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Supplementary Methods

Site-directed mutagenesis. Site-directed mutagenesis in the sequence coding for α-helix 12 (H12) in β-tubulin was performed by polymerase chain reaction (PCR) using the overlap extension method (Ho *et al*, 1989). The PCR primers used for mutagenesis are listed in Supplementary Information, Table SI.

Construction of plasmids. To amplify *TUB1* and *TUB2* containing each promoter region, we first performed PCR using chromosomal DNA as template with the following PCR primers; TUB1-NotI_F (5′-CAGCGGCCGCGACACATTTGGTGTACATAC-3′) plus TUB1-SalI_R (5′-GAGTCGACCAATTCGACGGCAAGGCGGG-3′), and TUB2-NruI_F (5′-AATCGCGATACAGTCTTGCCGTCCAG-3′) plus TUB2-SmaI_R (5′-AACCCGGGACCGCTTCCACAATCATC-3′), respectively. The amplified fragment containing *TUB1* was cloned into pGEM-T-Easy vector (A1360, Promega) to produce pSU93, and the fragment containing *TUB2* was cloned into pCR2.1-TOPO vector (K4500-01, Invitrogen) to produce pSU30. To construct pSU205, the *Not*I fragment of pSU93 containing *TUB1* was inserted into the *Not*I site and the *Nru*I/*Sma*I fragment of pSU30 containing *TUB2* was inserted into the *Sma*I site of pRS316. To construct pSU207, the *Not*I/*Sal*I fragment of pSU93 containing *TUB1* was inserted between the *Not*I and *Sal*I sites of pRS314. pSU251 was constructed as follows: The *Nru*I/*Sma*I fragment of pSU30 containing *TUB2* was first inserted between the blunt-ended *Sal*I and *Sma*I sites of pRS315. Then, the *Nco*I/*Hin*dIII fragment of this plasmid was swapped for the *Nco*I/*Hin*dIII fragment containing A19K, T23V, G26D, N227H, and Y270F mutations. To construct pSU238 and pSU240, the *Hin*dIII/*Sma*I fragment of pSU251 was swapped for the *Hin*dIII/*Sma*I fragment containing E412A and E421A mutations, respectively. pSU278 was constructed as follows: The *Hin*dIII/*Sma*I fragment of pSU251 was first swapped for the *Hin*dIII/*Sma*I fragment containing ^{440}GDFGEEEEGEEEEGEEEEGEEEEGEEEEA mutation. Then, the partially digested *Xho*I/*Sma*I fragment of this plasmid was inserted between the *Xho*I and *Sma*I sites of pRS313.

Construction of pSU342 and pSU345 required several steps. First, a *GAL10* promoter region (*GAL10pro*) was amplified by PCR using chromosomal DNA as a template with the PCR primers, GAL10pro-NotI_F (5′-GCGGCCGCATCGCTTCGCTGATTAATTAC-3′) and GAL10pro-TUB1_R (5′-CATTAATACTAATAACTTCTCTCATTTATATTGAATTTTCAAAAATTC-3′). To

generate a *GAL10pro-TUB1* fusion gene, the fragment containing *GAL10pro* and 25-bp downstream from ATG of *TUB1*, and the plasmid pSU207 were used as templates for a second amplification with the PCR primers, GAL10pro-NotI_F and TUB1-SalI_R. Then to produce the plasmid pSU286, the *GAL10pro-TUB1* fusion gene was further cloned into the pGEM-T-Easy vector. The plasmid containing *GAL10pro-TUB2* fusion gene, pSU287, was constructed in a procedure similar to the construction of pSU286, using pSU251 as a template with the following PCR primers; GAL10pro-NotI, GAL10pro-TUB2_R (5′-GATATGAATGATTTCTCTCATTTATATTGAATTTTCAAAAATTC-3′), and TUB2-SmaI_R. Next, to produce the plasmids pSU337 and pSU339, the *Hin*dIII/*Sma*I fragment of pSU287 was swapped for the *Hin*dIII/*Sma*I fragment containing E410A and D417A mutations, respectively. The *Not*I/*Sal*I fragments of pSU286 was inserted between *Not*I and *Sal*I sites of pRS305 to generate pSU289 and finally, the *Not*I fragment of pSU337 and pSU339 was inserted into *Not*I site of pSU289 to produce pSU342 and pSU345, respectively.

