CHAPTER

Structure–Function Analysis of Yeast Tubulin

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CHAPTER OUTLINE

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Abstract

Microtubules play essential roles in a wide variety of cellular processes including cell division, motility, and vesicular transport. Microtubule function depends on the polymerization dynamics of tubulin and specific interactions between tubulin and diverse microtubule-associated proteins. To date, investigation of the structural and functional properties of tubulin and tubulin mutants has been limited by the inability to obtain functional protein from overexpression systems, and by the heterogeneous mixture of tubulin isotypes typically isolated from higher eukaryotes. The budding yeast, *Saccharomyces cerevisiae*, has emerged as a leading system for tubulin structure–function analysis. Yeast cells encode a single beta-tubulin gene and can be engineered to express just one of two alpha isotypes. Moreover, yeast allows site-directed modification of tubulin genes at the endogenous loci expressed under the native promoter and regulatory elements. These advantageous features provide a homogeneous and controlled environment for analysis of the functional consequences of specific mutations. Here, we present the techniques to generate site-specific tubulin mutations in diploid and haploid cells, assess the ability of the mutated protein to support cell viability, measure overall microtubule stability, and define changes in the specific parameters of microtubule dynamic instability. We also outline strategies to determine whether mutations disrupt interactions with microtubule-associated proteins. Microtubule-based functions in yeast are well defined, which allows the observed changes in microtubule properties to be related to the role of microtubules in specific cellular processes.

INTRODUCTION

Microtubules are cytoskeletal polymers composed of αβ-tubulin heterodimers that exhibit highly dynamic growth behavior, which allows for rapid rearrangement of microtubule networks to support diverse cellular tasks (Desai & Mitchison, 1997). Specific mutations in tubulin genes result in a spectrum of congenital neurological disorders in humans (Cederquist et al., 2012; Jaglin et al., 2009; Tischfield, Cederquist, Gupta, & Engle, 2011). Moreover, the pathology of Alzheimer's and Parkinson's disease has been at least in part attributed to microtubule dysfunction (Brandt, Hundelt, & Shahani, 2005; Lei et al., 2010). The organization and dynamics of microtubule polymer are also essential for proper function of the mitotic spindle and fidelity of chromosome segregation. Indeed, small molecules that interfere with microtubule dynamics are a powerful class of antimitotic agents and represent a proven strategy in the treatment of various cancers (Jordan, 2002). Therefore, understanding the structural and functional properties underlying the regulation of microtubule dynamics is of great interest for human health.

Studies directed toward elucidating the structure–function relationship of tubulin and microtubules have been challenging. The folding of tubulin polypeptides into native heterodimers requires interactions with chaperones and protein cofactors (Lewis, Tian,

Vainberg, & Cowan, 1996), which has precluded expression in bacterial systems. Moreover, unbalanced levels of α- and β-subunits produce toxic effects in eukaryotes (Katz, Weinstein, & Solomon, 1990), while overexpression hinders folding and produces nonfunctional aggregates (M. L. Gupta, unpublished results). Consequently, the large-scale expression of exogenous tubulin has not been successful to date.

Conventionally, *in vitro* assays addressing microtubule function and regulation by microtubule-associated proteins have largely utilized tubulin purified from mammalian brain tissue. This method yields large amounts of protein; however, the material obtained is not homogeneous as the majority of higher eukaryotes express multiple α- and β-tubulin isotypes. Additionally, site-specific mutagenesis of tubulin expressed from the native locus is not possible or feasible in most higher eukaryotes. Thus, purified brain tubulin is a heterogeneous mixture that confounds the analysis of particular isotypes or mutations.

The budding yeast, *Saccharomyces cerevisiae*, is a powerful tool for systematic analysis of the functional consequences of tubulin mutations. Budding yeast encodes a single β-tubulin, *TUB2* (Neff, Thomas, Grisafi, & Botstein, 1983), and two α-tubulin isotypes, *TUB1* and *TUB3* (Schatz, Pillus, Grisafi, Solomon, & Botstein, 1986), which all share ~75% sequence conservation with human homologues. Furthermore, yeast can tolerate deletion of *TUB3*, which can clarify *in vivo* readouts by eliminating influence of multiple isotypes and confer a source of homogeneous tubulin for *in vitro* analyses (Bode, Gupta, Suprenant, & Himes, 2003). In budding yeast, site-specific mutations can be inserted into the endogenous locus. Mutant proteins are expressed under the control of the native promoter and regulatory elements, overcoming challenges and complexity of mammalian systems. This approach eliminates difficulties associated with exogenous expression and overexpression that may lead to nonphysiological results.

Budding yeast has been utilized for over 2 decades to study structural and functional properties of tubulin and microtubules. Alanine-scanning mutagenesis of α- and β-tubulin uncovered clusters of charged amino acids crucial for structural and functional integrity of tubulin and microtubules (Reijo, Cooper, Beagle, & Huffaker, 1994; Richards et al., 2000). Directed mutagenesis demonstrated the relationship between GTP hydrolysis and dynamic instability *in vivo* and *in vitro* (Anders & Botstein, 2001; Davis, Sage, Dougherty, & Farrell, 1994) and revealed the contributions of microtubule dynamics to assembly and positioning of the mitotic spindle (Gupta et al., 2002; Huang & Huffaker, 2006). Moreover, studies of yeast tubulin have been important in defining binding sites of anticancer therapeutics (Gupta, Bode, Georg, & Himes, 2003) and for discriminating among proposed models for their interactions with microtubules (Entwistle et al., 2012). Recently, structure–function analysis of yeast tubulin has been key for elucidating the molecular etiology of a class of human neurological disorders that result from missense mutations in distinct tubulin isotypes (Cederquist et al., 2012; Jaglin et al., 2009; Tischfield et al., 2010).

