

The structured core of human β tubulin confers isotype-specific polymerization properties

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Diversity in cytoskeleton organization and function may be achieved through variations in primary sequence of tubulin isotypes. Recently, isotype functional diversity has been linked to a “tubulin code” in which the C-terminal tail, a region of substantial sequence divergence between isotypes, specifies interactions with microtubule-associated proteins. However, it is not known whether residue changes in this region alter microtubule dynamic instability. Here, we examine recombinant tubulin with human β isotype IIB and characterize polymerization dynamics. Microtubules with β IIB have catastrophe frequencies approximately threefold lower than those with isotype β III, a suppression similar to that achieved by regulatory proteins. Further, we generate chimeric β tubulins with native tail sequences swapped between isotypes. These chimeras have catastrophe frequencies similar to that of the corresponding full-length construct with the same core sequence. Together, our data indicate that residue changes within the conserved β tubulin core are largely responsible for the observed isotype-specific changes in dynamic instability parameters and tune tubulin’s polymerization properties across a wide range.

Introduction

Microtubules, polymers of α/β tubulin subunits, carry out a wide range of functions in eukaryotes (Desai and Mitchison, 1997; Nogales, 2001). The tubulin gene family expanded substantially in higher eukaryotes, and the expression of different isotypes can vary according to cell identity and stage of development (Ludueña, 2013). For example, flies encode four α and four β tubulin isotypes, whereas humans encode at least seven α and eight β tubulin isotypes that can have distinct expression profiles (Ludueña and Banerjee, 2008). In particular, β II and β III are the major β tubulins in the brain (Banerjee et al., 1988), and β VI is limited to the hematopoietic cell lineage (Wang et al., 1986; Leandro-García et al., 2012). Furthermore, recent in vivo studies have revealed that β tubulin isotypes have noninterchangeable roles in development. In *Drosophila melanogaster*, when a testes-specific β tubulin isotype was replaced with a β tubulin isotype not normally expressed in the male germline, axoneme assembly and meiosis were no longer supported (Hoyle and Raff, 1990). In mice, embryonic knockdown of neuronal β III expression led to neural migration defects that could not be rescued by expression of other β tubulin isotypes (Saillour et al., 2014). Together, these studies suggest that having multiple tubulin isotypes can be important for achieving diversity in function.

Differences in amino acid sequence of tubulin isotypes can affect two important aspects of tubulin function: the binding to microtubule associated proteins (MAPs) and the dynamics of microtubule polymer assembly. Numerous studies examining

MAP interactions have focused on the ~ 25 amino acids that comprise the C-terminal tail of tubulin, where many posttranslational modifications are found and where isotype sequence differences are concentrated (Westermann and Weber, 2003; Verhey and Gaertig, 2007). Thus it has been proposed that tubulin’s C-terminal tail may establish a “tubulin code” to direct unique interactions with MAPs (Verhey and Gaertig, 2007). In contrast, it is unclear whether residue changes in tubulin’s C-terminal tail sequence can directly affect microtubule polymerization dynamics. Studies using native tubulin isolated from bovine brain have shown that α/β tubulin dimers with different β isotype composition have distinct polymerization properties (Banerjee et al., 1992; Lu and Ludueña, 1994; Panda et al., 1994), which are partially altered after limited proteolysis by subtilisin (Lu and Ludueña, 1994). However, because of challenges in generating human tubulins with modified amino acid sequence from recombinant sources, the basis of the observed changes in polymerization dynamics between tubulin isotypes is still unknown.

Here, we purify recombinant tubulin heterodimers that have human β tubulin isotype IIB (β IIB) and provide the first characterization of its biochemical properties and assembly dynamics. We quantify parameters of dynamic instability and compare them to those of isotype III (β III) heterodimers that we have recently examined (Ti et al., 2016). Further, we generate chimeric tail-swapped tubulins by fusing the C-terminal

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Abbreviations used in this paper: MAP, microtubule-associated protein; TCEP, tris(2-carboxyethyl)phosphine; TEV, Tobacco Etch virus; TIRF, total internal reflection fluorescence.

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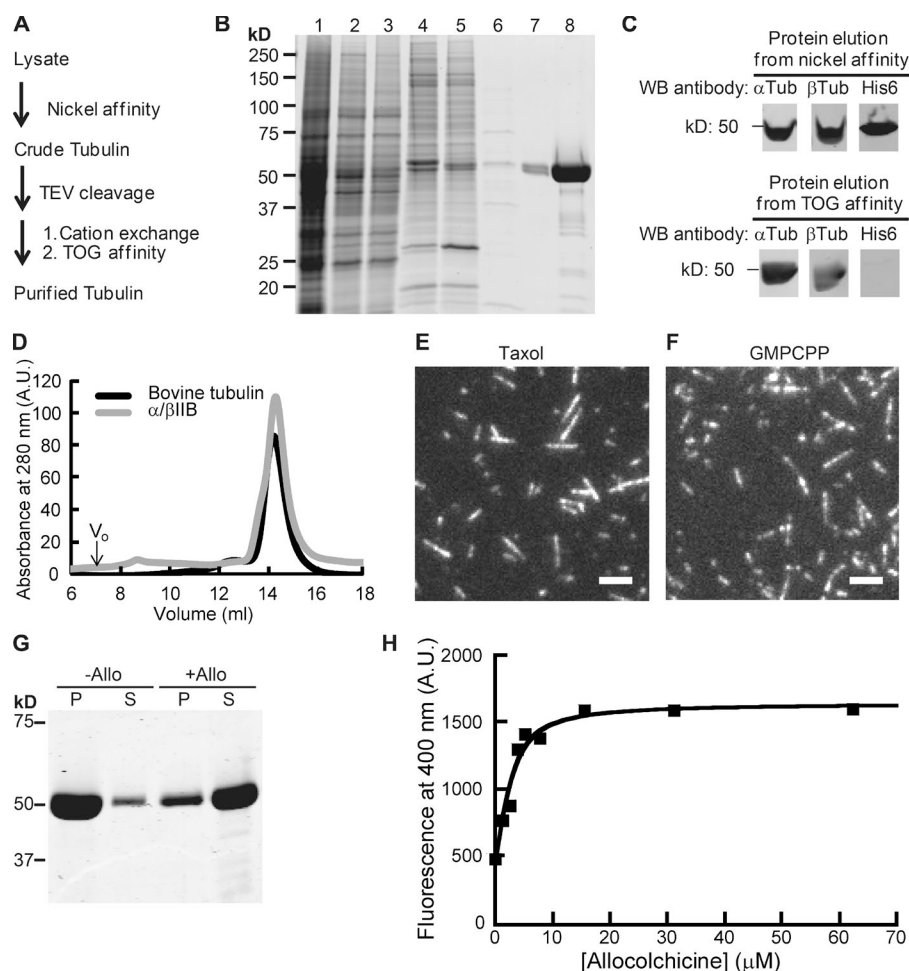