Construction of mutant strains and growth conditions. The mutant strains and plasmids used are listed in Supplementary Information, Table SII. All the yeast strains were derivatives of YPH499 (Sikorski and Hieter, 1989). To impart Taxol binding ability to yeast tubulin, five amino acids responsible for Taxol binding were introduced into yeast β-tubulin (Gupta *et al*, 2003). SUY104, SUY120, SUY121, and SUY148 strains were obtained by plasmid shuffling (Boeke *et al*, 1984). SUY152 and SUY153 strains were constructed by integrating the plasmids pSU342 and pSU345 into *leu2* locus of SUY148, respectively. All the strains were first cultured in the YPD medium (2% glucose; Sherman, 1991) at 30 ˚C until the growth of the cells reached the late-log phase. For SUY152 and SUY153 strains, cells were transferred to YPG medium (3% galactose) and cultured for another 8 h to induce the expression of the tubulin mutated in H12 (Burke *et al*, 1989).

Purification of tubulin from yeast cell lysate. The initial stages of the purification procedure were based on the procedure of Davis *et al* (1993), and all the steps were performed at 4 ˚C. Cells collected from 6 L of culture (~40 g wet weight) were disrupted in PME buffer (100 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), 10 mM $MgSO_4$, 2 mM EGTA, 1 mM GTP; pH 6.8) using a French pressure cell (Model 5501; Ohtake works Co.) and the cell lysate (15-20 mg/ml of soluble protein in total volume of ~80 ml) was incubated with a 2 ml of DEAE-Sepharose resin (DEAE

Sepharose Fast Flow, Amersham Biosciences) for 30 min. The resin was loaded into a 5 × 0.5-cm syringe column, washed with PME buffer containing 0.12 M NaCl, and eluted with the same buffer containing 0.25 M NaCl. This crude tubulin fraction was diluted three-fold with PME buffer and further fractionated by FPLC on a Mono-Q column (HR 10*10, Amersham Biosciences) through a 0.1-1.0 M NaCl gradient (Supplementary Information, Figure S1). Depending on the net charge of the tubulin dimer, tubulin was eluted between 0.2 and 0.4 M NaCl. Only the major fraction was collected, concentrated to 3-5 mg/ml by ultra-filtration (UFV5BTK00, Millipore), and polymerized into microtubules in the presence of 0.5 mM GTP at 30 ˚C for 1 h. The polymerized material was centrifuged through glycerol cushion and the pellet was suspended at 4 ˚C in ~30 μl of BRB buffer (80 mM PIPES, 5 mM $MgCl_2$, 1 mM EGTA; pH 6.8) containing 1 mM GTP. After being left on ice for 30 min, the aggregated protein was removed by brief centrifugation. The supernatant tubulin fraction (0.5-1.0 mg/ml in a volume of 20 to 30 μl) was rapidly frozen in liquid nitrogen and was stored at –80 ˚C until use. The purity of tubulin, examined by SDS-PAGE analysis using Sigma SDS (L-5750; Best *et al*, 1981), was >95% (Supplementary Information, Figure S2).

Mass spectrometry determination of the tubulin proteolytic fragments and of the full-length tubulin polypeptides was performed with electrospray ionization/ion trap mass spectrometer with liquid chromatography (LCQ-DECA XP; Thermo Electron) and quadrupole-TOF mass spectrometer (QSTAR; AB/MDS SCIEX), respectively.

Preparation of other proteins. Native kinesin was purified from porcine brain according to the method previously described (Kojima *et al*, 1997). HK560cys was purified and labelled at its C-terminal cystein residue with Cy3 (PA23031, Amersham Biosciences; Vale *et al*, 1996). Polarity-marked microtubules were produced using the sea urchin sperm axonemes (Tanaka-Takiguchi *et al*, 1998). One-headed kinesin heterodimer was engineered and purified as described (Hancock and Howard, 1998).