Here, we present techniques to study the structural and functional consequences of tubulin mutations using the budding yeast *S. cerevisiae*. This genetically tractable system provides a well-controlled environment to relate the consequences of tubulin

mutations on microtubule dynamics and interactions with specific regulatory proteins, to distinct microtubule-dependent processes.

22.1 REAGENTS AND EQUIPMENT

General equipment

- Temperature-controlled waterbath
- 30 °C incubator and shaker
- Thermocycler
- Tabletop centrifuge
- Tabletop microfuge
- Spectrophotometer
- Dissection microscope with micromanipulator
- Replica plate apparatus with sterile velvet towels
- Fluorescence microscope equipped with a 63 × high N.A. objective, CCD camera
- Microscope slides and coverglass
- SDS-PAGE/western blot apparatus and supplies
- Multichannel pipette or "frogger"
- Sterile wooden toothpicks
- 15-ml Conical tubes
- Culture tubes and flasks
- PCR tubes
- 96-Well plates

General reagents

- Yeast-extract peptone dextrose (YPD) media (1% yeast extract, 2% peptone, 2% glucose)
- YPD plates (YPD, 2% agar)
- Synthetic complete (SC) media (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.2% appropriate amino acid mix (Sunshine Science Products, CA))
- SC plates (SC, 2% agar)
- Sporulation plates (1% potassium acetate, 0.1% Bacto-yeast extract, 0.05% glucose, 2% agar)
- YNB plates (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agar)
- For more details on preparing yeast media, see Treco and Lundblad (2001)
- Single-stranded salmon sperm carrier DNA; for preparation protocol, see Becker and Lundblad (2001)
- Adenine, NaOH, KCl, lithium acetate (LiAc), glycerol, polyethylene glycol MW 3350, benomyl, DMSO (Sigma, St. Louis, MO)
- Restriction enzymes (see Table 22.1)
- TE buffer (10 mM Tris, 1 mM EDTA, pH 8)

Table 22.1 Plasmids used for genetic manipulation of tubulin in yeast

Plasmid name	Tubulin gene	Linked prototrophic marker	Antibiotic selection	Digest using	Digestion products (~bp)	Desired digestion product (bp)	Reference
pCS6	*TUB1*	*TRP1*	*Ampicillin*	*Kpn*I, *Sal*I	3500, 4500	4500	Gift from C. Sage and K. Farrell
pCS3	*TUB2*	*URA3*	*Ampicillin*	*Sac*I, *Sph*I, *Pvu*II	2300, 2500, 5000	5000	Sage, Davis, Dougherty, Sullivan, and Farrell (1995)
pMG1	*TUB2–6× His*	*URA3*	*Ampicillin*	*Sac*I, *Sph*I, *Pvu*II	2300, 2500, 5000	5000	Gupta, Bode, Dougherty, Marquez, and Himes (2001)
pAFS125	*GFP–TUB1*	*URA3*	*Ampicillin*	*Stu*I	8500	8500	Straight, Marshall, Sedat, and Murray (1997)
pMG3	*GFP–TUB1*	*LEU2*	*Ampicillin*	*Kas*I	8500	8500	Gupta et al. (2002)
pMG130	*CFP–TUB1*	*URA3*	*Ampicillin*	*Stu*I	8500	8500	Cederquist et al. (2012)

- Agarose
- 10 mM dNTP mix
- Taq polymerase and buffer
- Zymolyase (Zymoresearch, CA)
- Valap (equal weights of Vaseline (petrolatum), lanolin, and paraffin wax)
- Polyclonal anti-GFP antibody (Applied Biological Materials, Canada)

22.2 INTRODUCING TUBULIN MUTATIONS INTO YEAST

The most common method used for structure–function studies of yeast tubulin leverages homologous recombination-mediated gene replacement to introduce desired mutations into tubulin genes (Fig. 22.1). Directed mutagenesis is performed *in vitro* using standard molecular biology techniques. Following gene replacement, the modified tubulin is expressed as a single copy under control of the native promoter and regulatory elements.

Another useful approach is the "plasmid shuffle" in which a plasmid containing mutated tubulin is exchanged in the cell for a plasmid carrying a wild-type copy: described in Sikorski and Boeke (1991). This approach offers increased throughput relative to gene replacement. However, the number of gene copies cannot be tightly controlled using plasmids, which may confound interpretation of cell-based assays. Thus, we focus here on the strategy of direct gene replacement.

22.2.1 Preparation of modified tubulin DNA

Specific mutations are introduced into a plasmid containing the tubulin open reading frame (ORF) flanked by upstream and downstream genomic sequences and a prototrophic marker cassette. The prototrophy gene is inserted several hundred bases downstream from the stop codon and positioned not to interfere with neighboring genes. Site-directed mutagenesis can be accomplished with commercially available systems such as QuikChange (Agilent Technologies). Three commonly used starting plasmids are described in Table 22.1. In pMG1, *TUB2* has been tagged with 6 × His to aid in purification of mutated tubulin (Gupta et al., 2002; Johnson, Ayaz, Huddleston, & Rice, 2011).

> *Note:* It is essential to sequence the entire tubulin gene and promoter region after mutagenesis to ensure additional, unwanted mutations did not occur during *in vitro* manipulation.