Figure 1. Purification of recombinant α/β IIIB tubulin heterodimers. (A) Purification scheme. (B) SDS-PAGE analysis (1, lysate; 2, supernatant; 3 and 4, nickel affinity: flow-through (3); elution (4); 5, TEV-digested protein elution from nickel affinity column (a band corresponding to the added TEV protein is at ~25 kD); 6–8, TOG affinity: flow-through (6); elution (7), 20 \times amount in lane 7 (8); Coomassie stain). (C) Western blot (WB) analyses. Full blots are provided in Fig. S1 A. (D) Elution profiles from size-exclusion chromatography. Peak volume: 14.4 ml (α/β IIIB); 14.3 ml (bovine tubulin, used as reference). Void volume (V_0) is 7 ml. A.U., arbitrary units. (E and F) TIRF images of taxol-stabilized (E) or GMPCPP (F) microtubules. Bars, 3 μ m. (G) SDS-PAGE analysis of tubulin sedimentation in the presence of allocolchicine (+Allo) or 3% DMSO control (–Allo). Pellet (P) and supernatant (S) fractions are indicated. (H) Representative equilibrium binding curve for α/β IIIB with allocolchicine from one experiment is shown. $K_d = 1.8 \pm 0.42 \mu$ M ($n = 3$, mean \pm SD). Fig. S1 C shows data averaged from all three experiments and fitted to a single curve.

tail domain of one isotype to the core of the other and use these proteins to dissect the basis of isotype-specific changes in dynamic instability.

Results and discussion

Purification of recombinant α/β IIIB tubulin heterodimers

To determine the biochemical and polymerization properties of human β tubulin isotypes, we purified recombinant tubulin heterodimers using a protocol we have recently developed (Ti et al., 2016). This three-step procedure generated affinity tag–free recombinant protein (Fig. 1, A–C; and Fig. S1 A). We then used mass spectrometry to confirm the presence of human β IIIB and showed that heterodimers contained an approximately equimolar mixture of human and insect α tubulins that are ~97% identical by sequence (Fig. S1 B). Efforts to tag both α and β tubulin with different affinity tags led to a substantially reduced protein yield, and the strategy was not pursued further. We find our multistep approach suitable for comparing tubulins with different β isotype compositions, as done in this study. Hereafter, we refer to the purified recombinant tubulin as α/β IIIB, highlighting the specific purified human β tubulin isotype.

We assessed the recombinant α/β IIIB tubulin using two approaches. First, size-exclusion chromatography indicated that α/β IIIB existed as a stable dimer in solution (Fig. 1 D) and eluted at a similar volume to bovine tubulin purified using standard

methods (Al-Bassam et al., 2006; Gell et al., 2011). Second, we examined α/β IIIB in the presence of compounds that stabilize or destabilize microtubules. We found that the protein polymerized to form microtubules in the presence of the drug taxol (Fig. 1 E) and the slowly hydrolyzing GTP analogue, GMPCPP (Fig. 1 F). Colchicine and related analogues bind soluble tubulin at an interface between the α and β subunits and inhibit tubulin polymerization (Ravelli et al., 2004). In particular, we examined the binding of our recombinant α/β IIIB protein to a colchicine analogue, allocolchicine, which fluoresces only upon binding soluble tubulin and allows for a direct readout of the interaction (Hastie, 1989; Medrano et al., 1989). We first confirmed that allocolchicine can inhibit the assembly of α/β IIIB microtubules (Fig. 1 G). We next determined equilibrium binding and found that α/β IIIB tubulin binds allocolchicine with a low-micromolar affinity ($K_d = 1.8 \pm 0.42 \mu$ M, $n = 3$; Figs. 1 H and S1 C), similar to that reported for bovine brain tubulin (Rice et al., 2008). Together, these data indicate that our recombinant α/β IIIB protein has overall properties similar to those of bovine tubulin purified using conventional methods.

Polymerization properties of recombinant α/β IIIB tubulin heterodimers

To analyze polymerization dynamics of recombinant α/β IIIB, we used a total internal reflection fluorescence (TIRF)-based single-filament assay (Fig. 2 A). As a template for microtubule formation, we used GMPCPP-stabilized seeds assembled from α/β IIIB tubulin. We then applied solutions of soluble tubulin

