Plp1p does not affect the rate of β-tubulin degradation. The levels of β-tubulin are the same in tub3Δ and tub3Δ plp1Δ cells and essentially identical to wild type levels (data not shown),

**A role for Plp1p in microtubule morphogenesis**
The data suggest that *PLP1* functions not in controlling tubulin expression levels but in folding β-tubulin. Our results suggest that Plp1p function is relatively specific for β-tubulin. If Plp1p were equally important for α-tubulin folding, the suppression of modest levels of undimerized β-tubulin would not occur. Instead, *plp1Δ* has no apparent effect on α-tubulin folding.

In *plp1Δ* cells, a substantial fraction of the total tubulin is aggregated rather than in functional heterodimer. Such aggregates can be understood as arising due to inefficient folding of nascent β-tubulin. Consequently, a proportion of both tubulins is undimerized and so the proteins tend to form aggregates (Abruzzi et al. 2002). That Plp1p affects the proportion of tubulin in functional heterodimer provides a rationale for an activity that, under certain circumstances, is deleterious to the cell.

Where along the β-tubulin folding pathway does Plp1p act? Two steps in that pathway in yeast have been defined by the role of the TRiC chaperonin and the prefoldin complex. The function of Plp1p can be clearly distinguished from the proteins participating in each of those steps. First, unlike *plp1Δ*, defects in the chaperonin components have deleterious effects on microtubules. Second, deletion of the prefoldin component Pac10 makes cells supersensitive to microtubule depolymerizing drugs and are not viable without the minor α-tubulin gene *TUB3*; *plp1Δ* rescues both these phenotypes of *pac10Δ*. A third possible point of action is in the formation of heterodimers. The properties of Plp1p are also distinct from those of the yeast homologs of proteins that mediate heterodimer formation *in vitro*. Those proteins, although not essential in *S. cerevisiae*, may participate in a salvage pathway to rescue dissociated heterodimers (Fleming et al. 2000).
The data suggest that Plp1p functions at an early step in folding. One possibility is that it facilitates the efficient transfer of nascent β-tubulin polypeptides from the ribosome to the cytosolic chaperonin (Figure 2-10). As a consequence, in plp1Δ cells a substantial fraction of tubulin polypeptides is released into the cytoplasm. There, they can still interact with the GimC/PFD complex or the cytosolic chaperonin to be folded, although with lower efficiency. This defect can explain how plp1Δ rescues the phenotypes of tub3Δ and pac10Δ cells, both their benomyl supersensitivity and their synthetic lethal interaction with rbi2Δ: because plp1Δ cells bring less β-tubulin to the chaperonin, less β-tubulin is properly folded and therefore toxic. The same explanation applies to the protection by plp1Δ against the large excess of β-tubulin produced by a strong inducible promoter.

pac10Δ plp1Δ double mutants are much more resistant to β-tubulin overexpression than wild type cells (Figure 2-5), despite the fact that pac10Δ alone is much more sensitive. This apparent contradiction can be explained in part in terms of a role for the GimC/prefoldin complex in recruiting free β-tubulin to the TRiC for folding (Vainberg et al. 1998). In addition, it is important to note that the GimC/PFD complex seems to act asymmetrically on α- and β-tubulin; α-tubulin levels are decreased more than β-tubulin levels. Thus, in the absence of the GimC/PFD complex, there is less folded α-tubulin than β-tubulin, leading to an excess of β-tubulin that is folded, functional, and toxic. Plp1p, however, acts specifically on β-tubulin, with the consequence that pac10Δ plp1Δ mutants – although they accumulate undimerized α-tubulin – have an even larger pool of β-tubulin that is undimerized.
Figure 2-10. Model.
Plp1p increases the efficiency of folding of newly synthesized β-tubulin polypeptides at a step distinct from GimC/prefoldin and TriC.
Insight into the nature of this excess β-tubulin is provided by the fact that increasing the levels of α-tubulin either by low copy plasmid or by high-level expression driven by a strong inducible promoter does not further rescue the benomyl phenotypes of pac10Δ plp1Δ. In contrast, the toxic β-tubulin in pac10Δ cells is completely suppressed by overexpressed α-tubulin. This result, and the aggregation of tubulin in plp1Δ cells, demonstrate that excess β-tubulin can be in different conformations, depending in part upon whether or not Plp1p is present.

**Summary**

The data reported here suggest that Plp1p acts in the β-tubulin folding pathway to help create heterodimerizable β-tubulin. In the absence of PLP1, sufficient β-tubulin is made and folded correctly to support cell viability; however, some is in an aggregated form. It is the reduced efficiency of folding β-tubulin that promotes aggregate formation in plp1Δ cells and so rescues them from excess β-tubulin. The precise role and interactions of Plp1p, and the nature of the toxic form of β-tubulin, are among the interesting questions that remain.
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Chapter 3

The interactions between the pathways for tubulin

folding and expression in *S. cerevisiae*
ABSTRACT

This chapter characterizes an interaction between the pathways for tubulin folding and the regulation of tubulin expression. Cells that are pac10Δ plp1Δ have much less folded and functional β-tubulin than even plp1Δ cells, and also upregulate β-tubulin through increasing transcription. The upregulation of β-tubulin RNA is dependent on the putative transcription factor Cin5p. In the absence of CIN5, pac10Δ plp1Δ cells have decreased tubulin heterodimer levels, down to approximately 20% that of wild type. The heterodimer levels are also decreased from pac10Δ plp1Δ cells suggesting that the limiting factor in heterodimer formation in pac10Δ plp1Δ cin5Δ is β-tubulin. The pac10Δ plp1Δ cin5Δ cells grow normally, but have mitotic defects such as abnormal nuclear positioning and short anaphase spindles. The Cin5p dependent upregulation of β-tubulin may be a mechanism to maintain tubulin heterodimer levels and so sustain normal microtubule function.
INTRODUCTION

Microtubules are involved in many critical functions in the cell. In budding yeast, the essential functions of microtubules include segregating chromosomes and positioning the nucleus during mitosis. The consequences of improper microtubule function include aneuploidy and lethality. To ensure proper microtubule function and formation, there are many regulatory steps acting both before and after a microtubule is formed. The work in this chapter is focused on the interactions between the pathways involved in tubulin folding and expression and how they relate to proper microtubule function.

Tubulin polypeptides must be folded for functionality and heterodimer association. In budding yeast, the two α-tubulin proteins, Tub1p and Tub3p, and the single β-tubulin protein, Tub2p are folded by the cytosolic chaperonin, TriC (Dunn et al. 2001; Ursic and Culbertson 1991). A nonessential complex, GimC/PFD, acts in conjunction with the chaperonin and is thought to prevent premature release of unfolded polypeptides and possibly deliver released unfolded polypeptides to the chaperonin (Geissler et al. 1998; Siegers et al. 1999; Vainberg et al. 1998). Once the tubulin polypeptides are folded, they can then form an α-β-tubulin heterodimer. Tubulin cofactors have been found in vitro to incorporate tubulin polypeptides into heterodimer (Lewis et al. 1996; Tian et al. 1997). However, in budding yeast, these cofactors are not essential (Archer et al. 1995; Geiser et al. 1997; Hoyt et al. 1997; Stearns et al. 1990) and may act in a pathway to reclaim dissociated heterodimers (Fleming et al. 2000). Therefore, in yeast heterodimer formation either depends upon other factors or is not catalyzed.
Free β-tubulin is toxic in yeast. For example, high-level overexpression of β-tubulin results in microtubule disassembly and subsequent cell death (Burke et al. 1989; Weinstein and Solomon 1990). In contrast, excess α-tubulin produced from high-level overexpression does not cause lethality and has only negligible consequences. Free β-tubulin can arise in various ways, such as through differential expression of the tubulin genes or through release of the α-β-tubulin heterodimer. For example, a deletion of the minor α-tubulin gene TUB3 has excess β-tubulin as well as a decreased level of tubulin heterodimers. Cells deleted for TUB3 have an enhancement of chromosome missegregation and sensitivity to the microtubule depolymerizing drug benomyl (Schatz et al. 1986a; Schatz et al. 1986b).

As mentioned above, the dissociation of the heterodimer can lead to increases in free β-tubulin and can have harmful consequences. An α-tubulin mutant, tub1-724 results in a weakened α-β-tubulin heterodimer, releasing folded β-tubulin more readily than a wild type heterodimer (Vega et al. 1998). tub1-724 is lethal in the cold and in the presence of the microtubule depolymerizing drug benomyl. The lethality is most likely the result of free β-tubulin produced upon microtubule depolymerization and subsequent heterodimer dissociation.

Mutating a member of the GimC/PFD complex, PAC10, can also alter tubulin levels. In a pac10Δ cell, tubulin polypeptide levels are reduced and there is an excess of β-tubulin compared to α-tubulin (Alvarez et al. 1998; Geissler et al. 1998). Interestingly, tubulin RNA levels are also decreased in pac10Δ, but to the same extent (M. Magendanz and F. Solomon unpublished). Thus, the decrease in the ratio of α- to β-tubulin is due to differences at the level of the polypeptide. The free β-tubulin in pac10Δ
cells causes the increased sensitivity to benomyl and the cold (Alvarez et al. 1998; Geiser et al. 1997; Geissler et al. 1998). Furthermore, pac10Δ cells are synthetically lethal with other mutations that alter tubulin levels such as tub3Δ (Alvarez et al. 1998).

I have found that a mutation in the gene PLP1 can bypass the toxicity of β-tubulin by creating less β-tubulin that is functional and toxic. Thus, plp1Δ rescues toxicity by reducing the pool of toxic β-tubulin (Chapter 2). For example, plp1Δ can rescue the synthetic lethality of pac10Δ tub3Δ double mutants. The pac10Δ tub3Δ cells are likely dead because of an excess of β-tubulin. Furthermore, plp1Δ can even suppress the lethality of high-level overexpression of β-tubulin, especially in combination with a mutation of PAC10.