Microtubule gliding assay and image analyses. For microtubule gliding assay (Hancock and Howard, 1998), 6 μl of casein solution (10 mg/ml of casein (#07319-82, Nacalai Tesque) in 10 mM Tris, 0.1 M NaCl) was first introduced to a flow chamber prepared from a coverslip and a glass slide that were spaced by a two-sided scotch tape (dimensions 9 mm × 9 mm × ~80 μm), left for 3 min, and the chamber was washed with 12 μl of motility assay buffer (MA buffer; 10 mM PIPES, 5 mM K-Acetate, 2 mM $MgSO_4$, 1 mM EGTA, 0.1 mM EDTA, pH 6.8). Subsequently, 6 μl of MA buffer containing native kinesin was introduced into the chamber and left for 5 min. Finally,

the yeast microtubules diluted in MA buffer (10-50 μg/ml in a volume of 6 μl), supplemented with 10 μM Taxol (T-7402, Sigma) and 1 mM ATP, were infused into the chamber, and the interaction of these yeast microtubules with kinesin on the glass surface was observed under the dark field microscope (BX50: Oil iris objective lens, ×50, NA = 0.5-0.90; Olympus) at 25 ± 1 ˚C. The images were projected onto an image intensified CCD camera (C3077-70 and C8600-05; Hamamatsu Photonics) and stored in a digital video recorder (DR20; Sony). The kinesin density on the glass surface was calculated from the concentration (determined by Coomassie Brilliant Blue; Bradford, 1976) and the molecular mass of kinesin (390 kDa; Kuznetsov *et al*, 1988), and the geometry of the flow chamber, assuming that all the kinesin molecules in solution were adsorbed on the surface of the chamber (total surface area = 162 mm^2) with an even distribution on both sides.

TIRFM. To observe motility of HK560-Cy3 in single molecule motility assay, we used an objective-type TIRFM (Tokunaga *et al*, 1997), based on an inverted microscope (IX-70, Olympus) equipped with an oil-immersion objective (PlanApo, ×100, NA = 1.4; Olympus). HK560-Cy3 and BODIPY-FL-labelled microtubule were illuminated with a DPSS laser at wavelengths of 532 nm (CDPS532M-050; JDS Uniphase) and 473 nm (Model DPBL-9020; SUWTEC), respectively. Fluorescent images were acquired by a GenIV image intensifier (VS4-1845; Video Scope) coupled with a SIT camera (C2400-08; Hamamatsu Photonics) at video rate.

Image Analysis of Single Molecule Motility Assay. Run-length analysis of HK560-Cy3 was essentially performed as described earlier (Inoue *et al*, 2001). Attachment and detachment times and positions were determined for single fluorescent spots interacting with the microtubule, and these values were converted to run length and velocity. The mean run length was determined by fitting of the cumulative probability distribution of the data as described (Thorn *et al*, 2000), in which the lower limit of the run included in the analysis was set to 0.3 μm. Since the photobleaching rate of Cy3 fluorophore was significantly lower than the detachment rate of kinesin from the microtubule (0.03-0.05 s^{-1} and 0.45-0.83 s^{-1} for the rate of photobleaching and the rate of kinesin detachment, respectively), the run length was not corrected for photobleaching. The mean velocity was calculated by fitting the Gaussian distribution.

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Supplementary Table SI Oligonucleotides used for mutagenesis.

Primer name	Sequence
TUB2_F2	5'-TGGTAGAACACTCTGATG-3'
TUB2-SmaI_R	5'-AACCCGGGACCGCTTCCACAATCATC-3'
TUB2-E410A_F	5'-CTCT**GCT**GCTGAATCTAATATGAATG-3'
TUB2-E410A_R	5'-CATATTAGATTCAGC**AGC**AGAGAATTC-3'
TUB2-E412A_F	5'-GGCT**GCT**TCTAATATGAATGATCTGG-3'
TUB2-E412A_R	5'-CATTCATATTAGA**AGC**AGCCTCAGAG-3'
TUB2-D417A_F	5'-GAAT**GCT**CTGGTTAGCGAATACCAAC-3'
TUB2-D417A_R	5'-GTATTCGCTAACCAG**AGC**ATTCATATTAG-3'
TUB2-E421A_F	5'-GTTAGC**GCT**TACCAACAATACCAAGAG-3'
TUB2-E421A_R	5'-GTATTGTTGGTA**AGC**GCTAACCAGATC-3'

TUB2_F2 and TUB2-SmaI_R were used as outer primers. The codons targeted for mutagenesis are indicated in bold and underlined.