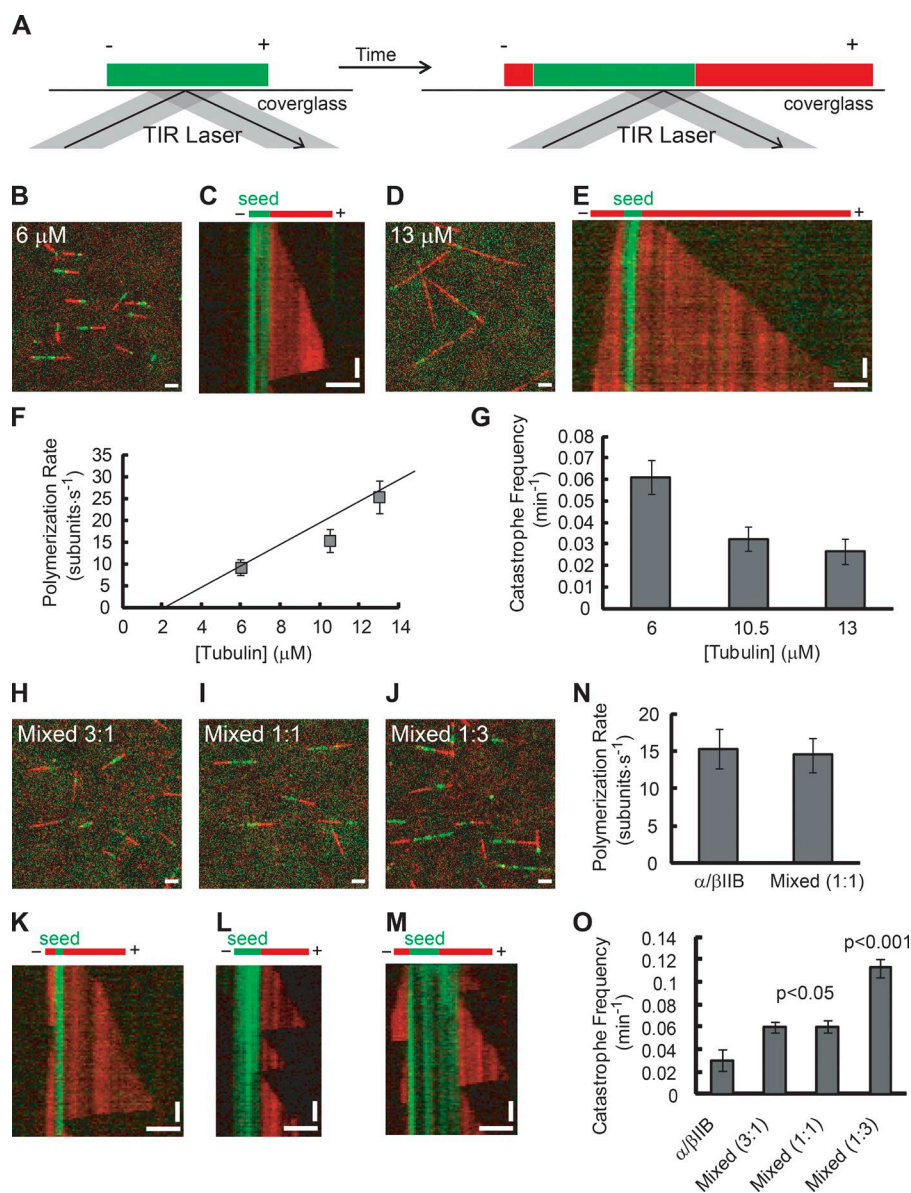


Figure 2. Single-filament TIRF analysis of α/β IIB tubulin polymerization properties. (A) TIRF assay schematic. Microtubule extensions (red) and GMPCPP seeds (green) are shown with plus ends (+) and minus ends (−) indicated. TIRF image overlays (B and D) and kymographs (C and E) of microtubule extensions (red) growing from seeds (green; total tubulin: 6 μ M [B and C] and 13 μ M [D and E]). (F and G) Plus-end polymerization rates (F) and catastrophe frequencies (G) for microtubules composed of α/β IIB at different total tubulin concentrations. The data were pooled from at least three independent experiments. Overlay images (H–J) and kymographs (K–M) of mixed α/β IIB and α/β III microtubules (α/β IIB: α/β III ratio, 3:1 [H and K], 1:1 [I and L], or 1:3 [J and M]; total tubulin, 10.5 μ M). (N and O) Plus-end polymerization rate (N) and catastrophe frequency (O) for mixed microtubules (α/β IIB: α/β III ratio, 1:1, total tubulin, 10.5 μ M) with α/β IIB shown for reference. The data were pooled from at least two independent experiments. Bars: (horizontal) 3 μ m; (vertical) 2 min. Error bars are SD. For catastrophe frequency (f_{cat}), SD were calculated as f_{cat}/\sqrt{n} (assuming a Poisson distribution), where n is the number of catastrophe events. Table 1 summarizes these measurements.

and observed microtubule assembly off of these seeds over a range of tubulin concentrations (Fig. 2, B and D). To quantify dynamic instability parameters, we generated kymographs from the time-lapse images of growing microtubules (Fig. 2, C and E). Even at the lowest tubulin concentration used (6 μ M), we observed growth off of all filaments examined. Microtubule elongation occurred primarily at only one end of the seed, which we designate the plus end. In contrast, assembly of microtubule polymer was rarely observed off of the minus ends at this concentration and only occasionally observed at the highest concentration used (13 μ M); therefore, we did not quantify the polymerization properties of tubulin at the minus end.

We determined the mean polymerization rate at plus ends of microtubules assembled from α/β IIB tubulin and found that this rate increased with free tubulin concentration (Fig. 2 F and Table 1). The measured growth rates were close to the reported values for α/β III (Ti et al., 2016) and purified bovine brain tubulin (Walker et al., 1988). We then fitted the data to a simple 1D model (Oosawa, 1970) whose slope and intercept are the apparent association (k_+) and dissociation (k_-) rate

constants of tubulin subunits, respectively ($k_+ = 1.9 \pm 0.5 \mu\text{M}^{-1} \text{s}^{-1}$ and $k_- = 2.6 \pm 4.2 \text{s}^{-1}$).

We next measured the frequency of catastrophe, the transition from a state of filament growth to a state of rapid shrinkage. Microtubules assembled from α/β IIB heterodimers underwent a catastrophe event infrequently, and we observed a moderate decrease in catastrophe frequency as tubulin concentration was increased from 6 to 13 μ M (Fig. 2 G). We measured a frequency of $0.03 \pm 0.006 \text{min}^{-1}$ at a tubulin concentration close to physiologic levels (10.5 μ M). We rarely observed rescue events (the transition from rapid shortening to relatively slow growth) under our experimental conditions and did not analyze this parameter. When we compared our analysis of recombinant α/β IIB tubulin with that of recombinant β tubulin isotype 3 (α/β III; Ti et al., 2016), we observed key differences. Notably, the catastrophe frequency for α/β IIB was 1.5- to 3-fold lower than that of α/β III at all tubulin concentrations tested ($P < 0.02$ at each concentration; Ti et al., 2016).