Plp1p most likely acts at an early step of β-tubulin folding to create folded and functional β-tubulin (Chapter 2). The absence of PLP1 causes an increased amount of tubulin present in an aggregated and nonfunctional form. Plp1p action is distinct from that of the GimC/PFD complex, the chaperonin, or the putative tubulin heterodimerization cofactors. The function of Plp1p likely defines a novel step of tubulin folding.

The results presented in this chapter demonstrate a connection between the folding of tubulin and its expression. There is a precedent for tubulin RNA regulation in animal cells. The addition of microtubule depolymerizing drugs such as nocodazole results in a feedback response decreasing tubulin RNA levels. In contrast, the addition of heterodimer crystallizing drugs such as vinblastine may cause an increase in tubulin RNA levels (Ben-Ze'ev et al. 1979; Cleveland 1983). The decrease of β-tubulin was shown to be due to enhanced degradation of the β-tubulin RNA associated with polysomes.
(Pachter et al. 1987; Theodorakis and Cleveland 1992; Yen et al. 1988). The mechanism for the downregulation of α-tubulin is unclear; there may be a transcriptional component (Theodorakis and Cleveland 1992). The upregulation of tubulin RNA levels was not characterized.

The work presented below is an analysis of how the tubulin folding pathway regulates tubulin expression in yeast. Surprisingly, pac10Δ plp1Δ double mutants have reduced microtubule phenotypes but greater excess β-tubulin relative to pac10Δ cells. The increase of β-tubulin can be explained by increased RNA levels. The apparent contradiction of having more free β-tubulin but healthier cells can be explained by analysis of the pool of β-tubulin in pac10Δ plp1Δ cells. The data show that there is less functional and toxic β-tubulin in this cell, and suggest that upregulating β-tubulin expression may serve to increase the amount of heterodimerizable β-tubulin and so help to sustain microtubule function.

In this chapter, I characterize an upregulation of β-tubulin RNA in S. cerevisiae. I propose that the mechanism to upregulate β-tubulin in pac10Δ plp1Δ mutants exists to increase levels of tubulin heterodimers beyond a certain threshold. Without the upregulation, tubulin heterodimer levels are decreased down to 20% that of wild type, but with the upregulation, the levels are increased to 30%. The 10% difference in tubulin polypeptides may allow normal microtubule function. Without the β-tubulin increase, the cells have a nuclear positioning defect and have very short microtubules. Surprisingly however, cells can survive and grow normally with 20% the amount of tubulin heterodimers.
MATERIALS AND METHODS

Strains, Media, and cell morphology

All yeast strains (Table 1) are derivatives of FSY 182, FSY183, and FSY184 (WEINSTEIN and SOLOMON 1990). We used standard yeast manipulation methods and media (GUTHRIE and FINK 1991; SHERMAN et al. 1986; SOLOMON et al. 1992). Budding morphology was determined by counting the number of large-budded cells, medium-budded, small-budded, and unbudded cells. Nuclear positioning was analyzed as described previously (SCHATZ et al. 1988). For growth curves, cultures started at 0.25 X 10^7 and counted every 2 hours for 12 hours total.

Gene Disruptions

CIN5 was deleted using a PCR based method (LONGTINE et al. 1998). Deletion of PAC10 and PLP1 was previously described ((ALVAREZ et al. 1998) and Chapter 2). We HA-tagged the chromosomal CIN5 gene using the pFA6a-3HA-kanMX6 module (LONGTINE et al. 1998). MAD2 was disrupted using a construct containing URA3 called pRC10-1 (a gift from A. Murray). This construct was digested with XhoI and HindIII and transformed into a diploid heterozygous for pac10Δ, homozygous for plp1Δ, and heterozygous for cin5Δ.

Immunoblotting

We followed standard procedures for immunoblotting (SOLOMON et al. 1992), using anti-α–tubulin antibody #345 (SCHATZ et al. 1987) and anti-β-tubulin antibody #206 (BOND et
al. 1986) at a dilution of 1/3500. α- and β-tubulin protein levels were normalized to CPY using anti-CPY antibody #1410 at a dilution of 1:5000. Immunoblot detection was performed as previously described (ABRUZZI et al. 2002).

**Gel Filtration Chromatography**

We used FSY183 (wild type haploid), SSY38 (plp1::hisG haploid), SSY43 (pac10::HIS3 plp1::hisG haploid), and SSY262 (pac10::HIS3 plp1::hisG cin5::kan⁸ haploid) cells to determine the state of tubulin polypeptides in each strain. Gel filtration chromatography was performed as described previously (ABRUZZI et al. 2002). Briefly, cell extracts were obtained through French Press cell lysis and applied to a 300S Sephacryl column. 1 ml fractions were collected and analyzed by western blot for the presence of α- and β-tubulin.

**Synthetic Lethal Interactions**

SSY339 diploid pac10::HIS3/++; plp1::hisG/plp1::hisG; cin5::kan⁸/++; mad2::URA3/++ cells were sporulated. Genotypes were determined through analysis of auxotrophic markers. χ² test was used to determine if the 4 pac10Δ plp1Δ cin5Δ mad2Δ cells were statistically significant. The expected value would be that pac10Δ plp1Δ cin5Δ mad2Δ would arise just as frequently as plp1Δ, or 24 times. However, this was not the case and the hypothesis that plp1Δ is just as likely to survive as pac10Δ plp1Δ cin5Δ mad2Δ was rejected with a p value of <=.005.
**Chromatin Immunoprecipitation**

Chromatin Immunoprecipitation assay (ChIP) was performed as described (APARICIO et al. 1997). Anti-HA antibody 12CA5 (Roche) was used to immunoprecipitate tagged Cin5p. PCR products using 28 cycles were resolved on 2% agarose gels. Primer sequences of *TUB2* regions are available upon request.

**Ribonuclease Protection Assay**

Total yeast RNA was prepared using a modified hot phenol extraction procedure (AUSUBEL et al. 1989). Antisense RNA probes for *TUB1, TUB2, and CPY* RNAs were obtained using the Maxiscript In Vitro Transcription Kit from Ambion. The RPA11 kit from Ambion was used to perform the assay using 10ug of yeast RNA. The protected fragments were run on a urea polyacrylamide denaturing gel. The gel was dried and analyzed using phosphoimaging (Storm System from Molecular Dynamics) and quantitated using Imagequant software.

**RNA decay assay**

Cells were grown to early log phase of 1.0 X 10⁷. Thiolutin (very generous gift of A. Jacobson) was added to a final concentration of 8ug/ml. Samples were taken every 20 minutes for 2 hours and harvested for RNA. RNase protection assay was performed on each sample.
Immunofluorescence and Spindle Measurements

Cells were fixed and prepared for immunofluorescence as previously described (SCHATZ et al. 1988). Spindle lengths were measured using Openlab 3.02 software. Late Anaphase/Telophase spindles were observed looking at large-budded cells with two divided nuclei.
RESULTS

β-tubulin RNA levels are upregulated in pac10Δ plp1Δ cells

In chapter 2, I reported that the ratio of β− to α−tubulin increases in pac10Δ plp1Δ relative to pac10Δ. The amount of excess β-tubulin is approximately 30% in pac10Δ cells and around 41% in pac10Δ plp1Δ cells. Since both Pac10p and Plp1p affect protein folding, these quantitative differences could be a consequence of changing protein stability. However, we previously found that pac10Δ cells have decreased tubulin RNA levels (Magendantz and Solomon unpublished), suggesting the possibility that the excess β-tubulin in pac10Δ plp1Δ cells is due to increased levels of RNA.

I used RNase protection assay to measure tubulin RNA levels (Figure 3-1). The levels of TUB1 and TUB2 were normalized to an internal control, carboxypeptidase Y (CPY). I found that α-tubulin RNA levels in pac10Δ plp1Δ are comparable to pac10Δ cells: 74 ± 10% of wild type in pac10Δ plp1Δ and 70 ± 15% of wild type in pac10Δ. However, β-tubulin RNA levels are dramatically increased in pac10Δ plp1Δ compared to pac10Δ: 127 ± 16% in pac10Δ plp1Δ and 81 ± 13% in pac10Δ. Therefore, the increase of β-tubulin polypeptides in pac10Δ plp1Δ cells is likely due to an increase in β-tubulin RNA.

Increase of β-tubulin RNA levels is not due to a slower rate of message degradation

There is a precedent for β-tubulin regulation in animal cells, mediated through regulation of the rate of message degradation (Cleveland 1989).
Figure 3-1. RNA levels.
Total RNA levels from wildtype, *plp1 Δ*, *cin5 Δ*, *pac10 Δ*, *pac10 Δ plp1 Δ*, and *pac10 Δ plp1 Δ cin5 Δ* cells were analyzed for levels of *TUB1*, *TUB2*, and *CPY* using RNAsese protection assay (see Materials and Methods). Mean values of tubulin message levels were normalized to *CPY* RNA; the wild type values are defined as 1.0. Data presented are the average of 5 experiments.
For this reason, I asked if β-tubulin messages are degraded at the same rate in wild type, pac10Δ, and pac10Δ plp1Δ cells. I added a drug called thiolutin to each of the cultures to inhibit RNA polymerase II and halt transcription. I then collected samples every 20 minutes after addition of the drug and performed RNase protection assay to measure message decay. All three cell types have a similar rate of β-tubulin message decay, with a half life of approximately 30 minutes (data not shown). It is difficult to determine if the downregulation of tubulin RNA in pac10Δ is due to enhanced degradation because the difference of levels may be too small to use this assay. However, the increase of β-tubulin RNA levels in pac10Δ plp1Δ cannot be ascribed to increased message stability.