To generate linear DNA for yeast transformation, digest >5 μg plasmid as described in Table 22.1, verify complete digestion by agarose gel electrophoresis, and gel-purify the appropriate fragment (Table 22.1).

22.2.2 Transformation of modified tubulin DNA into yeast

Tubulin is an essential gene. Thus, introducing a nonfunctional mutant into haploid cells may select for unwanted suppressor mutations. Thus, unless a mutation has been previously verified as nonlethal, the mutated gene should be transformed into a diploid strain and then sporulated to generate a haploid.

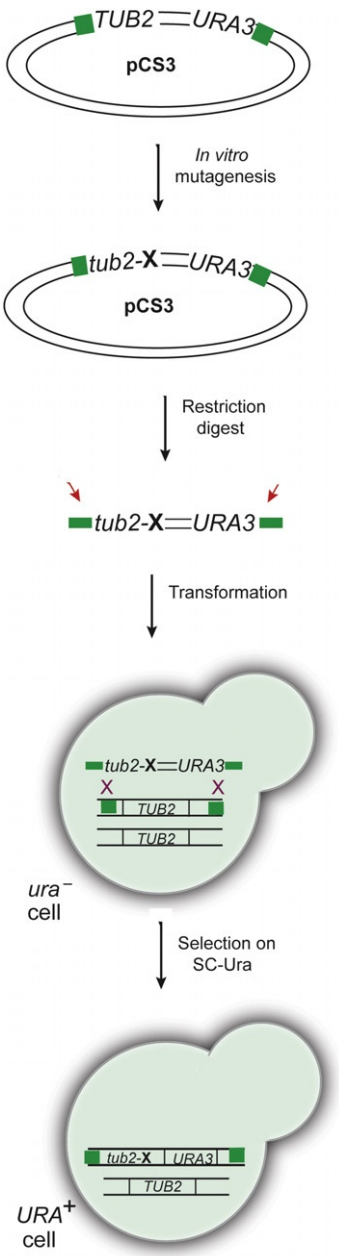

FIGURE 22.1

Schematic of direct gene replacement of yeast β-tubulin. Green shading and red arrows indicate homologous regions flanking β-tubulin ORF exposed by restriction digest. Purple "X" denotes regions of homologous recombination. See text for details.

To transform yeast with the linear DNA fragment containing modified tubulin, grow a 5-ml yeast culture in YPD media overnight at 30 °C with shaking/rotating. The following day, dilute the near-saturated culture 1:50 into fresh media and continue shaking until cells enter log phase; 4 h is usually sufficient with typical growth rates. Use 10–20 ml of culture per transformation. Harvest cells by centrifuging 5 min at $\sim 1000 \times g$ and wash by resuspending in 1/10 original volume of sterile 0.1 M LiAc in TE buffer. Harvest cells again and resuspend in 1/100 original volume of 0.1 M LiAc in TE. For each transformation, combine in a sterile 15-ml tube the purified linear DNA fragment containing the mutated tubulin gene (≥ 0.5 µg in <30 µl) with 10 µl single-stranded salmon sperm carrier DNA. Subsequently, add 100 µl LiAc-treated yeast cells and mix gently. Add 0.7 ml sterile 40% PEG, 0.1 M LiAc in TE and mix well. Incubate for 30 min at 30 °C with shaking. Heat-shock the cells for 20 min in a 42 °C waterbath. Add 10 ml YPD and allow cells to recover for 2 h at 30 °C with shaking. Harvest cells, resuspend in ~0.5 ml YPD, spread onto appropriate selection plates, and incubate at least 2 days at 30 °C to allow colony growth.

When colonies reach sufficient size, potential transformants must be taken through two rounds of single-colony isolation on appropriate selection media to generate clonal strains. To isolate single colonies, streak cells across a fresh plate with a sterile loop or wooden toothpick to obtain sparse colony growth. For long-term storage, isolated clones should be grown in YPD or appropriate selection media, resuspended in sterile 15% glycerol, and stored at −80 °C in cryovials. To revive, transfer a small scraping from the still frozen tube onto a fresh plate using a sterile wooden applicator.

Note: After several freeze-thaw cycles, incubate salmon sperm DNA aliquots at 95 °C for 10 min and cool on ice to enrich single-stranded fragments.

22.2.3 Verification of tubulin mutations in yeast

Although fragment-mediated gene replacement is efficient, it is possible for the prototrophic marker to integrate into the genome independently from the tubulin ORF. Thus, it is essential to verify that the isolated clonal strain carries the desired mutation before proceeding with analyses. Verification can be accomplished by PCR amplification and sequencing of the tubulin locus. Primers should anneal ~50 bases outside the tubulin ORF. Genomic template DNA is readily obtained using commercial products, for example, the Yeast DNA Extraction Kit (Pierce). Colony-based PCR can be a useful alternative when dealing with many samples, although it is less robust than PCR from purified genomic DNA. Using a sterile pipette tip, resuspend a small portion of a fresh colony in 10 µl of 20 mM sterile NaOH in a PCR tube. Incubate in a thermocycler for 10 min at 95 °C. Use 3 µl of the NaOH-treated cells as a template in 25-µl reactions and perform PCR using standard conditions. Sequence the PCR product to verify presence of the mutation. If the mutation is in a diploid strain, the sequencing chromatogram should reflect the presence of both the wild-type and mutated alleles as a double peak at the position corresponding to the mutation (Fig. 22.2).