Tubulin in cells is a mixture of multiple isoforms of β tubulin. In particular, bovine brain tubulin has been shown to be

a mixture of at least four β isotypes ($\beta 2$, $\beta 3$, $\beta 4$, and $\beta 1$ detected at 58%, 25%, 13%, and 3%, respectively; Banerjee et al., 1988). Therefore, we mixed α/β IIB and α/β III heterodimers and analyzed polymerization dynamics (Fig. 2, H–O). At equal ratios of α/β IIB and α/β III tubulin (10.5 μ M total tubulin), microtubules readily polymerized off of GMPCPP seeds (Fig. 2 I). The polymerization rate of these mixed microtubules (14 ± 2 subunit \cdot s $^{-1}$) was close to that of α/β IIB microtubules (15 ± 3 subunit \cdot s $^{-1}$; Fig. 2 N) and α/β III microtubules (Ti et al., 2016) at the same tubulin concentration. This suggests that the two isotypes can indeed copolymerize. If they did not, the expected polymerization rate would be ~ 7 –8 subunit \cdot s $^{-1}$, as the effective concentration would be 5.25 μ M for each isotype. The catastrophe frequency at filament plus ends was 0.06 ± 0.01 min $^{-1}$ at 10.5 μ M total tubulin concentration (Fig. 2 O). This value is intermediate between that for α/β IIB microtubules (0.03 ± 0.01 min $^{-1}$; Fig. 2 G) and that for α/β III microtubules (Ti et al., 2016). Together, our data indicate that the dynamics of microtubules assembled from mixed tubulin populations depends on the contribution of each isotype, and that mixing gives a catastrophe frequency intermediate between what is observed for either isotype alone.

Characterization of recombinant tubulins containing human β tubulin chimeras

To examine whether residue changes within tubulin's C-terminal tail, which are proposed to specify interactions with MAPs (Sirajuddin et al., 2014), also confer the observed isotype-specific catastrophe frequencies, we generated chimeric β tubulin constructs with the C-terminal tails swapped (Fig. 3 A). We designated the core region (aa 1–427) to be equal to the length of tubulin protein resolved in a recent structural study (Alushin et al., 2014), and the tail region to be the remaining C-terminal amino acids (aa 428–445 in β IIB and 428–450 in β III). We coexpressed one of the two chimeric β tubulins fused to a cleavable hexahistidine tag along with human α isotype 1B in insect cells and generated the

following two heterodimers: α/β IIB-tail-III (β IIB core and β III tail) and α/β III-tail-IIB (β III core with β IIB tail; Fig. 3 B).

Using our purification protocol, we generated affinity tag-free recombinant chimeric tubulin heterodimers (Fig. 3, C and D; and Fig. S2 A) that were of similar purity to that of α/β IIB. Size-exclusion chromatography analysis indicated that the α/β IIB-tail-III and α/β III-tail-IIB proteins existed as stable dimers in solution and eluted at a volume similar to the full-length α/β IIB (Figs. 3 E and 1 D). As with the full-length α/β IIB tubulin, we analyzed the chimeric proteins using a TIRF microscopy-based assay. We showed that both chimeric tubulins assembled readily into microtubules in the presence of taxol (Fig. 3 F) and GMPCPP (Fig. 3 G). These experiments indicated that the chimeric tubulins formed stable dimers and polymerized under standard conditions.

Polymerization properties of recombinant tubulins containing β tubulin chimeras

To determine the intrinsic dynamic properties of recombinant tubulin heterodimers containing chimeric β tubulins, we used the same single-filament TIRF assays used to analyze the full-length α/β IIB construct (Fig. 2 A). We first used GMPCPP-stabilized seeds assembled from α/β IIB-tail-III and applied solutions composed of different concentrations of α/β IIB-tail-III onto the seeds. In separate experiments, we examined growth of α/β III-tail-IIB tubulin off of α/β III-tail-IIB seeds.

In the case of α/β IIB-tail-III tubulin, we frequently observed growth at only one of the two ends of the seed (Fig. 4, A–D), as was noted for full-length α/β IIB. At the plus ends, the rate of polymerization for microtubules assembled from α/β IIB-tail-III increased with greater concentrations of free tubulin (Fig. 4 I and Table 1). The apparent k_+ (2.0 ± 0.4 μ M $^{-1}$ s $^{-1}$) and k_- (5.5 ± 2.7 s $^{-1}$) for α/β IIB-tail-III were close to those measured for α/β IIB and α/β III (Ti et al., 2016).

In contrast, in a solution of α/β III-tail-IIB tubulin, assembly frequently occurred off both ends of seeds at all tubulin

Table 1. Plus end dynamic instability parameters for full-length wild-type tubulins and chimeric tubulins

Total tubulin concentration and recombinant protein	Polymerization rate (subunit \cdot s $^{-1}$)		Catastrophe frequency (min $^{-1}$)	
	Mean \pm SD	n	Mean \pm SD	n
6 μ M				
α/β IIB	9 \pm 2	70	0.06 \pm 0.008	60
α/β IIB-tail-III	6 \pm 1	87	0.08 \pm 0.008	92
α/β III-tail-IIB	8 \pm 2	37	0.12 \pm 0.02	58
α/β III*	8 \pm 3	77	0.10 \pm 0.01	105
10.5 μ M				
α/β IIB	15 \pm 3	70	0.03 \pm 0.006	33
α/β IIB-tail-III	14 \pm 3	81	0.03 \pm 0.005	37
α/β III-tail-IIB	17 \pm 2	88	0.10 \pm 0.01	115
α/β III*	16 \pm 6	75	0.10 \pm 0.01	104
Mixed (3:1) (α/β IIB: α/β III)	ND		0.05 \pm 0.008	41
Mixed (1:1) (α/β IIB: α/β III)	14 \pm 2	79	0.06 \pm 0.007	65
Mixed (1:3) (α/β IIB: α/β III)	ND		0.12 \pm 0.009	153
13 μ M				
α/β IIB	25 \pm 4	52	0.03 \pm 0.006	20
α/β IIB-tail-III	21 \pm 3	49	0.008 \pm 0.003	9
α/β III-tail-IIB	19 \pm 3	53	0.08 \pm 0.01	62
α/β III*	19 \pm 4	57	0.09 \pm 0.01	68