**A deletion of CIN5 suppresses the benomyl sensitivity of pac10Δ plp1Δ**

The rate of β-tubulin message degradation does not change in pac10Δ plp1Δ cells, suggesting that the increase in tubulin RNA is due to enhanced transcription. I directly tested whether a transcription factor that has microtubule phenotypes, Cin5p (FERNANDES et al. 1997; HOYT et al. 1990) plays a role in tubulin RNA regulation. I deleted the entire open reading frame of CIN5 in different genetic backgrounds and asked if this changed the benomyl sensitivity of the strains. The benomyl super sensitivity of pac10Δ cells is suppressed by a deletion of PLP1 at low (10ug/ml and below) but not high benomyl concentrations (15ug/ml and above, see Chapter 2). Deletion of CIN5 partially rescues the benomyl sensitivity of pac10Δ plp1Δ on plates containing benomyl at 5, 10 and 15ug/ml (Figure3-2). A deletion of CIN5 in an otherwise wild type background does not confer either benomyl resistance or sensitivity at any concentration of drug. Thus, by deleting CIN5 in pac10Δ plp1Δ, the cells can survive better in the presence of the microtubule depolymerizing drug benomyl.
**Figure 3-2.** *cin5Δ* suppresses the benomyl phenotypes of *pac10Δ plp1Δ*.
Serial dilutions of saturated yeast cultures of wildtype, *cin5Δ*, *pac10Δ plp1Δ* and *pac10Δ plp1Δ cin5Δ* cells were plated to rich media (YPD) and media containing 15 ug/ml of benomyl. Wild type and *cin5Δ* cells grow equivalently. *pac10Δ plp1Δ* cells are benomyl sensitive but can be suppressed by an additional deletion of *CIN5*. 
A deletion of CIN5 does not rescue the cold sensitivity of an α-tubulin mutant with a destabilized heterodimer

To determine if cin5Δ can rescue cells from toxic β-tubulin newly released from the heterodimer, I asked if cin5Δ can rescue an α-tubulin mutant with a destabilized heterodimer, tub1-724. This mutation in α-tubulin forms a weakened heterodimer with β-tubulin and is most likely cold sensitive because of the production of free and toxic β-tubulin (VEGA et al. 1998). I found that a deletion of CIN5 in strains containing this α-tubulin allele does not rescue the cold or benomyl sensitivity (Figure 3-3). The results suggest that cin5Δ does not rescue cells from free β-tubulin that is folded and toxic and may act specifically in response to a signal in pac10Δ plp1Δ cells.

Presence of Cin5p causes increased β-tubulin RNA levels in pac10Δ plp1Δ

To determine if Cin5p has a role in tubulin RNA regulation, I measured RNA levels using the RNase protection assay in different strain backgrounds (Figure 3-1). RNA levels of α- and β-tubulin in pac10Δ plp1Δ cin5Δ are 80 ± 5% and 87 ± 11% of wild type levels, respectively. These levels are comparable to RNA levels of pac10Δ and the β-tubulin levels are not elevated as they are in pac10Δ plp1Δ cells. The levels of α- and β-tubulin RNA in cin5Δ cells are comparable to wild type, 105 ± 6% and 107 ± 11% respectively. The tubulin RNA levels of plp1Δ and plp1Δ cin5Δ are also comparable to wild type levels (Figure3-1). Thus, expression of Cin5p is necessary for increased levels of β-tubulin in pac10Δ plp1Δ cells but does not cause an affect on β-tubulin message levels in wild type or plp1Δ cells.
Figure 3-3. A deletion of CIN5 does not rescue the cold or benomyl sensitivity of the α-tubulin mutant tub1-724.
Saturated cultures of strains containing deletions of the genomic copies of TUB1 and TUB3 covered with a wild type TUB1 plasmid or tub1-724 plasmid with and without deletions of CIN5 were diluted and plated to rich media (YPD) or media containing 15ug/ml of benomyl and grown at either 30 degrees or 18 degrees Celsius.
Cin5p binds the β-tubulin promoter

Cin5p is classified as an AP-1 like transcription factor. The enhanced levels of β-tubulin in pac10Δ plp1Δ may be due to increased transcription by Cin5p. If this is the case, Cin5p could be bound to β-tubulin promoter regions. I used Chromatin Immunoprecipitation (ChIP) to determine if Cin5p does indeed bind β-tubulin promoter regions. CIN5 was HA-tagged in wild type, pac10Δ, plp1Δ, and pac10Δ plp1Δ cells. After crosslinking proteins to the DNA, Cin5p was immunoprecipitated with anti-HA antibody. By ChIP, Cin5p is bound to a specific region of the TUB2 promoter in each of the different genetic backgrounds, from −8 to −250 bps from the start (Figure 3-4). Cin5p is not bound to a region of the promoter from −300 to −500 bps from the start site. Since Cin5p dependent upregulation of TUB2 occurs only in pac10Δ plp1Δ cells, but the protein immunoprecipitates with the β-tubulin promoter in all backgrounds, another factor may be involved in the differential regulation.

α- and β-tubulin polypeptide levels are decreased to 20-40% that of wild type in pac10Δ plp1Δ cin5Δ cells

To my surprise, tubulin polypeptide levels do not correlate well with RNA levels when CIN5 is deleted in pac10Δ plp1Δ. As shown earlier, pac10Δ plp1Δ cin5Δ mutants have tubulin RNA levels similar to pac10Δ, down to 80-87% of wild type, but the polypeptide levels are much lower. The α-tubulin levels are 25 ± 8% of wild type and the β-tubulin levels are 40 ± 15% of wild type in pac10Δ plp1Δ cin5Δ cells (Figure 3-5). Significantly, the deletion of CIN5 on its own does not alter polypeptide levels: α-tubulin levels are 92 ± 15% and β-tubulin levels are 97 ± 19%.
Figure 3-4. Cin5p binds the TUB2 promoter.
Cin5 was HA-tagged in wild type, plp1Δ, pac10Δ, and pac10Δ plp1Δ cells. Chromatin immunoprecipitation was performed using an anti-HA antibody. The immunoprecipitates were analyzed by PCR to determine if DNA at the promoter region of TUB2 was present. Only a certain region of the promoter immunoprecipitated with Cin5p (between -8 and -250). A tagged polymerase subunit (RPB3-HA) was used as a positive control and an untagged strain was used as a negative control.
Furthermore, I have previously shown that plp1Δ cells have tubulin polypeptide levels equivalent to that of wild type; these levels are the same for plp1Δ cin5Δ cells (Figure 3-5).

There is also a significant but less dramatic difference between tubulin RNA and protein levels in pac10Δ plp1Δ cells. As noted earlier, the β-tubulin RNA is significantly increased to 127 ± 16% in pac10Δ plp1Δ cells but the protein levels are 92 ± 3%, a drop of about 35%. α-tubulin levels are also decreased to 49 ± 5% from the RNA levels of 74 ±10%, decreasing by approximately 25%. Therefore, since both Pac10p and Plp1p are involved in tubulin folding, some of the misfolded tubulin protein may be degraded.

**α/β-tubulin heterodimer levels are decreased in pac10Δ plp1Δ cin5Δ**

The fraction of tubulin present in an α/β-tubulin heterodimer was determined using gel filtration columns. As previously shown, in wild type cells, approximately 95% of tubulin is in heterodimer, but in plp1Δ about ~50% of the tubulin is in heterodimer and 50% is found in the void volume of the column and is likely aggregated (Chapter 2). In pac10Δ plp1Δ cells, there is an even smaller proportion of the tubulin eluting from the column as heterodimer (Figure 3-6). The fraction of tubulin present in heterodimer is ~30% of the amount of tubulin present in heterodimer in a wild type cell. In contrast, the tubulin present in pac10Δ plp1Δ cin5Δ cells is mostly in heterodimer form. However, pac10Δ plp1Δ cin5Δ cells have such low tubulin levels that the amount of tubulin in heterodimer is ~20% that of wild type. This is the lowest tubulin heterodimer level demonstrated to be sufficient for survival.
**Figure 3-5. Tubulin protein levels in different mutant backgrounds.**

Extracts from wild type, *cin5Δ, plp1Δ, plp1Δ cin5Δ, pac10Δ, pac10Δ plp1Δ*, and *pac10Δ plp1Δ cin5Δ* were analyzed for levels of Tub1p, Tub2p, and carboxypeptidase Y (CPY) by immunoblotting. Mean values of tubulin polypeptides are normalized to CPY. Wild type values are defined as 1.0. Data presented are the averages of at least 5 experiments.
Figure 3-6. Gel filtration columns to determine heterodimer fractions.
Cell extracts from wild type, plp1Δ, pac10Δ plp1Δ, and pac10Δ plp1Δ cin5Δ cells were analyzed by gel filtration chromatography. Each fraction was assayed for Tub1p and Tub2p by immunoblotting. The graphs plot the percentage of protein in each fraction. Solid lines represent α-tubulin and dashed lines represent β-tubulin.
Although *pac10Δ plp1Δ cin5Δ* cells have low heterodimer levels, they grow at a normal rate. Surprisingly, *pac10Δ plp1Δ cin5Δ* cells do not have a growth defect. Growth rates of different cell types were determined by a 12 hour time course. At time zero, cell cultures were diluted to early log phase in rich media and counted every two hours. Growth curves are shown in Figure 3-7. Over the period of 12 hours, wild type cells divided 4.8 times. Cells deleted for *CIN5* divided 4.6 times, and *pac10Δ plp1Δ* cells divided 3.6 times. The *pac10Δ plp1Δ cin5Δ* cells grew similarly to wild type, 4.7 divisions in 12 hours. Thus, cells with heterodimer levels down to 20% that of wild type have a normal rate of growth.