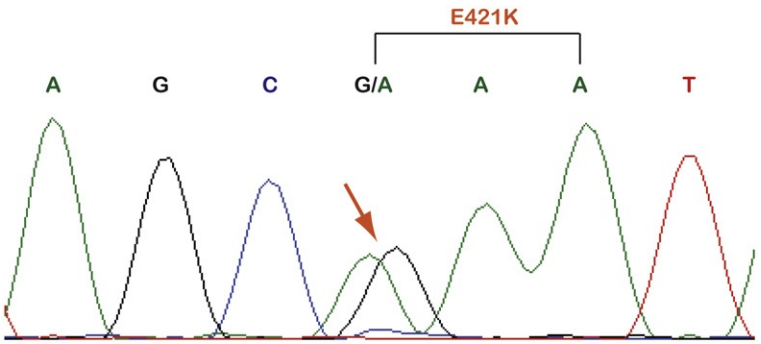

FIGURE 22.2

Sequencing chromatogram from a diploid strain heterozygous for *tub2–E421K* mutant tubulin. Double peak (arrow) indicates the presence of both wild-type and mutant alleles.

Note: We recommend sequencing the full gene of interest to exclude the possibility of additional mutations in the final strain.

22.3 ANALYSIS OF TETRAD VIABILITY

Linking genetic alterations to phenotypic changes through tetrad dissection and subsequent analysis of spores is fundamental for determining the functional consequences of specific mutations. In response to nitrogen starvation and in the presence of a nonfermentable carbon source, diploid yeast cells sporulate. During this process, meiotic segregation gives rise to four haploid daughter cells or spores. Once spores have formed, the intact anucleate mother cell collapses around them forming a mature tetrahedral ascus (Fig. 22.3A). Microdissection and germination of the spores from a single tetrad yields four haploid strains where a given genetic locus originating from the pair of homologous parent chromosomes is represented in two of the four strains.

Mutations that disrupt the function of essential genes, such as *TUB1* and *TUB2*, typically exhibit recessive lethality and produce a characteristic pattern of two viable and two inviable spores (2+/2−). The surviving spores contain the wild-type version of the gene, which is reflected by the phenotype. Alternatively, viability of all four spores is indicative of less harmful mutations and allows analysis of the resultant haploids that contain only the mutated tubulin. In certain cases, spore viability may digress from the classic "all or nothing" 2+/2− hypothesis. With regard to tubulin mutations, low viability can result from severe disruption of meiotic chromosome segregation, which is a microtubule-dependent process. However, the survival of none, one, or three spores can also indicate the presence of independent genomic variations that influence spore viability, for example, suppressor mutations.

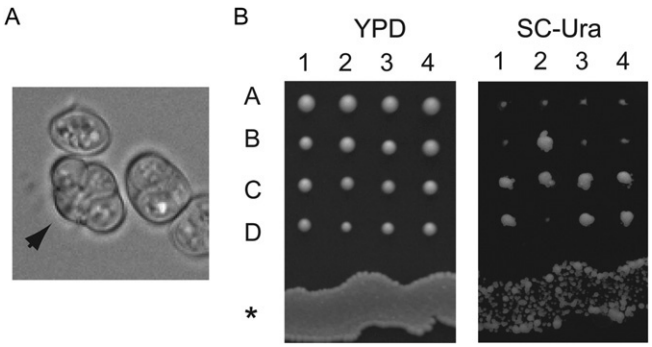

FIGURE 22.3

Yeast tetrad dissection and spore viability analysis. (A) Diamond-shaped tetrad (arrow) consisting of four haploid spores encapsulated by the ascus. (B) Cells heterozygous for a *TUB2* mutation linked to uracil prototrophy (*tub2–URA3*) were sporulated, Zymolyase-treated, and spread across a dissection plate (asterisk). Four tetrads (1–4) were dissected and the individual spores placed in positions A–D. All spores grew on YPD demonstrating the mutation is nonlethal. Replica-plating onto SC-Ura revealed two spores contained the mutated *tub2–URA3* allele (growth) and two contained the wild-type allele (no growth).

Assessing spore viability in independently created diploid mutants or elevated chromosome missegregation rates in the surviving mutant spores can help discriminate between these possibilities.

22.3.1 Sporulation

Grow yeast strains overnight on YPD agar plates and then transfer onto sporulation plates as a patch using a sterile toothpick. Verify sporulation progress after 3–4 days by picking a small amount of cells off the plate and scoring for the presence of asci. The appearance of dyads/triads is usually a positive indicator of sporulation progress. Formation of mature tetrads can take approximately a week depending on the genetic background of the yeast strain. Proceed with dissection when sufficient tetrads have formed.

Note I: Dissection is facilitated when sporulation efficiency is $>20\%$. Dissection is possible, albeit more challenging, when tetrad frequency is lower.
Note II: Tetrads can be stored at 4 °C for an extended period of time.

22.3.2 Microdissection of haploid spores

Each complete ascus encapsulates four spores in what typically appears as a pyramidal or diamond-shaped structure (Fig. 22.3A). To isolate spores, the ascus is digested with Zymolyase. Pick up a small amount of sporulated culture using a toothpick or pipette tip and resuspend in 40 µl of filtered 0.8 M KCl, 25 mM Tris, pH 7.5 buffer, containing 0.4 mg/ml Zymolyase. Incubate 10 min in a 37 °C waterbath and then dilute with 400 µl YPD to inhibit the reaction. Mark a line through the center of

an YPD or appropriate selection plate. While holding the plate nearly vertical, place ~20 µl of Zymolyase-treated cells at the top and allow the drop to run across the surface along the line. Let the plate dry ~10 min, then, using a dissection microscope with attached micromanipulator, move a complete tetrad to the first of four clearly marked locations, separate the spores, and place one spore in each of the three remaining positions (Fig. 22.3B). Repeat until a sufficient number of tetrads have been dissected. Micromanipulators for yeast dissection typically have well-marked grids clearly denoting the four positions for depositing the spores. Each plate can accommodate ~10–20 tetrads. Incubate at 30 °C until the spores form colonies. For a description of the microdissection technique, see Treco and Winston (2008).