Measurements are from data presented in Fig. 2 (F, G, N, and O) and Fig. 4 (I and J). Mean \pm SD are shown. n represents the number of filaments analyzed (for polymerization rate) or catastrophe events (for catastrophe frequency). *, data from Ti et al. (2016); ND, not determined.

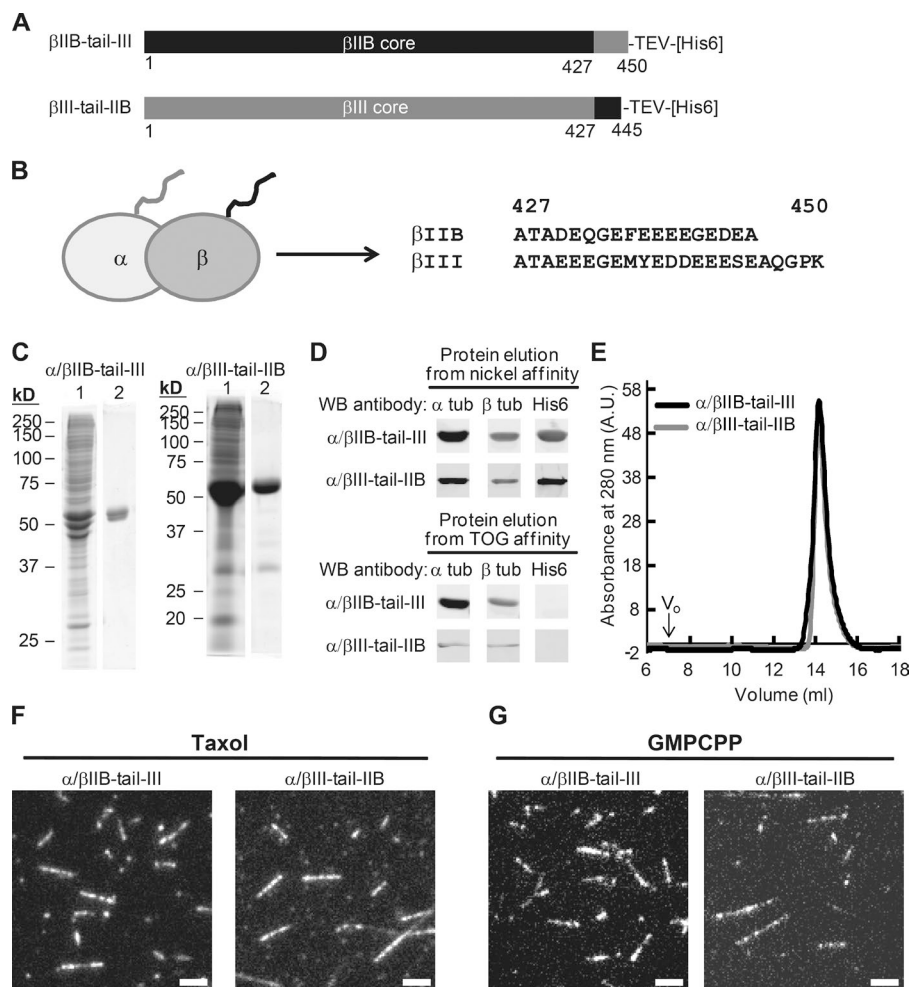


Figure 3. Design and characterization of chimeric β tubulin constructs. (A) Design of tail-swapped β tubulin constructs, with amino acid sequence derived from α/β IIB (black) and α/β III (gray). (B) Schematic of tubulin heterodimer indicating β tubulin C-terminal tail (black). Amino acid sequences from the C terminus of β IIB and β III are shown. (C) SDS-PAGE analysis (1, nickel affinity elution; 2, TOG affinity elution; Coomassie stain). (D) Western blot (WB) analysis. Full blots are provided in Fig. S2 A. (E) Protein elution profiles from size-exclusion chromatography. Peak volume: 14.2 ml (α/β IIB-tail-III); 14.1 ml (α/β III-tail-IIB). Void volume (V_0) is 7 ml. A.U., arbitrary units. (F and G) TIRF images of taxol-stabilized (F) or GMPCPP (G) microtubules. Bars, 3 μ m.

concentrations (Fig. 4, E–H). This was consistent with what we found for full-length recombinant α/β III, which grows frequently at both seed ends under similar experimental conditions (Ti et al., 2016). To compare rates between isotypes, we focused on the faster-growing plus end. The polymerization rate of microtubules assembled from α/β III-tail-IIB tubulin also increased with increasing concentrations of free tubulin (Fig. 4 I). The apparent k_+ ($1.8 \pm 0.5 \mu\text{M}^{-1} \text{s}^{-1}$) and k_- ($2.4 \pm 4.2 \text{s}^{-1}$) for α/β III-tail-IIB were close to those of both full-length α/β IIB and full-length α/β III. These data indicate that each of the chimeric β tubulin constructs can elongate into microtubule polymer at rates close to those measured for each of the full-length wild-type proteins α/β IIB and α/β III.