*pac10Δ plp1Δ cin5Δ* cells are more cold sensitive than *pac10Δ*

Mutations that affect microtubule formation are often cold sensitive. For example, deletion of the member of the GimC/PFD complex, *PAC10* was previously characterized as being cold sensitive (Geiser et al. 1997). A deletion of *PAC10* causes cells to grow slowly at 18 °C and lower. However, *pac10Δ plp1Δ cin5Δ* cells grow even more slowly in the cold (Figure 3-8). In contrast, cells that are *cin5Δ, plp1Δ*, or *plp1Δ cin5Δ* are not cold sensitive and grow just as rapidly as wild type cells at those temperatures (Figure 3-8) (Flanary et al. 2000). Cells that are *pac10Δ cin5Δ* or *pac10Δ plp1Δ* are equivalently cold sensitive as *pac10Δ* cells. The increase in cold sensitivity of cells with the genotype *pac10Δ plp1Δ cin5Δ* suggest that increased tubulin levels may be needed to maintain normal survival in the cold.
Figure 3-7. Growth curve of \textit{pac10\Delta plp1\Delta cin5\Delta} cells.
Overnight cultures of wild type, \textit{cin5\Delta}, \textit{pac10\Delta plp1\Delta}, and \textit{pac10\Delta plp1\Delta cin5\Delta} cells were diluted to early log phase at time zero and then counted every two hours for twelve hours. For each strain, the log of cell number was plotted at each time point.
Figure 3-8. Cold sensitivity of different mutants.
Serial dilutions of saturated yeast cultures were plated onto rich plates and grown at 30 and 13 degrees Celsius.
The spindle checkpoint is required in pac10Δ plp1Δ and pac10Δ plp1Δ cin5Δ cells

If a mitotic spindle is not properly formed such that chromosomes are not attached and aligned correctly during mitosis, the spindle checkpoint halts the cell cycle preventing chromosome missegregation. I asked if the spindle checkpoint is required in strains with lower tubulin heterodimer levels. I knocked out one of the major components of the spindle checkpoint, MAD2, in a diploid cell homozygous for plp1Δ, and heterozygous for pac10Δ and cin5Δ. I then sporulated and dissected these cells. The colonies that grew from the spores were different sizes, either large, small, or the size of a pin prick.

I dissected 34 tetrads and analyzed 106 large and small colonies for the auxotrophic markers that replaced the genes of interest to determine their genotypes (Figure 3-9). There were 74 large colonies, 32 small colonies, and 28 pin-sized colonies. I found that the small colonies were either pac10Δ plp1Δ or pac10Δ plp1Δ cin5Δ. Their small colony size is explained by the previous report that pac10Δ cells have a germination defect (GEISER et al. 1997). The large colonies were plp1Δ, plp1Δ cin5Δ, plp1Δ mad2Δ, or plp1Δ cin5Δ mad2Δ. No large or small colonies were present with the genotype of pac10Δ plp1Δ mad2Δ. However, 4 large colonies were the genotype of pac10Δ plp1Δ cin5Δ mad2Δ.

Previous studies have shown that mad2Δ cells can acquire suppressors that allow them to grow rapidly (HARDWICK et al. 1999). To determine whether the 4 colonies of this genotype are significant, I applied a χ² test. This test is a statistical tool to determine the probability of obtaining an observed result by chance.
Figure 3-9. The spindle checkpoint is required in \textit{pac10Δ plp1Δ} and \textit{pac10Δ plp1Δ cin5Δ} mutants.
Diploid \textit{pac10Δ/+; plp1Δ/plp1Δ; cin5Δ/+; mad2Δ/+} cells were sporulated and dissected. Spores were analyzed for auxotrophic markers replacing the knocked out genes to determine genotypes. Each genotype was counted and analyzed. No \textit{pac10Δ plp1Δ mad2Δ} colonies were recovered and only 4 \textit{pac10Δ plp1Δ cin5Δ mad2Δ} colonies grew. *By $\chi^2$ test, 4 colonies were not statistically significant.
The genotype of \( plp1\Delta \) should occur just as frequently as \( pac10\Delta \ plp1\Delta \ \text{cin5}\Delta \ \text{mad2}\Delta \). There were 24 \( plp1\Delta \) colonies and only 4 \( pac10\Delta \ plp1\Delta \ \text{cin5}\Delta \ \text{mad2}\Delta \) colonies. From these results I found that the presence of the 4 colonies was not statistically significant with a probability of obtaining the deviation by chance of \(<< 0.5\% \). The four colonies likely acquired suppressors, allowing survival. Thus, both \( pac10\Delta \ plp1\Delta \) and \( pac10\Delta \ plp1\Delta \ \text{cin5}\Delta \) cells require the spindle checkpoint.

\textbf{pac10\Delta \ plp1\Delta \ cin5\Delta \ cells have a mitotic defect}

Budding yeast have various bud sizes depending upon the stage of the cell cycle. An asynchronous population of cells grown in early log phase was scored for percent of cells in the population with no buds, small, medium, or large buds (Figure 3-10). The large-budded fraction represents the cells in G2/M of the cell cycle. Wild type cells have a total of 14% large-budded cells in an asynchronous population. 13% of \( plp1\Delta \) cells and 22% of \( pac10\Delta \ plp1\Delta \) cells are large-budded. The number of large-budded cells is increased in \( pac10\Delta \ plp1\Delta \ cin5\Delta \) cells to 44% of an asynchronous population.

The increase in number of cells in G2/M phase of the cell cycle in \( pac10\Delta \ plp1\Delta \ \text{cin5}\Delta \) and the requirement for the spindle checkpoint suggests that the cells may be delayed at the metaphase/anaphase transition. To address this possibility, nuclear position was analyzed in the population of large-budded cells (Figure 3-11). 70% of wild type large-budded cells have two nuclei that are clearly separated into mother and daughter cells, and separated from the neck. In 19%, the nuclei have their DNA straddling the neck between mother and daughter cells. Only 11% have a single nucleus located in one cell.
<table>
<thead>
<tr>
<th></th>
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<th>S</th>
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<th>G2/M</th>
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<td>15%</td>
<td>15%</td>
<td>13%</td>
</tr>
<tr>
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<td>45%</td>
<td>12%</td>
<td>21%</td>
<td>22%</td>
</tr>
<tr>
<td>$pac10\Delta plp1\Delta cin5\Delta$</td>
<td>30%</td>
<td>7%</td>
<td>19%</td>
<td>44%</td>
</tr>
</tbody>
</table>

**Figure 3-10. In an asynchronous culture, $pac10\Delta plp1\Delta cin5\Delta$ mutants have more cells in G2/M.**

Early log phase asynchronous cultures of wild type, $plp1\Delta$, $pac10\Delta plp1\Delta$, and $pac10\Delta plp1\Delta cin5\Delta$ cultures were counted for bud size. Large-budded populations represent cells in G2/M of the cell cycle. At least 300 cells were counted for each genotype.
<table>
<thead>
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<th>19%</th>
<th>70%</th>
<th>0%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>73%</td>
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<td>0%</td>
</tr>
<tr>
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<td>16%</td>
<td>75%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><em>pac10Δ</em> <em>plp1Δ cin5Δ</em></td>
<td>47%</td>
<td>21%</td>
<td>24%</td>
<td>8%</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3-11.** *pac10Δ* *plp1Δ cin5Δ* large-budded cells have improperly positioned nuclei.

Wild type, *plp1Δ*, *pac10Δ* *plp1Δ*, and *pac10Δ* *plp1Δ cin5Δ* cells were analyzed for nuclear positioning. Percentages were based on counts of at least 200 large-budded cells for each genotype.
In pac10Δ plp1Δ cin5Δ cells, the nuclear positions were different. Of the large budded cells, 44% have two nuclei, one in the mother and one in the daughter. In approximately half of those, the nuclei were well separated from each other and from the mother-bud neck. The other half had nuclei that are very close to the neck. This position is rarely seen in wild type cells. 47% of pac10Δ plp1Δ cin5Δ large-budded cells have a single nucleus straddling the neck, and 15% have a single nucleus in one of the cells. Interestingly, 8% of cells had two nuclei localized to one of the cells, showing a defect in nuclear segregation. The nuclear positioning of plp1Δ and pac10Δ plp1Δ cells is similar to that of wild type cells. In conclusion, pac10Δ plp1Δ cin5Δ cells have a nuclear positioning defect characterized by an increase in the percent of large-budded cells with a single nucleus straddling the neck.

Besides having a nuclear positioning defect, the spindle lengths are greatly reduced in pac10Δ plp1Δ cin5Δ cells. Spindle lengths were measured for large-budded cells with two distinct nuclei (Figure 3-12). Wild type cells have a spindle length varying from 7 to 11μm with an average spindle length of 9μm. pac10Δ plp1Δ cin5Δ cells have a varying range of spindle length from 4 to 8μm with an average and median length of 5μm. This length is sufficient to span the distance through the neck to segregate chromosomes, but is much shorter than normal Anaphase spindles.
A. Wild type Cells

FITC

DAPI

DIC

B. pac10Δ plp1Δ cin5Δ

FITC

DAPI

DIC

C. Spindle Lengths

% of total

'Length' in Microns

Wild type

pac10Δ plp1Δ cin5Δ
Figure 3-12. Spindle lengths are shorter in pac10Δ plp1Δ cin5Δ compared to wild type.
Immunofluorescence of wild type (A) and pac10Δ plp1Δ cin5Δ (B) cells. Microtubule structures were identified by anti-tubulin antibodies and nuclei staining using DAPI. * represents a large-budded cell with divided nuclei. (C) Graph shows % of large-budded cells with split nuclei at each spindle length in microns. Wild type is represented in green and pac10Δ plp1Δ cin5Δ in purple.
DISCUSSION

The data presented demonstrate a pathway for upregulation of β-tubulin levels to maintain normal microtubule function. In wild type cells, increases in β-tubulin levels are harmful to the cell and an upregulation could not be tolerated. However, I have shown that pac10Δ plp1Δ cells can survive excess β-tubulin because most of the tubulin is not present as a heterodimer but is in a nonfunctional and nontoxic form (Chapter 2). Thus, increasing β-tubulin can be tolerated, and may be beneficial to pac10Δ plp1Δ cells. In pac10Δ plp1Δ cells, the absence of a gene required for upregulation, CIN5, causes mitotic delays and short spindles.