Note I: Handle Zymolyase-treated cells gently. Avoid vortexing or vigorous pipetting.
Note II: The concentration of Zymolyase and duration of digestion required may vary by strain, thus optimization is recommended for best results.
Note III: For statistical significance, we recommend assessing the viability of at least 25 tetrads per mutation.

22.3.3 Genotype analysis of haploid spores

To determine the effects of a specific mutation, the phenotype of the haploid spores must be correlated with genotype. In the protocols presented above, the mutated α- or β-tubulin is tightly linked to tryptophan or uracil prototrophy, respectively, and the dissection plate should be replica-plated onto SC-Trp or SC-Ura selection media to determine whether spores contain the mutated tubulin gene.

Common replica-plating devices consist of a ring that is used to spread a sterile velvet towel over a Petri dish-sized circular pedestal. Gently press the dissection plate onto the spread velvet towel. Subsequently, press the selection plate onto the same towel and mark the orientation with respect to the dissection plate. As an example, spores from a heterozygous β-tubulin mutant diploid that grow on SC-Ura plates inherited the mutant allele whereas spores that fail to grow contain the wild-type allele (Fig. 22.3B).

Note: If haploid spores containing the mutant allele are viable, they should be saved for analysis with respect to the wild-type sister spores.

22.3.4 Mating-type analysis

Interpretation of tetrad dissection results depends on the fidelity of meiotic segregation and the reliable separation of spores from complete tetrads. Tetrad dissection can be verified by following 2+/2− segregation of independent heterozygous markers and/or haploid mating type. Mating-type tester strains are prototrophic for common laboratory markers and auxotrophic for an uncommon marker. Upon conjugation with laboratory strains, the auxotrophic markers in both haploid strains are complimented and the resultant diploid can grow on minimal media.

With a single sterile velvet towel, replica-plate the colonies on a tetrad dissection plate onto two YPD plates. Using clean velvets, now replica-plate a lawn of *MAT***a** tester cells onto the first YPD plate. Repeat with a lawn of *MAT*α testers on the second YPD plate. Incubate the YPD plates for 8–24 h at 30 °C to allow conjugation, then replica-plate each onto minimal media (YNB), and grow overnight. Only newly formed diploids with complimented markers will grow on minimal media. Growth after mating with *MAT***a** tester cells indicates that the spore in the corresponding position is *MAT*α haploid. Conversely, growth after mating with *MAT*α testers denotes a *MAT***a** spore. A properly dissected, valid tetrad will generate two *MAT***a** and two *MAT*α spores. If this is not the case, the tetrad should not be considered for analysis.

Note: To prepare mating-type tester lawns, spread ~200 µl saturated culture onto YPD plates, incubate overnight at 30 °C, and store in a sealed container at 4 °C up to several months.

22.4 ASSESSING MICROTUBULE STABILITY BY DRUG SENSITIVITY

Challenging yeast cells with the microtubule destabilizing drug benomyl is a common method to assess changes in microtubule stability *in vivo*. Benomyl supersensitivity is indicative of less stable microtubules while improved resistance implies increased stability. For example, the β-tubulin C354S substitution resulted in robust benomyl resistance. The increased microtubule stability was corroborated by direct visualization of green fluorescent protein (GFP)-labeled microtubule dynamics and confirmed to be independent of any potential changes to benomyl binding and/or regulatory proteins by purifying the mutated tubulin and measuring microtubule dynamics *in vitro* (Gupta et al., 2002). Overall, the benomyl plate assay is representative of changes in microtubule stability *in vivo*.

22.4.1 Benomyl plate assay

Prepare 5-ml overnight cultures in YPD. The following day, dilute 1:50 in fresh media and grow to log phase (4–6 h). Measure optical density of the cultures at 600 nm (OD600) and equalize cell concentration by adjusting volumes with YPD. In a 96-well plate, prepare logarithmic serial dilutions of each culture in YPD. With a multichannel pipette or a "frogger," spot 3 µl of each dilution onto the assay plates. We typically use plates with 0, 4, 6, 9, 12, 15, 18, and 21 µg/ml benomyl. Incubate plates at 24 °C for 72 h. A larger dynamic readout is consistently obtained by conducting this assay at 24 °C rather than 30 °C.

Drug sensitivity is scored as the concentration that inhibits cell growth. Spots where high-density cultures were deposited often have minor but visible growth at high drug concentrations. Growth inhibition becomes more obvious where low-density cultures were spotted and individual colonies are dispersed.

Note I: Preparations of benomyl plates can vary. It is essential to include a nonmutated strain and, if possible, strains with known sensitivity or resistance as controls.
Note II: To prepare benomyl plates, autoclave the YPD and agar with a stir bar, cool to ~65 °C, and then add 10 mg/ml benomyl in DMSO dropwise while stirring.