Supplementary Table SII Yeast strains and plasmids used in this study.

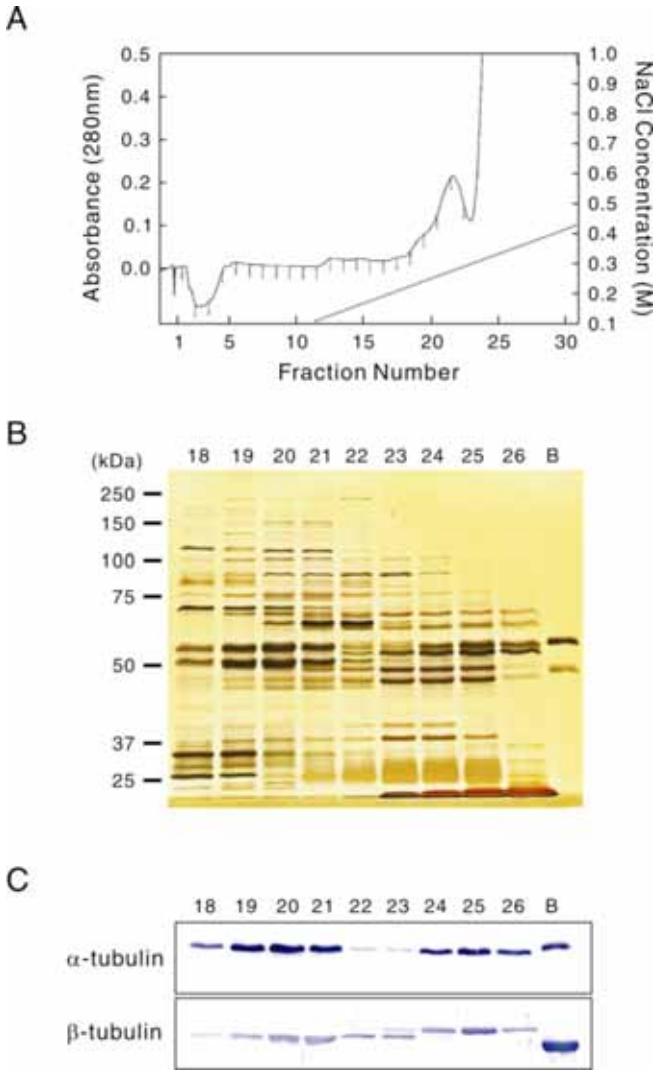
Designation	Description	Reference
Yeast Strains		
YPH499	*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1*	Ref. (1)
SUY57	*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, tub1::natNT2, tub2::hphMX4, tub3::ADE2, pSU205*	This study
SUY104	*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, tub1::natNT2, tub2::hphMX4, tub3::ADE2, pSU207, pSU251*	This study
SUY120	*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, tub1::natNT2, tub2::hphMX4, tub3::ADE2, pSU207, pSU238*	This study
SUY121	*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, tub1::natNT2, tub2::hphMX4, tub3::ADE2, pSU207, pSU240*	This study
SUY148	*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, tub1::natNT2, tub2::hphMX4, tub3::ADE2, pSU207, pSU278*	This study
SUY152	*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, tub1::natNT2, tub2::hphMX4, tub3::ADE2, pSU207, pSU278, LEU2::GAL10pro-TUB1-GAL10pro-tub2^{tax}-E410A*	This study
SUY153	*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, tub1::natNT2, tub2::hphMX4, tub3::ADE2, pSU207, pSU278, LEU2::GAL10pro-TUB1-GAL10pro-tub2^{tax}-D417A*	This study
Plasmids		
pRS305	*LEU2*-based integration plasmid	Ref. (1)
pRS313	*HIS3*-based CEN plasmid	Ref. (1)
pRS314	*TRP1*-based CEN plasmid	Ref. (1)
pRS315	*LEU2*-based CEN plasmid	Ref. (1)
pRS316	*URA3*-based CEN plasmid	Ref. (1)
pSU205	pRS316 carrying *TUB1* and *TUB2*	This study
pSU207	pRS314 carrying *TUB1*	This study
pSU251	pRS315 carrying *TUB2^{tax*}*	This study
pSU238	pRS315 carrying *tub2^{tax}-E412A*	This study
pSU240	pRS315 carrying *tub2^{tax}-E421A*	This study
pSU278	pRS313 carring *tub2^{tax}-plusE*[§]	This study
pSU342	pRS305 carrying *GAL10pro-TUB1-GAL10pro-tub2^{tax}-E410A*	This study
pSU345	pRS305 carrying *GAL10pro-TUB1-GAL10pro-tub2^{tax}-D417A*	This study