A pathway to upregulate β-tubulin

By measuring tubulin RNA levels, I found that β-tubulin RNA levels increase in pac10Δ plp1Δ cells, beyond even wild type levels (Figure 3-1). In contrast, α-tubulin RNA levels are not enhanced, but are similar to pac10Δ. Since β-tubulin RNA levels are decreased in pac10Δ, there is a substantial increase from pac10Δ to pac10Δ plp1Δ. By measuring β-tubulin message decay, I found that the upregulation in pac10Δ plp1Δ is not due to a more stable message. Accordingly, I have focused on the possibility of RNA upregulation through enhanced transcription.

A putative transcription factor, Cin5p is involved in the upregulation of β-tubulin in pac10Δ plp1Δ cells. Disrupting CIN5 in pac10Δ plp1Δ resulted in β-tubulin RNA levels comparable to pac10Δ, not pac10Δ plp1Δ (Figure 1). Furthermore, Cin5p does bind to the β-tubulin promoter suggesting that it could act directly to upregulate β-tubulin.
The β-tubulin upregulation pathway could exist to prevent mitotic problems in \( pac10\Delta plp1\Delta \) cells. By deleting \( CIN5 \), β-tubulin upregulation does not occur in \( pac10\Delta plp1\Delta \) and the cells have low tubulin heterodimer levels and mitotic phenotypes. The triple mutant has a larger fraction of large-budded or G2/M cells in an asynchronous population. These large-budded cells also have a nuclear positioning defect and shorter spindles. In contrast, \( pac10\Delta plp1\Delta \) cells that have increased β-tubulin and heterodimer levels do not display mitotic defects.

**The specificity of the β-tubulin upregulation pathway**

The upregulation of β-tubulin is specific to the \( pac10\Delta plp1\Delta \) genotype. The deletion of \( CIN5 \) in wild type or \( plp1\Delta \) does not cause any changes in β-tubulin RNA levels. Furthermore, \( cin5\Delta \) rescues the benomyl sensitivity of \( pac10\Delta plp1\Delta \), but is not benomyl resistant on its own (Figure 3-2) or in combination with \( plp1\Delta \) (data not shown). A deletion of \( CIN5 \) also does not rescue the benomyl sensitivity or cold sensitivity of an α-tubulin mutant \( tub1-724 \). This mutation compromises the α/β-tubulin heterodimer, and more readily releases toxic β-tubulin when the microtubule is depolymerized in the cold (VEGA et al. 1998). Thus, \( cin5\Delta \) does not rescue the consequences of free β-tubulin released from the heterodimer, but rescues specifically \( pac10\Delta plp1\Delta \). The rescue of benomyl sensitivity is most likely due to the differences in RNA upregulation since \( pac10\Delta plp1\Delta \) upregulates β-tubulin and may have slightly more free β-tubulin than \( pac10\Delta plp1\Delta cin5\Delta \).

**The regulation of Cin5p**

Cin5p may act directly to cause an upregulation of β-tubulin in \( pac10\Delta plp1\Delta \) cells. Cin5p is a putative transcription factor and has a conserved domain called a bZIP
that contains a basic region that interacts with DNA (FERNANDES et al. 1997). CIN5 is also called YAP4 because it is in a family of specific bZIP proteins called yeast AP-1 like transcription factors. Cin5p may promote transcription to upregulate β-tubulin.

Interestingly, by ChIP, Cin5p bound to the promoter region of TUB2 even if it did not cause an upregulation of β-tubulin RNA (Figure 3-4). For example, β-tubulin levels in a wild type cell do not depend upon Cin5p, but Cin5p is bound to the TUB2 promoter region. This result is similar to a result from the original study identifying CIN5 as a transcription factor. Fernandes et al found that in vitro, Cin5p binds a site they termed the Yap site (1997). However, Cin5p does not promote transcription of genes containing this typical binding site. From these assays, Fernandes et al. propose that Cin5p is transcriptionally inert because it may need to act in combination with other proteins to activate transcription. In pac10Δ plp1Δ, Cin5p may act with a cofactor to promote transcription. That cofactor may not be present or activated unless signaled, perhaps by low tubulin heterodimer levels in pac10Δ plp1Δ.

**Discrepancy between polypeptide and RNA levels**

Surprisingly, the tubulin RNA levels do not exactly match the amount of protein in pac10Δ, pac10Δ plp1Δ, and pac10Δ plp1Δ cin5Δ cells. In pac10Δ cells, the ratio of α-to β-tubulin protein decreases compared to the RNA levels, suggesting that α-tubulin may be degraded because it is not properly folded in the absence of a functional GimC/PFD complex. In pac10Δ plp1Δ cells, both α- and β-tubulin protein levels are decreased relative to the RNA levels. I have shown that Plp1p is involved in β-tubulin folding (Chapter 2) and the combination of its absence and a deletion of the GimC/PFD complex may create a substantial amount of protein that is more prone to degradation as
well as aggregation. Cells that are \textit{pac10Δ plp1Δ cin5Δ} also have decreased levels of \(\alpha\)-and \(\beta\)-tubulin. To our surprise, the amount of protein is decreased substantially, down to 20-40\% that of a wild type cell. In \textit{pac10Δ plp1Δ cin5Δ} cells, there is a tubulin folding problem, but \(\beta\)-tubulin is not upregulated, creating even less \(\beta\)-tubulin to stabilize and bind \(\alpha\)-tubulin.

\textbf{Tubulin heterodimer levels and the upregulation pathway}

The upregulation of \(\beta\)-tubulin may be a response to maintain tubulin heterodimer levels because \(\beta\)-tubulin is likely the limiting factor for tubulin heterodimer formation. In the absence of the upregulation, \textit{pac10Δ plp1Δ cin5Δ} cells have approximately 20\% of the amount of tubulin heterodimers, as opposed to the approximately 30\% of heterodimer found in \textit{pac10Δ plp1Δ}. The upregulation could allow the \(\sim\)10\% difference in tubulin heterodimer levels. From Chapter 2, we know that the limiting factor for heterodimer formation is \(\beta\)-tubulin in \textit{pac10Δ plp1Δ} since high-level overexpression of \(\alpha\)-tubulin does not rescue the microtubule phenotypes. Excess \(\alpha\)-tubulin without a partner is likely degraded (\textsc{Katz} et al. 1990). Thus, the upregulation of \(\beta\)-tubulin, even inefficiently folded as in \textit{pac10Δ plp1Δ}, may stabilize some of the \(\alpha\)-tubulin, increasing the amount of heterodimer in the cell.

A similar method of regulation exists in the iron uptake pathway in yeast. Iron is essential for cells, but can cause toxicity in excess. The cell regulates iron levels through a low-affinity (\textsc{Dix} et al. 1997; \textsc{Dix} et al. 1994) and a high-affinity pathway (\textsc{Dancis} et al. 1990; \textsc{Dancis} et al. 1992). The genes involved in the high-affinity uptake are regulated in part by the transcription factor Atf1p (\textsc{Yamaguchi-Iwai} et al. 1995; \textsc{Yamaguchi-Iwai} et al. 1996). When iron is depleted from the cell, Atf1p mediated
expression is induced. Similarly, Cin5p may induce transcription of β-tubulin further when the levels of β-tubulin are limiting for heterodimer formation. Otherwise, when heterodimer levels are not limiting, Cin5p is not active and the levels of β-tubulin are not enhanced.

We have previously found that the cell does not upregulate tubulin levels in response to half the amount of β-tubulin in the cell (Katz et al. 1990). The amount of tubulin heterodimers, most likely 50%, may not be at the threshold level to elicit an increase of tubulin RNA. pac10Δ plp1Δ cin5Δ cells have substantially lower levels and may be below the threshold level. Furthermore, pac10Δ plp1Δ cells also have a folding defect that protects them from the consequences of excess β-tubulin and so can tolerate a higher amount of undimerized β-tubulin (Chapter 2). The increase in protection may allow the cell to upregulate β-tubulin RNA, even beyond wild type levels. The cell may have a sensing mechanism to respond to an inadequate amount of folded β-tubulin and act by upregulating the transcription of TUB2.

Summary: The data presented suggest that there is a pathway that upregulates β-tubulin in response to low tubulin heterodimer levels in pac10Δ plp1Δ cells. In the absence of Cin5, the upregulation is abolished and the cells have tubulin heterodimer levels down to 20%. These cells also have mitotic defects associated with abnormal spindle formation. The interactions of Cin5p and the other genes involved in the upregulation are among the interesting questions that remain.
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Chapter 4

Conclusions and Future Directions
The results in this thesis suggest that the tubulin folding pathway interacts with tubulin expression to give normal microtubule function. I have identified a gene product \textit{PLP1} that is a novel step in β-tubulin folding that allows proper heterodimer formation. The absence of this gene leads to a decrease in functional and folded β-tubulin. A deletion of \textit{PLP1} in combination with a mutation of the GimC/PFD component, \textit{PAC10}, lowers the amount of functional and folded β-tubulin, thus decreasing the amount of toxic β-tubulin in the cell. I have found evidence for a feedback response to upregulate β-tubulin messages in \textit{pac10Δ plp1Δ} cells. This upregulation maintains heterodimer levels at approximately 30% of wild type. In the absence of the putative transcription factor Cin5p the upregulation of tubulin RNA is abolished, and heterodimer levels fall to approximately 20% of wild type. This upregulation of β-tubulin, and therefore of heterodimer levels, may be needed to maintain proper microtubule function, since the \textit{pac10Δ plp1Δ cin5Δ} triple mutant has short spindles and an enrichment of cells at G2/M. Taken together, these results suggest that the cell upregulates β-tubulin when increasing β-tubulin can be well tolerated and the levels of heterodimer are sufficiently low.