22.5 DIRECT ANALYSIS OF MICROTUBULE DYNAMICS *IN VIVO*

Live-cell imaging of cells expressing GFP-tagged α-tubulin allows individual astral microtubules to be tracked at high temporal and spatial resolution. This approach determines how microtubule behavior and parameters of dynamic instability are altered by specific tubulin mutations.

22.5.1 Yeast strains for *in vivo* microtubule dynamics analysis

Yeast strains are typically engineered to express an exogenous copy of α-tubulin fused to GFP under the *TUB1* promoter (*pTUB1–GFP–TUB1*) because GFP–tubulin alone does not support cell viability. Two commonly used integrating plasmids, pAFS125 and pMG3, are described in Table 22.1. To integrate *GFP–TUB1*, transform cells with ~1 μg linearized plasmid and plate on appropriate SC-Ura or SC-Leu media. Identify positive transformants by visually inspecting for GFP expression. Generate clonal strains of the positive transformants by two rounds of single-colony isolation on selection media.

Note: If strains destined for *in vivo* microtubule analysis are not auxotrophic for uracil and leucine, they may be made so by disrupting the *URA3* or *LEU2* gene using "marker swap" cassettes (Cross, 1997).

22.5.2 Maintaining comparable *GFP–TUB1* expression levels

Linearized plasmids can potentially integrate as tandem repeats, introducing multiple copies of *pTUB1–GFP–TUB1*. Anecdotal evidence also suggests that increased GFP–Tub1 is correlated with increased microtubule stability. Thus, it is advised to ensure that strains used for the *in vivo* analysis of microtubule dynamics have comparable levels of GFP–Tub1.

A common and practical method of selecting strains with similar *GFP–TUB1* expression is to compare ~10 transformants after streaking for single colonies. Prepare the strains for microscopy as described in the following section. With identical excitation intensity and exposure times, compare the relative fluorescence signal from individual astral microtubules among the strains. Typically, >50% will have microtubules with similar fluorescence intensity, while a minority display noticeably increased intensity. Select strains representative of the group with the lower basal level of GFP–Tub1 fluorescence, which should be comparable to other strains

created by this method. Alternately, a haploid strain containing *GFP–TUB1* can be crossed with haploid strains containing nonlethal tubulin mutations. The resultant diploids are sporulated and dissected to generate control and experimental strains, both containing an identical integration of *GFP–TUB1*. If necessary, GFP–Tub1 levels among strains can be directly compared by western blot as described below.

22.5.3 Preparation of samples for microscopy

Prepare 5-ml overnight cultures at 30 °C by inoculation from fresh plates into SC, or appropriate SC-based selection media, with 0.2 mM adenine. The following day, dilute cultures to OD600 ~0.02 in the same media and grow with shaking at room temperature 4–6 h to reach log phase. Pellet 1.5 ml for 1 min at 13,000 × *g* and resuspend in 30–50 µl of fresh media. Sediment an additional 1.5 ml if a higher cell density is required for imaging. Prepare a fresh sample from the log-phase culture after 20–30 min.

> *Note*: Yeast strains auxotrophic for adenine (*ade*$^-$) require imaging media supplemented with adenine to avoid high background fluorescence.

Prepare microscope slides with agarose pads (Fig. 22.4). Add 1.2% agarose in SC media, boil briefly in a microwave, and pipet 40 µl onto the center of a microscope slide. Let stand for ~2 s and place another slide on top of the agarose and let cool 2 min. Remove the top slide by gently rotating ~60° and slowly sliding it away from the bottom slide. Deposit 1.5 µl of resuspended yeast sample onto the pad and apply a coverglass (22 × 22 × 1 mm). Do not apply strong pressure as this may damage the cells. Seal the coverglass edges with melted valap to prevent drying during imaging.

> *Note I*: To prepare valap, slowly melt ingredients (Vaseline, lanolin, and paraffin 1:1:1 w/w/w) using low setting on a hot plate and mix thoroughly using a disposable heat-resistant container and stirring devise if possible. Pour into small bottles, let cool, and cap for storage.
> *Note II*: Valap is difficult to remove from microscope optics and will degrade image quality. Do not allow valap to get onto microscope optics.
> *Note III*: To apply, dip a wooden- or cotton-tipped applicator into melted valap and spread quickly along the edges of the microscope coverglass.
> *Note IV*: Use caution when heating valap for extended periods. The vapor will resolidify on nearby surfaces.
> *Note V*: Valap is only recommended for time-lapse imaging exceeding 2–3 min.

22.5.4 Microscopy and analysis of microtubule dynamics

Microtubules in live yeast are typically imaged with an automated microscope equipped with a 63× Plan Fluor 1.4 N.A. objective, a filter set optimized for GFP, and a cooled CCD camera. Typically, time-lapse series encompassing eight z-series images with a step-size increment of 0.75 µm are captured at ~8 s intervals. To minimize photobleaching, the light source should be reduced using neutral density filters and the exposure time increased, yet still allow z-series to be acquired in