We next analyzed catastrophe frequency, the dynamic instability parameter that differs between α/β IIB and α/β III. For α/β IIB-tail-III microtubules, the catastrophe frequencies were close to those of the full-length α/β IIB at the same tubulin concentrations (Fig. 4 J and Fig. 2 G). Next, we measured the catastrophe frequencies of microtubules assembled from α/β III-tail-IIB tubulin and found them to be ~1.5- to 3-fold higher than those of α/β IIB-tail-III microtubules over a range of tubulin concentrations (Fig. 4 J and Table 1). These catastrophe frequencies of α/β III-tail-IIB microtubules were close to the reported catastrophe frequencies of microtubules assembled from full-length α/β III (Fig. 4 J and Table 1). Together, these data indicate that the amino acid substitutions within the structured core are crucial for establishing isotype-specific parameters of dynamic instability.

Our studies indicate that microtubules assembled from each of the two tubulin isotypes can have substantially different catastrophe frequencies. The suppression of catastrophe in α/β IIB microtubules observed in vitro is on the order of what can be achieved by regulatory proteins in cells, such as TPX2, and by microtubule-stabilizing drugs, such as taxol (Mohan et al., 2013; Wiczorek et al., 2015). Human tubulins β IIB and β III differ in amino acid identity at ~9% of residues within the ~450-aa polypeptide, and the short ~25-residue C-terminal tail carries a large fraction (15 of 42) of the total residue changes (Fig. S3 A). Our analyses of chimeric tail-swapped tubulins suggest that of these 42 nonidentical residues, those within the structured core of tubulin are largely responsible for the different dynamics. Recent studies examining the effects of residue mutations in tubulin's intermediate domain (Geyer et al., 2015) and kinesin-binding site (Ti et al., 2016) are beginning to reveal how subtle allostery within the tubulin heterodimer affects microtubule assembly dynamics. Given that there are 27 amino acid differences between the β IIB and β III isotypes within the tubulin core, and that each may affect long-range communication across the dimer, structure or sequence alone is not likely to help prioritize which residues should be examined. Additional studies will be needed to identify which of the 27 amino acid differences, alone or in combination with other residues, specify the observed differences in dynamic instability. Studies in *Drosophila* have revealed how the exogenous expression of tubulin isotypes from another insect species can alter

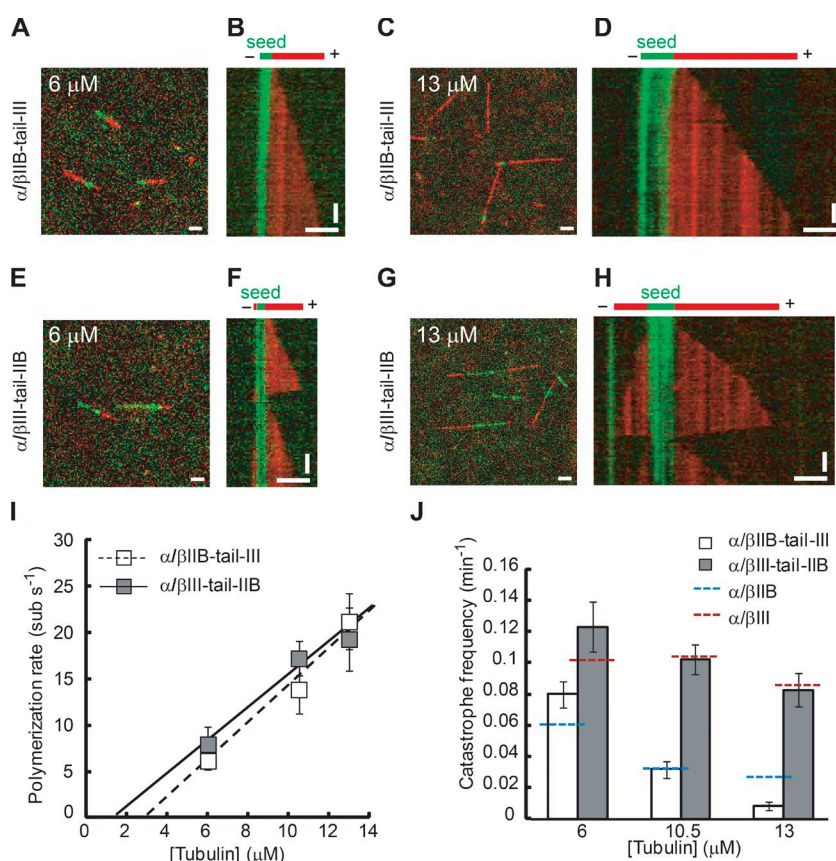


Figure 4. Single-filament TIRF analysis of chimeric β tubulins. TIRF image overlays (A, C, E, and G) and kymographs (B, D, F, and H) showing microtubule extensions (red) growing from GMPCPP seeds (green) assembled with α/β IIB-tail-III (A–D) or α/β III-tail-IIB (E–H). (I and J) Plus-end polymerization rates (I) and catastrophe frequencies (J) for chimeric α/β IIB-tail-III or α/β III-tail-IIB microtubules at different free tubulin concentrations. Catastrophe frequency measurements for full-length α/β IIB (blue dashed line) and α/β III (red dashed line) are shown for reference. The data were pooled from at least three independent experiments. Error bars are SD. For catastrophe frequency (f_{cat}), SD were calculated as f_{cat}/\sqrt{n} (assuming a Poisson distribution), where n is the number of catastrophe events. Bars: (horizontal) 3 μ m; (vertical) 2 min. Table 1 summarizes these measurements.

microtubule protofilament number in cells (Raff et al., 1997). Our recombinant expression system will help dissect whether these effects are caused by intrinsic properties of tubulin isoforms and whether differences in protofilament number can also regulate dynamic instability.

There are five β tubulins expressed in the brain (Banerjee et al., 1988; Leandro-García et al., 2010). The core sequence of β IIB shares the least amount of similarity with that of β III compared with the other isoforms expressed in the tissue (Leandro-García et al., 2010; Fig. S2 B). We hypothesize that these two tubulin isoforms establish the range of potential catastrophe frequencies of neuronal microtubules. In addition, our data indicate that recombinant α/β IIB and α/β III tubulin can copolymerize and form microtubules comprising mixed isoforms, which have catastrophe frequencies intermediate between those measured for microtubules composed of either β tubulin isoform alone. Based on intrinsic polymerization properties alone and a simple model (Verde et al., 1992), the mean length of microtubules assembled from α/β IIB would be approximately three times longer than those assembled from α/β III (see Materials and methods), and the mean length of mixed microtubules would be intermediate. Thus, microtubule dynamics could be tuned to have different catastrophe frequencies by varying the ratio of different isoforms in cells. Additional functional specialization would come through interactions with MAPs and via posttranslational modifications.