There are many opportunities to further explore the tubulin folding pathway through Plp1p and the β-tubulin upregulatory pathway through Cin5p as well as the convergence of the two pathways. Both pathways must have other proteins that are involved and currently unidentified. Furthermore, aspects of the convergence of these two pathways are also unknown. In the rest of this chapter, I will describe questions that remain and possible experiments to address them.
Plp1p localization and Binding Partners

I have proposed a model that Plp1p acts at an early step of tubulin folding, possibly at the ribosome to promote folding of β-tubulin. I know that Plp1p has a novel role in tubulin folding because the loss-of-function phenotypes are distinct from any of the known components of the folding pathway. Furthermore, I found that a fraction of Plp1p in the cell is polysome-associated (Chapter 2). The site of action of Plp1p may be at the ribosome either to prevent aggregation or to promote folding in collaboration with the chaperonin or a ribosome-associated chaperone. I have also found that Plp1p may act specifically on folding β-tubulin and not α-tubulin. Thus, there may be some specificity with polysome binding.

The interaction of Plp1p with the ribosome could be further explored. The polysome gradient (Chapter 2) shows that Plp1p does bind actively translating ribosomes because it binds polysomes and not just ribosomes. However, I would like further evidence of this interaction, taking advantage of a temperature sensitive mutation in the gene PRT1. Prt1p is a subunit of the translation initiation factor EIF3 (NARANDA et al. 1994). The temperature sensitive mutation inhibits translation (KEIERLEBER et al. 1986), decreasing protein synthesis by 95-97% after 15 minutes at the restrictive temperature (WELCH and JACOBSON 1999). Furthermore, the polysomes dissociate, becoming monosomes easily distinguished in a sucrose gradient. I would like to ask whether Plp1p shifts in this gradient at the restrictive temperature, either to a monosome peak or to an even lighter fraction of the gradient. From my EDTA control, where the addition of EDTA breaks up ribosomes and Plp1p shifts to lighter fractions, I predict that the prtl-1 mutation will give the same result.
The question remains whether Plp1p only acts at ribosomes translating β-tubulin messages, since Plp1p seems to act specifically for β-tubulin folding and not α-tubulin. To address this question, I would like to immunoprecipitate ribosomes with nascent β-tubulin chains and ask if Plp1p is bound. I would use the polosme specific fractions to IP out the N-terminus of α- and β-tubulin using N-terminal specific antibodies we have prepared and ask if these ribosomes are enriched for Plp1p as compared to α-tubulin containing ribosomes. If there is enrichment of Plp1p for ribosomes translating β-tubulin messages compared to α-tubulin, there must be some mechanism for recognizing particular sequences of mRNA or polypeptide.

If Plp1p does bind only β-tubulin translating polysomes, a specificity factor may exist to recognize these ribosomes. For this specificity, the factor would have to either bind TUB2 RNA or the beginning to the nascent chain. The yeast three-hybrid technique can be used to find proteins that bind TUB2 mRNA. This system (Sengupta et al. 1996) takes advantage of a coat protein from bacteriophage MS2 that recognizes a 21-nt RNA stem loop. These 21 nucleotides can be fused to TUB2 to make a fusion RNA. The MS2 coat protein is fused to a DNA binding domain with its corresponding DNA binding site placed upstream of a reporter gene. A library of proteins fused to a transcriptional activation domain is then screened. If a protein from the library binds TUB2 RNA, it should activate transcription of the reporter gene. Isolating TUB2 RNA binding proteins could lead to further knowledge of the genes involved in regulating the translation and folding of Tub2p.

Plp1p could bind other proteins that contribute to its function. To ask what proteins Plp1p interacts with, I would like to immunoprecipitate Plp1p and determine
what proteins are bound. This is a tricky endeavor because I have tried to tag Plp1p in the past. The protein is small and any tag larger than 3HAs abolishes Plp1p’s function. To avoid this, I tried to do a yeast-2-hybrid with Plp1p but did not pull out any true interactions. We may have to make an antibody to Plp1p. Direct tests to ask if Plp1p binds other tagged proteins may be fruitful— for example, asking if Plp1p binds tagged components of different polysome-associated chaperones. Plp1p does not seem to directly bind the cytosolic chaperonin through coimmunoprecipitation with a tagged subunit.

**Other proteins involved in promoting functional β-tubulin**

A major difference between pac10Δ plp1Δ and pac10Δ plp1Δ cin5Δ is that the levels of β-tubulin in the latter are not upregulated. Furthermore, pac10Δ plp1Δ cin5Δ cells have a somewhat compromised spindle but do seem to have the minimal level of tubulin for microtubule function. Thus, this strain could be a useful tool to find other proteins needed for β-tubulin regulation.

I am proposing a screen to find proteins involved in β-tubulin expression, folding, and heterodimer formation. pac10Δ plp1Δ cin5Δ cells can tolerate excess β-tubulin, even from high induction off of a galactose inducible promoter (data not shown). Thus, a single plasmid copy of β-tubulin in this cell should be well tolerated. I would like to screen for mutations or overexpressed genes that make the plasmid containing the β-tubulin gene essential in pac10Δ plp1Δ cin5Δ cells. Mutant genes could be involved in folding or in producing heterodimerizable β-tubulin polypeptides, or involved in the regulation of β-tubulin expression. Thus, in their absence, less functional β-tubulin would
be present, pushing the cell below the threshold of β-tubulin required for viability. The overexpression screen may uncover negative regulators of β-tubulin transcription, folding, or heterodimerization. Conversely, increasing the amount of a negative regulator could cause an increase in its activity, thus preventing normal β-tubulin expression or function.

**Regulation of Cin5p-dependent transcription of β-tubulin**

Cin5p binds to the promoter of *TUB2* and may be the transcription factor responsible for increasing β-tubulin RNA levels in *pac10Δ plp1Δ* (Chapter 3). However, Cin5p also binds the *TUB2* promoter in wild type cells that do not have enhanced levels of *TUB2* RNA due to Cin5p, suggesting that another factor is involved in regulating *TUB2* transcription in *pac10Δ plp1Δ* that may interact with Cin5p. Since Cin5p is not altered by a modification in *pac10Δ plp1Δ* cells (data not shown), a cofactor could be involved in regulating its activity. To identify a cofactor, an immunoprecipitation of Cin5p could be done comparing bands present in *pac10Δ plp1Δ* and not present in wild type or *plp1Δ* strains. These bands could then be analyzed by mass spectroscopy. Of course, there is the risk that the cofactor is always present, but is modified differentially in the distinct cell types and would not be identified by this assay. However, isolating proteins bound to Cin5p would still be interesting and may lead to other genes involved in regulating *TUB2* expression.

One of the most interesting questions that remains is the identity of the signal that upregulates β-tubulin transcription in *pac10Δ plp1Δ* cells. The two main differences between *pac10Δ plp1Δ* and wild type cells are that the levels of heterodimer are
substantially decreased and that folded β-tubulin is the limiting factor. My current model is that low tubulin heterodimer levels cause an increase of β-tubulin, when this increase can be tolerated. The β-tubulin upregulation then allows more α-tubulin to be stabilized, increasing heterodimer levels. To test this model, I am making a strain with β-tubulin levels that are substantially decreased, but that have a normal folding pathway. I will then ask if there is a Cin5p dependent upregulation of β-tubulin in this strain. To decrease β-tubulin levels, I am placing the sole copy of β-tubulin under control of the TUB3 promoter. Tub3p only makes up about 15% of the amount of α-tubulin in this cell, so β-tubulin levels should be decreased to 15%. To determine if there is a Cin5p dependent transcriptional upregulation of β-tubulin, I am placing a reporter under control of the β-tubulin promoter and will measure the amount of RNA from this reporter gene in the presence and absence of Cin5p. If the reporter is upregulated, I can conclude that the cell increases β-tubulin in response to deficient levels of β-tubulin or tubulin heterodimers. If upregulation does not occur in this strain, poor β-tubulin folding may signal the increased β-tubulin RNA levels. Further experiments would be needed to determine if folding was involved in the upregulation.

Other genes are likely to be involved in the signal in pac10Δ plp1Δ cells to upregulate β-tubulins expression. I have identified Cin5p as part of this signal. Cin5p may be the downstream target of other proteins in a response to the amount of tubulin present in the cell, since it acts in the nucleus and tubulin folding occurs in the cytoplasm. To identify other proteins involved, I am proposing a screen using a reporter assay. In this screen, I would look for proteins that when overexpressed or deleted would cause an increase in β-tubulin expression. However, instead of using β-tubulin, I would substitute
a reporter gene under control of the TUB2 promoter and place TUB2 somewhere else in the genome. This reporter would have to vary by expression levels. I would first determine if LacZ would be a good reporter by asking if there is a difference in blue colony color in pac10Δ cells where TUB2 levels are decreased versus in pac10Δ plp1Δ where TUB2 levels are increased with a difference of about 50% between the two strains (Chapter 3). If there is an easily noticeable difference between the two, I would then perform the screen in a pac10Δ strain, looking for bluer colonies, or more β-galactosidase. As a positive control, I know that plp1Δ should create a bluer color. This screen may identify other proteins involved in the upregulation of β-tubulin RNA.

**Normal microtubule function with low tubulin levels**

I have shown that pac10Δ plp1Δ cin5Δ cells have tubulin heterodimer levels down to 20% that of wild type. The low amount of heterodimer is sufficient to allow the cell to grow at normal rates; however, there are other phenotypes. For example, the spindles are much shorter and there are nuclear positioning defects associated with more cells in G2/M phase of the cell cycle. These phenotypes suggest that the low tubulin levels affect normal microtubule function.