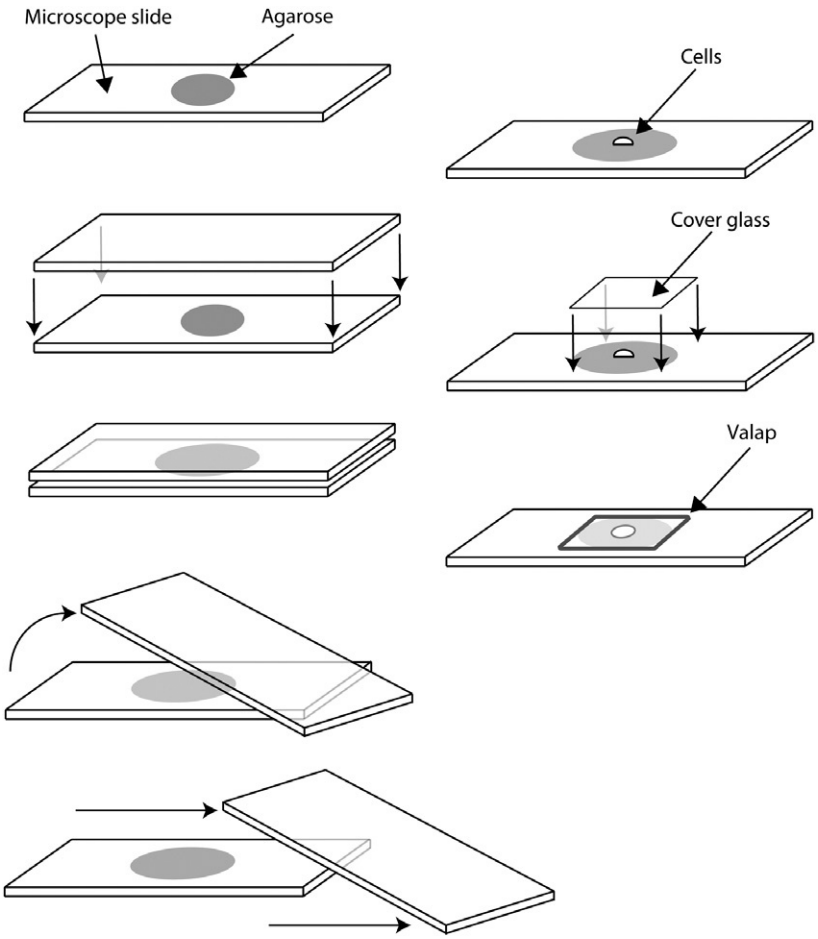

FIGURE 22.4

Preparation of microscope slides with agarose pads. See text for details.

<8 s. With these conditions, time lapses can be captured for as long as 8 min without significant photobleaching. Although precise measurement of microtubule lengths will be somewhat compromised, time lapses can be extended by reducing excitation intensity or exposure time and binning images 2×2.

Microtubule length can be measured using commercial (e.g., Slidebook/Metamorph) or public domain (ImageJ) software. Over subsequent time points, the lengths of individual microtubules are determined from the base of the microtubule, where they anchor to the spindle pole body, to the opposite tip of the microtubule. The measurement should accommodate the three-dimensional length by utilizing the z-focal planes. Care should be taken that z-series encompass the whole cell,

and only microtubules whose entire length remained within focus should be measured. The length of the microtubule throughout the time lapse is typically measured 2–3 times and the average graphed over time to construct a "lifetime history plot."

Using the lifetime history plots, microtubule polymerization and depolymerization events are typically identified as four or more consecutive time points spanning >0.5 μm with a linear regression fit of $R^2 \geq 0.85$. Attenuations, or pauses, are defined as four or more time points during which length change did not exceed ± 0.2 μm. Because the criteria for specific events generally require a sustained behavior, that is, multiple consecutive time points, brief periods for some microtubules will remain unclassified. Catastrophes are scored as transitions from polymerization or paused states into depolymerization, and rescues are scored as transitions from depolymerization into paused or polymerization states. To calculate catastrophe frequency, the number of catastrophe events is divided by the total observable lifetime spent in polymerization and paused states for all analyzed microtubules. Similarly, to determine rescue frequency, the number of rescues is divided by total time spent in depolymerization.

22.6 LOCALIZATION OF MICROTUBULE-ASSOCIATED PROTEINS *IN VIVO*

Budding yeast utilizes a conserved set of microtubule-associated proteins to regulate microtubule behavior during processes such as spindle positioning and spindle elongation. Here, we outline the use of quantitative fluorescence microscopy to determine whether specific mutations alter the interactions of microtubule-associated proteins with microtubules. The technique involves tagging the endogenous copy of a microtubule regulator with yellow fluorescent protein (YFP) and monitoring cyan fluorescent protein (CFP)–Tub1-labeled microtubules in the same cell.

22.6.1 Yeast strains for localization of microtubule-associated proteins

Engineer cells to express CFP–Tub1. A useful plasmid for this purpose is pMG130, which has the CFP variant Cerulean fused to *TUB1* (Table 22.1). Digest, integrate, and screen as described for *GFP–TUB1* above.

Fuse the protein of interest to YFP to allow colocalization with CFP-labeled microtubules. The simplest method utilizes fragment-mediated homologous recombination. A fragment containing YFP and a selection marker is amplified by PCR from a plasmid template. Many plasmids useful for this are commonly available (Janke et al., 2004). The forward and reverse PCR primers are designed to contain >40 bases of homology just prior to and after the stop codon, respectively, of the targeted gene. Potential transformants should be screened for YFP fluorescence and isolated as described for *GFP–TUB1*. Successful fusion with the desired gene can be verified by PCR using primers that anneal inside the gene of interest and YFP.

Note: The functionality of fusion proteins is frequently increased by introducing a soluble flexible linker of 6–8 amino acids between the two proteins.