Materials and methods

Purification of recombinant human tubulin

The cDNA encoding *Homo sapiens* α tubulin 1B (NP_006073.2) and β tubulin 2B (BC001352) were cloned into pFastBac Dual vector (Thermo

Fisher Scientific). For affinity purification, a sequence encoding a Tobacco Etch virus (TEV) protease site and hexahistidine tag was fused to the 3' end of the β tubulin isoform 2B cDNA sequence. We used the Bac-to-Bac system (Thermo Fisher Scientific) to generate recombinant baculovirus. HiveFive cells (Thermo Fisher Scientific), grown to $3.0\text{--}3.5 \times 10^6$ cells/ml in SF-900 II SFM (10902-096; Thermo Fisher Scientific) and supplemented with 1 \times antibiotic-antimycotic (15240-062; Thermo Fisher Scientific), were infected with P3 viral stocks. Cells were cultured in suspension at 27°C and harvested 60 h after infection. The following steps were performed on ice or at 4°C. We lysed cells in an equal volume of lysis buffer (50 mM Hepes, 20 mM imidazole, 100 mM KCl, 1 mM MgCl₂, 0.5 mM DTT, 0.1 mM GTP, 3 U/ml benzamide, and 1 \times protease inhibitor Roche Complete EDTA-free, pH 7.2) by dounce homogenizer (20 strokes) and centrifuged the homogenate at 55,000 rpm in a rotor (Ti70; Beckman Coulter) for 1 h. The supernatant was then filtered through a 0.22- μ m Millex-GP PES membrane (SLGP033RS; EMD Millipore) and loaded at 0.8 ml/min onto a 1-ml HisTrap HP column (17-5247-01; GE Healthcare) pre-equilibrated with lysis buffer. The column was washed with 25 ml lysis buffer and then eluted with nickel elution buffer (1 \times BRB80 [80 mM Pipes, 1 mM MgCl₂, and 1 mM EGTA], 500 mM imidazole, 0.1 mM GTP, and 1 mM DTT, pH 6.8). The fractions containing proteins were pooled, diluted 10-fold with TOG-column buffer (1 \times BRB80, 1 mM DTT, and 0.2 mM GTP, pH 6.8), mixed with 6 mg TEV protease, and incubated for 1 h on ice. The TEV-digested protein solution was loaded at 1 ml/min onto tandem chromatography columns consisting of a 1-ml HiTrap SP Sepharose FF column (17-5054-01; GE Healthcare) and a 1-ml TOG-affinity column (Widlund et al., 2012) containing TOG 1 and 2 domains. The columns were then washed with 10 ml TOG column buffer. We removed the 1-ml HiTrap SP Sepharose FF column and washed the 1-ml TOG-affinity column with 20 ml wash buffer 1 (1 \times BRB80, 1 mM DTT, 0.1 mM GTP, 10 mM MgCl₂, and 5 mM ATP, pH 6.8), 20 ml wash buffer 2

(1× BRB80, 1 mM DTT, 0.1 mM GTP, 0.1% Tween-20, and 10% glycerol, pH 6.8), and 10 ml TOG column buffer. The tubulin was eluted with TOG elution buffer (1× BRB80, 500 mM (NH₄)₂SO₄, 1 mM DTT, and 0.2 mM GTP, pH 6.8). The eluate containing tubulin was pooled, exchanged into storage buffer (1× BRB80, 5% glycerol, 1 mM DTT, and 0.2 mM GTP, pH 6.8), and concentrated to at least 3 mg/ml with an Amicon Ultra 50K MWCO centrifugal filter (UFC901024; EMD Millipore) within 2 h after elution from the TOG column. A typical preparation yielded 1.5 mg protein from 1 liter of cultured insect cells. The purified tubulin was snap-frozen with liquid nitrogen and stored at −80°C.

Mass spectrometry analysis

Mass spectrometry was performed essentially as described previously (Li et al., 2012). Dried protein samples were resuspended in LDS sample buffer (Thermo Fisher Scientific), reduced, alkylated, and separated on a 4–12% Bis-Tris gradient gel (Thermo Fisher Scientific), followed by in-gel trypsin digestion. Tryptic peptides were purified and analyzed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). To quantify the relative amounts of human and insect α tubulin in the final protein, we compared the signal intensities for each pair of unique peptides that differed by no more than one amino acid and took the mean for all of the peptide pairs. To examine the possibility of insect β tubulin copurifying in our final protein, we used a similar approach. Insect β tubulin was detected at low abundance, indicating that it is not a major contaminant of our final recombinant protein. We estimate this fraction of insect tubulin to constitute ~4% of the total β tubulin.

Microtubule sedimentation assay in the presence of allocolchicine

Allocolchicine was synthesized by the established method (Fernholz, 1950). Purified tubulin was preclarified by high-speed centrifugation in a TLA120.1 rotor (Beckman Coulter) at 90,000 rpm for 10 min at 4°C. Solutions of preclarified tubulin (13 μ M) were prepared in assay buffer (1× BRB80, 33.33% [vol/vol] glycerol, 1 mM GTP, and 1 mM tris(2-carboxyethyl)phosphine [TCEP]) containing 3% DMSO and 60 μ M allocolchicine or 3% DMSO alone. The reactions were incubated at RT for 30 min, followed by another 30-min incubation at 37°C, and then subjected to high-speed centrifugation in a TLA 120.1 rotor (Beckman Coulter) at 90,000 rpm for 10 min at 30°C. The supernatant was removed and saved for SDS-PAGE analysis. The pellet was rinsed with 40 μ l warm wash buffer (1× BRB80, 60% [vol/vol] glycerol, and 1 mM TCEP) and resuspended in 1× Laemmli sample buffer for SDS-PAGE analysis.