Another interesting question is whether microtubule dynamics are normal in this cell. The concentration of tubulin is significantly altered compared to a wild type cell and this may be below the critical concentration of tubulin needed for normal microtubule dynamics. To test this, I have established a collaboration with the Bloom lab to monitor microtubule dynamics using live cell microscopy.
The Bloom and Salmon labs have previously shown that \textit{S. cerevisiae} microtubules are dynamic during metaphase using a technique called fluorescence recovery after photobleaching (FRAP) (Maddox et al. 2000). A metaphase spindle in a cell expressing tubulin tagged with GFP is laser photobleached and then observed for recovery using time-lapse microscopy. Maddox et al found that 63\% of the bleached fluorescence recovers with an average of 53 seconds in a wild type cell. The 63\% correlates with the number of kinetochore microtubules (16/24) versus interpolar microtubules (8/24). I will measure the half-life of recovery in \textit{pac10Δ plp1Δ cin5Δ} cells compared to wild type. Either result- a change in microtubule dynamics or no change- would be interesting. If microtubule dynamics decreases, the result suggests that the cells have levels of tubulin below the critical concentration for normal dynamics. If microtubule dynamics remain the same, this suggests that a wild type cell has levels of tubulin above the critical concentration.
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Appendix I

Tubulin RNA regulation through overexpression of $\alpha$-tubulin
INTRODUCTION

The yeast *S. cerevisiae* is sensitive to imbalances of tubulin polypeptides that lead to an increased ratio of β-tubulin to α-tubulin. Perturbations that lead to an excess of free β-tubulin can cause severe microtubule defects, even cell death. For example, deletion of the minor α-tubulin gene, *TUB3* results in microtubule phenotypes such as an enhancement of chromosome missegregation (SCHATZ et al. 1986). High-level overexpression of β-tubulin leads to microtubule depolymerization and subsequent cell death (BURKE et al. 1989; WEINSTEIN and SOLOMON 1990). Work described in this thesis demonstrates that regulatory mechanisms exist at the level of the polypeptide and RNA to maintain tubulin levels.

We have shown that Pac10p, a protein with a known molecular role at the level of the tubulin polypeptide, may also have a role at the level of the tubulin RNA. The absence of *PAC10* results in decreased tubulin RNA levels ((SMITH 2000) and Chapter 3). Pac10p is a member of the GimC/PFD complex that is thought to help fold tubulin polypeptides along with the chaperonin (GEISSLER et al. 1998; VAINBERG et al. 1998). The absence of this gene leads to decreased levels of both α- and β-tubulin polypeptides, but α-tubulin levels are decreased more, changing the ratio of α- to β-tubulin (ALVAREZ et al. 1998; GEISSLER et al. 1998). Adelle Smith and Margaret Magendantz have shown that the RNA levels are also decreased in *pac10Δ* cells, although to the same extent.

This finding has interesting parallels to work done in animal cells. Tubulin RNA may be regulated by a feedback loop termed autoregulation. Tubulin levels are hypothesized to decrease when unpolymerized tubulin is present in the cell. Addition of
the microtubule depolymerizing drug colchicine, or injection of free tubulin polypeptides decreases the amount of both α- and β-tubulin messages (Ben-Ze'ev et al. 1979; Cleveland 1983). Cleveland and colleagues have shown that the downregulation of β-tubulin messages is posttranscriptional and cotranslational, needing the first four amino acids at the N-terminus (Pachter et al. 1987; Yen et al. 1988). They hypothesize that a ribonuclease may recognize the four amino acids and degrade the message as the ribosomes are translocating. However, the factor that recognizes the β-tubulin message has not been identified.

Our lab has shown that a different sort of autoregulation may also occur in S. cerevisiae. In the absence of the prefoldin component Pac10p, the tubulin folding pathway is compromised, leading to more unfolded tubulin (Chapter 3). It is possible that the decrease of tubulin RNA levels in pac10Δ cells may be a feedback response to an increase in unfolded α-tubulin polypeptides. However, we are currently unsure why there would be a response to unfolded α-tubulin. We are addressing one aspect of this question by asking if increasing unfolded α-tubulin causes a decrease in tubulin levels.
RESULTS

I would like to test directly if increasing tubulin polypeptides can result in decreased tubulin RNA levels. I overexpressed α-tubulin by using a plasmid copy of the minor α-tubulin gene TUB3 under the control of a galactose inducible promoter. I then used the RNAse protection assay to determine if TUB1 and TUB2 message levels were changed. Surprisingly, I found that the overexpression of Tub3p results in decreased TUB1 and TUB2 levels to 68 ±10% and 75 ± 6% respectively. (Figure A1-1) This level was similar to the decrease found in pac10Δ cells (Chapter 3).
Figure A1-1. *TUB1* and *TUB2* RNA levels decrease when *TUB3* is overexpressed.
Total RNA was collected after 6 hours of growth in galactose media from wild type cells with a control vector (YCpGAL) and wild type cells with a vector containing GAL promoted *TUB3*. The levels of *TUB1, TUB2,* and *TCM1* were analyzed by RNAse protection assays. Mean values (+/- standard deviation) of levels of tubulin messages were normalized to TCM1 RNA; the wildtype values are defined as 1.0. Data presented are the average of 6 experiments.
DISCUSSION

Interestingly, the overexpression of one of the tubulin genes, TUB3 can regulate the message levels of the other two tubulin genes, TUB1 and TUB2. By overexpressing TUB3, I was hoping to saturate the folding pathway to produce an excess of unfolded tubulin polypeptides and perhaps trigger a response similar to a pac10Δ cell. We do know that overexpression of α-tubulin leads to aggregated α-tubulin even when excess β-tubulin is present (ABRUZZI et al. 2002). Thus, the aggregated α-tubulin may represent an excess of unfolded α-tubulin in the cell, and it is likely that overexpressing α-tubulin does saturate the folding pathway.

We are currently unsure whether the decrease in tubulin RNA levels is due to a difference in message stability. As stated in Chapter 3, I performed a message decay experiment, comparing pac10Δ and wild type. However, the differences in levels of tubulin RNA are so small, that it may be beyond detection using this assay. I also tried promoter swapping experiments, but had many technical difficulties.

Since α-tubulin folding seems to require the GimC/PFD complex more than β-tubulin folding (see Chapter 2), there is a possibility that unfolded α-tubulin in pac10Δ triggers the downregulation of both α- and β-tubulin messages. We cannot test β-tubulin overexpression because it is toxic to the cell. However, from Chapter 3, increasing unfolded β-tubulin, for example in plp1Δ cells, does not cause a difference in tubulin RNA levels. Severe amounts of unfolded β-tubulin, causing low heterodimer levels, result in an upregulation of β-tubulin. Thus, it is likely that the decrease in tubulin RNA levels is a response to unfolded α-tubulin and not β-tubulin.
Interestingly, excess *TUB3* down regulates both *TUB1* and *TUB2*, showing that the recognition of the RNA is not specific to the exact tubulin gene that is overexpressed. We are currently unsure why the cell would decrease both α- and β-tubulin messages in response to excess α-tubulin. Perhaps the cell wants to allow more time to fold the tubulin polypeptides already translated.
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Appendix II

Interactions between *GRR1* and *PLP1*. 
INTRODUCTION

Through various screens, our lab has identified proteins that have known molecular roles at the level of the polypeptide but that are also involved in tubulin RNA regulation. A deletion of the gene encoding Pac10p, one of the GimC/PFD complex members, lowers tubulin RNA levels to 70-80% of wild type ((SMITH 2000), Chapter 3, and Appendix I). Similarly, deletion of the gene GRR1 also results in decreased tubulin RNA levels to 70-80% of wild type ((SMITH 2000) and Figure A2-2). Grr1p has a known molecular function as an F-box specificity factor for the SCF complex, targeting proteins for ubiquitination and subsequent degradation (SKOWYRA et al. 1997). Grr1p is involved in many processes including glucose repression, divalent cation transport, bud emergence, and cell cycle progression (BAILEY and WOODWORD 1984; BARRAL et al. 1995; CONKLIN et al. 1993; FLICK and JOHNSTON 1991; VALLIER et al. 1994). However, Grr1p’s involvement in RNA regulation has not been reported.

In our lab, GRR1 was isolated in a screen looking for mutations that are lethal in combination with a mutation in PAC10 (SMITH 2000). Both grr1Δ and pac10Δ result in lower tubulin RNA and polypeptide levels. The deletion of PAC10 also results in an altered ratio of α- to β-tubulin, producing excess β-tubulin. The deletion of GRR1 decreases the levels of tubulin equivalently, maintaining the ratio of α-to β-tubulin ((SMITH 2000) and Figure A2-3). The inviability of pac10Δ grr1Δ cells is likely due to an excess of β-tubulin compared to α-tubulin; a plasmid containing α-tubulin can rescue the lethality (my unpublished result).

The genes involved in the regulation of tubulin RNA levels in pac10Δ and grr1Δ are currently unknown. The synthetic lethality of pac10Δ and grr1Δ suggest that the
pathways are additive, and so may be independent. In the following section, I describe results analyzing the role of Plp1p in the pathway to decrease tubulin levels in grrIΔ.
RESULTS

*ppl*Δ suppresses the benomyl sensitivity of *grr1*Δ

A deletion of *PLP1* can suppress the benomyl sensitivity of *pac10*Δ at low benomyl concentrations. I decided to test if *plp1*Δ could also rescue the benomyl sensitivity of *grr1*Δ. Saturated cultures were serially diluted and plated to media containing varying concentrations of benomyl. At low benomyl concentrations, *plp1*Δ suppresses the benomyl sensitivity of *grr1*Δ, although not as effectively as the suppression of the sensitivity of *pac10*Δ by *plp1*Δ (Figure A2-1).