To increase fluorescence signal, proteins are often tagged with multiple tandem copies of YFP. A straightforward method to accomplish this uses a selectable, integrating plasmid containing tandem copies of YFP. A C-terminal fragment of the targeted gene, selected to contain >50 bp on either side of a restriction site unique to the plasmid, is cloned, without the stop codon, in-frame upstream of the YFPs. Plasmid digestion at the unique restriction site generates a linear fragment that will be targeted to the C-terminus of the endogenous locus. A major advantage of this approach is that tandem incorporation of multiple plasmid copies will simply generate additional C-terminal YFP regions, which lack the promoter and most of the ORF and, thus, are unlikely to have residual expression/activity. For an example in which the kinesin Kip3 was tagged by fusion with 3YFP (see Gupta, Carvalho, Roof, & Pellman, 2006).

The positive YFP-fusion transformants should be identified microscopically or by PCR as described above. The forward primer should anneal to a region of the tagged protein that was not cloned into the plasmid, and the reverse primer to the linker sequence between the tagged protein and the YFPs. This avoids complications from primer annealing to the repetitive sequences within the tandem YFPs. Generate clonal strains and confirm the YFP fusion before storing and/or using for analyses.

22.6.2 Maintaining equal levels of YFP-fusion proteins

Strains used for quantitative analyses must be verified by western blot to express equal amounts of the YFP-tagged protein. Prepare log-phase cultures in SC as described above.

> *Note*: We find improved consistency in quantitative imaging and western blots by preparing cultures under selective pressure for the tagged protein, for example, SC-Leu for cells containing *KIP3-3YFP-LEU2*.

Determine OD600 of the cultures, centrifuge 10 ml of cells, and resuspend in 1 ml of cold 0.1 M NaOH. Incubate 10 min on ice, centrifuge, and resuspend in ~200 µl SDS-PAGE loading buffer. The exact volume of loading buffer should reflect the OD600 ratio to maintain equal cell-to-volume ratios among the samples. Heat to 100 °C for 10 min, cool on ice, and centrifuge 10 min at 13,000 × *g*. Use equal volumes for western blotting. For low abundance fusion proteins, we find 4–12% Bis–Tris gradient gels (NuPAGE, Invitrogen) useful to concentrate and sharpen protein bands. GFP/CFP/YFP can be detected with polyclonal anti-GFP antibody (ABM). Actin (anti-β-actin, Abcam) can be used to verify equal loading.

22.6.3 Microscopy and analysis of microtubule-associated proteins

Prepare concentrated log-phase cells, mount on agarose pads, and image in the CFP and YFP channels, essentially as described for GFP–Tub1. The z-series should be acquired rapidly to minimize microtubule movements during imaging. Acquisition speeds can generally be improved by capturing the entire CFP z-series before switching filters to capture YFP, increased excitation intensity, a piezoelectric z-control, 2 × 2 binning, and increased efficiency with dedicated CFP/YFP filter sets rather

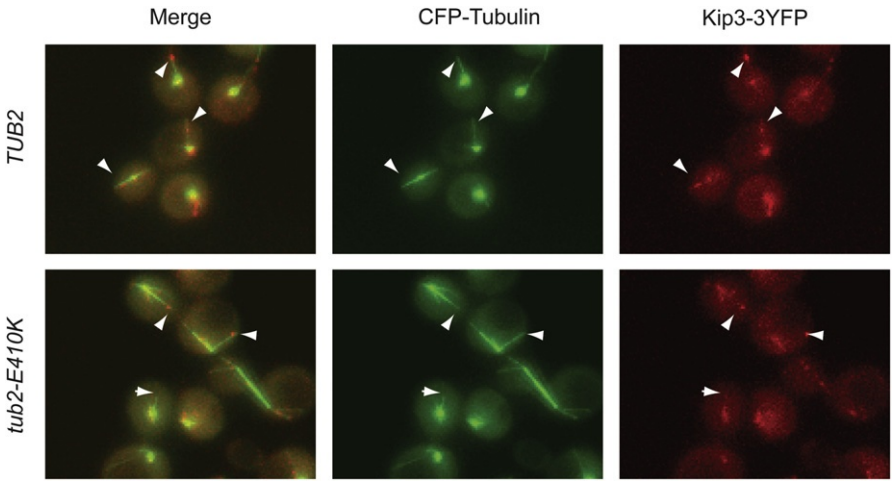

FIGURE 22.5

Microtubule plus-end localization of the kinesin Kip3 on microtubules bearing the β-tubulin E410K substitution (bottom row). Quantitative analysis determined that Kip3-3YFP at microtubule plus-ends (arrowheads) was reduced by 80% in the mutant strain relative to control cells (top row). Microtubules and Kip3 were visualized with CFP–Tub1 and Kip3-3YFP fusions, respectively (Cederquist et al., 2012).

than filter wheel with shared dichroic. Care must be taken that z-series include the entire cell. Capture all images with identical settings, for example, excitation intensity, exposure times, and microscope/camera configuration.

The precise localization and dynamics of different microtubule-associated proteins can vary extensively. Thus, a detailed discussion of analyses is prohibitive. Here, we describe an example where localization of Kip3-3YFP to microtubule plus-ends was quantified (Fig. 22.5). Z-series images were deconvolved and merged to create a single summed image with Slidebook software (Intelligent Imaging Innovations). A circular region of 12 pixels was designated over the plus-ends of visible microtubules, and the average YFP intensity was calculated with the same software. Cytoplasmic background signal was determined and subtracted by calculating the YFP intensity of an identical region placed in the cytosol near each microtubule plus-end. To determine average Kip3-3YFP localization, ~100 microtubules were measured on multiple days from three wild-type or mutated tubulin clones.

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