Binding of allocolchicine to tubulin

Purified tubulin was preclarified by high-speed centrifugation in a TLA120.1 rotor (Beckman Coulter) at 90,000 rpm for 10 min at 4°C. Preclarified tubulin (3 μ M) was mixed with increasing concentrations of allocolchicine (0, 1.3, 2.6, 3.9, 5.2, 7.8, 15.6, 31.2, or 62.4 μ M) in assay buffer (1× BRB80, 5% [vol/vol] glycerol, 1 mM GTP, and 1 mM TCEP). After 2 h of incubation at RT, the emission spectra of the reactions were collected from 360 to 420 nm with 5-nm increments using excitation at 310 nm. The measured fluorescence intensity at 400 nm was plotted on the vertical axis versus allocolchicine concentration on the horizontal axis. To determine the affinity of tubulin for allocolchicine, the unnormalized equilibrium binding curves were fitted with the following equation:

Fluorescence intensity at 400 nm =

$$\frac{(K_d + [Allo] + [\text{Tubulin}]) - \sqrt{(K_d + [Allo] + [\text{Tubulin}])^2 - 4 \times [Allo] \times [\text{Tubulin}]}}{2 \times [\text{Tubulin}]} \times (F_{\max} - F_{\text{Background}}) + F_{\text{Background}}$$

where K_d represents the dissociation constant for allocolchicine binding, F_{\max} represents the fluorescence intensity at plateau, and $F_{\text{background}}$ represents the fluorescence intensity of tubulin (62.4 μ M) in the absence of allocolchicine.

In vitro fluorescence microscopy

All experiments were performed on an Eclipse Ti microscope (Nikon) equipped with a NA 1.49 100× Plan Apo TIRF objective (Nikon). The microscope setup included a three-axis piezo-electric stage (Nano LP-200; Mad City Labs), an EM-CCD camera (iXon DU-897; Andor Technology), and two-color TIRF imaging optics (lasers: 488 nm [Spectra-Physics] and 561 nm [Cobalt]; filters: emission [FF01-520/35 and FF01-609/54; Semrock], dichroic [Di01-R488/561; Semrock]). Sample chambers were prepared by first cleaning 18 × 18-mm glass coverslips (thickness no. 1; Gold Seal Cover Glass) and 27 × 46-mm slides (40-80000-01; Buehler). To prevent nonspecific surface sticking, we then coated the surface of slides with nonbiotinylated PEG and the surface of coverslips with a mixture of biotinylated PEG and nonbiotinylated PEG according to standard protocols. To build flow chambers, we applied two strips of double-sided tape to a microscope slide and applied the coverslip. Sample chamber volumes were ~6–8 μ l.

To generate seeds for templated microtubule growth, we polymerized the recombinant tubulins at 12- μ M concentration along with 8 mol% Alexa Fluor 488 and biotin-labeled bovine tubulin in the presence of 2.5 mM GMPCPP. The polymerized GMPCPP seeds were immobilized on a coverslip by first coating the surface with NeutrAvidin. After a brief incubation with κ -casein to block nonspecific binding to the surface, a mixture of recombinant tubulin and 4 mol% X-rhodamine-labeled bovine tubulin was flowed into the TIRF chamber maintained at 30°C. Time-lapse images were acquired at a rate of one frame every 10 s for 15 min. All assays with dynamic microtubules were done in buffer containing 1× BRB80, 1 mM GTP, 4% glycerol, 0.2 mg/ml κ -casein, 0.2% methylcellulose, and oxygen scavenging mix (25 mM glucose, 40 mg/ml glucose oxidase, 35 mg/ml catalase, and 0.5% β -mercaptoethanol final concentration in reaction).

Image analysis was performed by creating kymographs from the time-lapse TIRF images of microtubules using ImageJ. The data were quantified by measuring the slope of the growing microtubule extension and using it to determine the mean growth speed for each filament. The mean polymerization rate was calculated from all microtubules analyzed for each condition. The mean and standard deviation are reported in the figures. To determine catastrophe frequency, we divided the total number of catastrophe events for all filaments by the total polymerization time (the sum of the time each GMPCPP seed was observed with a growing microtubule extension). The standard deviation was estimated as the catastrophe frequency divided by the square root of the number of catastrophe events. This assumes that catastrophe events are Poisson processes.

Estimating the mean length of microtubules

We used a model that described the probability of finding a microtubule of a given length x at any time, assuming four dynamic instability parameters: v_g (velocity of growth), v_s (velocity of shrinkage), f_{cat} (frequency of catastrophe), and f_{res} (frequency of rescue; Verde et al., 1992).

The length probability distribution can be described as

$$p(x) = \frac{1}{L} \times e^{-\frac{x}{L}}$$

From their derivations, the average length L is estimated by

$$L = \frac{v_s v_g}{(v_s f_{\text{cat}}) - (v_g f_{\text{res}})}$$

We make the simplifying assumption that $f_{res} = 0$; as we rarely observe rescue events. So this simplifies to

$$L = \frac{v_s v_g}{v_s f_{cat}} = \frac{v_g}{f_{cat}}$$

At 10.5- μ M tubulin concentration, the growth rates of α/β IIB microtubules and α/β III microtubules are approximately equal and the catastrophe frequencies are such that

$$f_{cat_a/\beta IIB} = \frac{1}{3} f_{cat_a/\beta III}$$

This implies that the length of α/β IIB microtubules would be approximately threefold greater than that of α/β III microtubules.

Online supplemental material

Fig. S1 shows the purification of recombinant α/β IIB tubulin heterodimers. Fig. S2 shows the purification of chimeric β tubulin heterodimers and tubulin isotype identity matrix. Fig. S3 shows β tubulin isotypes IIB and III alignment and a secondary structure topology map. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201603050/DC1>.

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