Tubulin RNA levels are increased in *grr1*Δ *plp1*Δ

Tubulin RNA levels were measured in wild type, *plp1*Δ, *grr1*Δ, and *grr1*Δ *plp1*Δ by the RNase protection assay. The levels of *TUB1* and *TUB2* were normalized to an internal control, carboxypeptidase Y (CPY). *grr1*Δ cells have reduced levels of tubulin RNA; α-tubulin at 74 ± 11% and β-tubulin levels at 79 ± 9.2% (Smith 2000 and Figure A2-2). As previously mentioned in Chapter 3, *plp1*Δ levels were equivalent to wild type. *grr1*Δ *plp1*Δ cells have tubulin RNA levels increased from *grr1*Δ cells; α-tubulin levels are 109 ± 9% and β-tubulin levels are 118 ± 10% (Figure A2-2). Thus, a deletion of *PLP1* rescues the reduced levels of tubulin RNA in *grr1*Δ cells.

Tubulin polypeptide levels are slightly increased in *grr1*Δ *plp1*Δ cells

Tubulin polypeptide levels were measured in wild type, *plp1*Δ, *grr1*Δ, and *grr1*Δ *plp1*Δ by immunoblotting. Levels of Tub1p and Tub2p were normalized to the internal control, CPY. *grr1*Δ cells have decreased levels of α- and β-tubulin polypeptides to 59 ± 11% and 68 ± 4% respectively (Smith 2000 and Figure A2-3). *plp1*Δ polypeptides are
Figure A2-1. Benomyl supersensitivity is reduced in *grr1Δ plp1Δ* mutants.
Saturated yeast cultures were diluted and plated to rich media (YPD + DMSO) and media containing 10μg/ml of benomyl.
Figure A2-2. Tubulin RNA levels in grr1Δ plp1Δ mutants are increased.

*TUB1*, TUB2, and *CPY* RNA levels were measured using RNAsase protection assay. Total RNA was collected in wild type, plp1Δ, grr1Δ, and grr1Δ plp1Δ. Mean values of levels of tubulin messages were normalized to *CPY* RNA; the wild type values are defined as 1.0. Data presented are the average of 3 experiments.
Figure A2-3. Protein levels are increased in the grr1Δ plp1Δ double mutants.
Excerpts from wild type, plp1Δ, grr1Δ, and grr1Δ plp1Δ were analyzed for levels of Tub1p, Tub2p, and CPY by immunoblotting. Mean values of tubulin polypeptides are normalized to CPY. Wild type values are defined as 1.0. Data presented are the averages of at least 3 experiments.
similar to wild type levels. \textit{grr1Δ plp1Δ} mutants have levels of tubulin polypeptides in between that of wild type and \textit{grr1Δ} cells; α-tubulin levels are 73 ± 6\% and β-tubulin levels are 80 ± 6\% (Figure A2-3). Thus, the polypeptide levels in \textit{grr1Δ plp1Δ} cells are somewhat reduced compared to the RNA levels, but this is also similar to \textit{grr1Δ} cells.

\textit{plp1Δ does not suppress all of the phenotypes of grr1Δ}

grr1Δ cells grow slowly compared to wild type cells. The growth defect is likely due to the inability of \textit{grr1Δ} cells to properly degrade the G1 cyclins Cln1p and Cln2p, increasing the amount of time in G1 phase of the cell cycle (BARRAL et al. 1995; SKOWYRA et al. 1997). The cells also have an elongated morphology due to a perturbed ability to switch from apical to isotropic growth (JAQUENAUD et al. 1998). As expected, a deletion of \textit{PLP1} does not rescue the slow growth phenotype (Figure A2-4) or morphology of \textit{grr1Δ} cells (data not shown). Thus, the deletion of \textit{PLP1} can only specifically rescue some of the tubulin related defects of \textit{grr1Δ} cells.

However, \textit{plp1Δ} does not suppress all of the tubulin-associated phenotypes of \textit{grr1Δ}. Interestingly, \textit{grr1Δ} cells are sensitive to overexpression of α-tubulin and do not grow with high-level induction of α-tubulin ((SMITH 2000) and Figure A2-5). A deletion of \textit{PLP1} does not rescue the sensitivity to excess α-tubulin (Figure A2-5).

\textbf{Plp1p levels are increased in grr1Δ cells}

The molecular role of Grr1p is to target proteins for ubiquitination and subsequent degradation. Thus, if Plp1p is a target of Grr1p, Plp1p levels may be increased in a \textit{grr1Δ} cell. To determine if Plp1p levels were increased in \textit{grr1Δ}, I tagged Plp1p in a wild type strain and a \textit{grr1Δ} strain and then measured levels of Plp1p by immunoblot. I found that the levels of Plp1p were 2.85 ± .77 times higher in \textit{grr1Δ} than a wild type cell (Figure
Figure A2-4. *plp1Δ* does not rescue the slow growth phenotype of *grr1Δ*.
Cell cultures of wild type, *grr1Δ*, *grr1Δ plp1Δ* were diluted to $0.5 \times 10^7$ in rich media at time zero and counted every three hours for twelve hours.
Figure A2-5. *plp1Δ* does not rescue the sensitivity of *grr1Δ* cells to excess α−tubulin.

*grr1Δ* and *grr1Δ plp1Δ* cells containing a control plasmid or a plasmid containing *TUB1* under a galactose inducible promoter were grown overnight in raffinose and then plated to galactose.
A2-6). Plp1p may be a target of Grr1p, but more ubiquitination experiments would be needed to support this conclusion. The data show that either directly or indirectly, the levels of Plp1p are different in the absence of Grr1p.

If increased levels of Plp1p result in the microtubule phenotypes of grr1Δ, overexpressing Plp1p in pac10Δ cells should cause lethality. I plated wild type and pac10Δ cells with a plasmid containing a galactose inducible PLP1 gene to plates containing both glucose and galactose (Figure A2-7). Overexpressing Plp1p did not cause lethality in a pac10Δ cell. Thus, although Plp1p levels are increased in grr1Δ, this increase is not sufficient to cause the microtubule phenotypes associated with grr1Δ in wild type cells.
Figure A2-6. Plp1p levels are increased in *grrlΔ* cells.

Extracts from wild type HA-tagged Plp1p cells and *grrlΔ* HA-tagged Plp1p cells were analyzed for levels of HA-Plp1p by immunoblotting. Mean values (+/- standard deviation) of the Ha-Plp1p. Polypeptides were normalized to CPY. The wildtype values are defined as 1.0.
Figure A2-7. Overexpression of Plp1p does not kill pac10 Δ.
Wild type and pac10Δ cells containing either a control vector or a vector containing PLP1 under the galactose inducible promoter were plated onto glucose and galactose plates.
DISCUSSION

A deletion of PLP1 can rescue some of the microtubule phenotypes of grr1Δ cells. The benomyl sensitivity of grr1Δ is suppressed by plp1Δ (Figure A2-1). This suppression is likely due to the increased levels of tubulin RNA and polypeptides in grr1Δ plp1Δ compared to grr1Δ (Figure A2-2 and A2-3). Thus, the absence of PLP1 rescues the levels of tubulin RNA in grr1Δ, suppressing the microtubule phenotypes.

Tubulin RNA levels in grr1Δ plp1Δ are completely rescued possibly beyond wild type levels. Similarly, the levels of β-tubulin in pac10Δ plp1Δ are also above wild type levels (Chapter 3). However, the polypeptide levels in grr1Δ plp1Δ are only somewhat rescued. Since grr1Δ cells also have lower polypeptide levels than RNA levels, the difference at the level of the polypeptide is not a contribution of the PLP1 deletion but is likely due to the deletion of GRR1. The rescue of tubulin RNA levels beyond wild type suggests that plp1Δ may be a bypass suppressor of the tubulin phenotypes of grr1Δ. Similarly, the increased β-tubulin levels in pac10Δ plp1Δ are likely due to an upregulation pathway responding to decreased heterodimer levels (Chapter 3). Thus, Plp1p may not directly regulate the tubulin RNA levels in grr1Δ; however, the absence of Plp1p could result in a response that leads to upregulation of tubulin RNA.

Not all of the tubulin related phenotypes of grr1Δ are rescued by plp1Δ. A deletion of PLP1 does not rescue the sensitivity of grr1Δ cells to excess α-tubulin. grr1Δ cells are the first cell type isolated that are sensitive to an excess of α-tubulin (SMITH 2000). I am intrigued by this phenotype, but currently do not understand what causes this sensitivity.
Interestingly, Plp1p may be a target of Grr1p for ubiquitination and degradation. The absence of *GRR1* leads to enhanced levels of Plp1p, suggesting that Grr1p may be needed as the specificity factor for the SCF complex to ubiquitinate Plp1p. However, an increase of Plp1p in *pac10Δ* cells does not cause lethality, and is thus not the target that causes the tubulin phenotypes in *grr1Δ*. Perhaps increasing levels of Plp1p in combination with increased levels of another protein or complex results in the microtubule phenotypes.

I looked for other genes involved in the *grr1Δ* tubulin phenotypes. I screened for other proteins that when overexpressed in combination with overexpressing Plp1p would be lethal in *pac10Δ* cells. Since *pac10Δ* and *grr1Δ* cells are synthetically lethal and Grr1p targets proteins for ubiquitination, the thought was that overexpressing Plp1p in combination with another protein would cause lethality. However, no other true candidates were found.

There is also the possibility that Plp1p is increased in *grr1Δ* cells but does not act with any other target to cause the microtubule phenotypes. In chapter 3, I described an upregulation of β-tubulin RNA levels in a *pac10Δ plp1Δ* cell through a feedback pathway. Similarly, deleting *PLP1* in *grr1Δ* cells could cause a feedback pathway, increasing tubulin RNA levels.

**Summary**

From these results, Plp1p may be a ubiquitination target of Grr1p, but upregulation of Plp1p is not sufficient to cause for the microtubule phenotypes associated with *grr1Δ*. A deletion of *plp1Δ* rescues the RNA levels of *grr1Δ* but this rescue may be
a result of indirect regulatory response. The issues that remain include finding the genes involved in the microtubule phenotypes of \textit{grr1}\Delta and understanding the response that causes an increase in tubulin levels in \textit{grr1}\Delta \textit{plp1}\Delta.
LITERATURE CITED