[*] *TUB2^{tax}; tub2-A19K-T23V-G26D-N227H-Y270F*

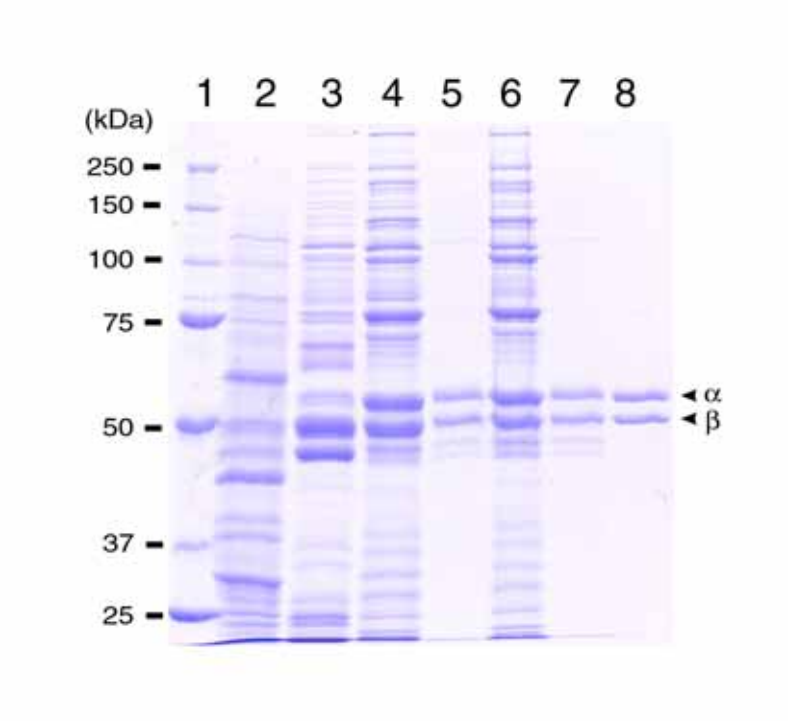
[§] *tub2^{tax}-plusE; tub2$^{tax\text{-}440}$GDFGEEEEGEEEEGEEEEGEEEEGEEEEA*

Reference

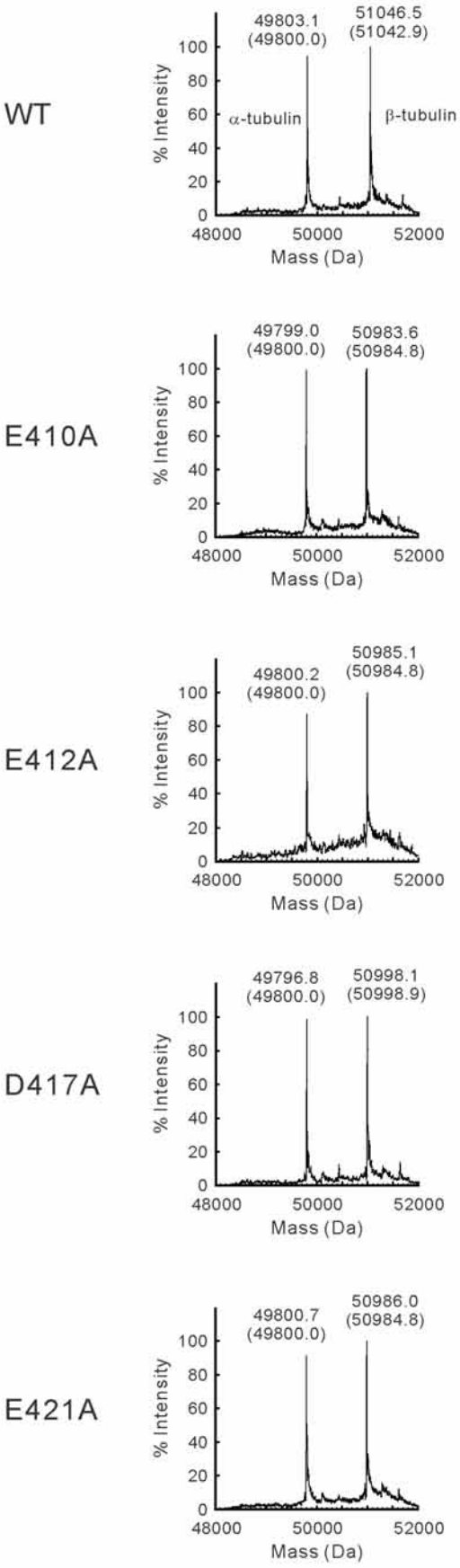
1. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19-27



Supplementary Figure S1 Separation of two species of tubulin dimers by MonoQ column. The DEAE-sepharose-purified crude tubulin fraction obtained from the cell lysates of the yeast strain SUY152 was further fractionated on a MonoQ column to separate the two types of tubulin dimers, one composed of Tub2^{tax}p-plusE and the other composed of Tub2^{tax}p-E410A. (**A**) An elution profile of the MonoQ column, each fractionated in 1 ml aliquots and monitored by UV absorption at 280 nm (*solid line*). Dashed line indicates the NaCl concentration. Each of the fractions (10 μl) eluted at 0.21-0.38 M NaCl (fraction no.18-26) were resolved by SDS-PAGE, and analyzed by Silver staining (**B**) and by Western blotting probed with either α- or β-tubulin-specific antibody (**C**). In both (B) and (C), the right most lane labelled as "B" contains 0.1 μg of porcine brain tubulin. The tubulin dimer containing Tub2^{tax}p-E410A was eluted at 0.25-0.27 M NaCl, whereas the dimer containing Tub2^{tax}p-plusE was eluted at 0.34-0.36 M NaCl.



Supplementary Figure S2 SDS-PAGE analysis of the protein fraction isolated from the yeast cell lysates (wild-type). Tubulin was purified through a couple of anion exchange column chromatographies followed by a cycle of polymerization and depolymerization. The fractions in each preparative process were loaded on a 7.5% SDS-PAGE gel and stained with Coomassie Blue. Lane 1, molecular weight standard; Lane 2, crude cell lysate (10 μg); Lane 3, the tubulin-containing fraction eluted from DEAE-sepharose (10 μg); Lane 4, the tubulin-containing fraction eluted from the MonoQ column (10 μg); Lane 5/6, the pellet (1 μg)/supernatant (10 μg) after the polymerization of microtubule and centrifugation at 200, 000× *g*; Lane 7/8, the pellet (1 μg)/supernatant (1 μg) after the depolymerization of microtubule and the brief centrifugation at 20, 000× *g*. The supernatant tubulin fraction was frozen in liquid nitrogen and stored at -80 ˚C until use.



Supplementary Figure S3 Representative deconvoluted electrospray ionization mass spectra of the full length α- and β-tubulin polypeptides in positive mode. In both wild-type and mutants, the molecular mass determined for each α- and β-polypeptides, as labelled on each mass peaks, coincides well with the theoretical masses of α- and β-tubulin without any posttranslational modification (shown in parentheses), indicating that both polypeptides primarily lack posttranslational modification.