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Autoinhibition of TBCB regulates EB1-mediated microtubule dynamics

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Autoinhibition of TBCB regulates EB1-mediated microtubule dynamics

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Abstract

Tubulin cofactors (TBCs) participate in the folding, dimerization, and dissociation pathways of the tubulin dimer. Among them, TBCB and TBCE are two CAP-Gly domain-containing proteins that interact and dissociate the tubulin dimer. Here we show how TBCB localizes at spindle and midzone microtubules during mitosis. Furthermore, the motif DEI/M-COO^- present in TBCB, which is similar to the EEY/F-COO^- element characteristic of EB proteins, CLIP-170, and α-tubulin, is required for TBCE–TBCB heterodimer formation and thus for tubulin dimer dissociation. This motif is responsible for TBCB autoinhibition, and our analysis suggests that TBCB is a monomer in solution. Mutants of TBCB lacking this motif are derepressed and induce microtubule depolymerization through an interaction with EB1 associated to microtubule tips. TBCB is also able to bind to the chaperonin complex CCT containing α-tubulin, suggesting that it could escort tubulin to facilitate its folding and dimerization, recycling or degradation.

Introduction

The cytoskeleton of eukaryotic cells is required for many essential cell processes such as motility, organelle and membrane structural integrity, intracellular trafficking, chromosome segregation, and cytokinesis [1]. Microtubules are complex polar polymers of the cytoskeleton that assemble from αβ-tubulin heterodimers. The heterodimers polymerize, forming protofilaments that associate laterally, forming the wall of a hollow cylinder, the microtubule[2,3]. Therefore, within the microtubule lattice, each single α-tubulin or β-tubulin subunit interacts with four other neighboring tubulin subunits. In fact, each α-tubulin subunit interacts with its β-tubulin partner inside of the heterodimer, with a second β-tubulin subunit from the preceding heterodimer in the protofilament, and laterally with two α-subunits from the two side protofilaments. Thus, the assembly of a microtubule, while preventing unwanted interactions, is a highly complex task that must be properly controlled to avoid critical errors.

Tubulin folding cofactors (TBCs) are a set of different proteins discovered a decade ago in the so-called "postchaperonin" tubulin folding pathway. TBCs are responsible for the achievement of the quaternary conformation of the αβ-heterodimer after tubulin monomers have reached their tertiary structure [4,5]. More recent studies have shown that in vivo, these proteins are implicated in microtubule dynamics through their ability to dissociate the tubulin heterodimer, and probably by controlling tubulin monomer quality and exchange (shuffling mechanism) [6-8].

TBCB and TBCE are two well-conserved α-tubulin interacting proteins that collaborate in the regulation of microtubule dynamics [6-9]. Both cofactors participate in the α-tubulin folding pathway and are required for cell survival [5, 10], playing important roles in vivo as revealed by the plethora of human disorders in which they are implicated. TBCE mutations cause a syndrome called hypoparathyroidism-retardation-dysmorphism, also known as the Sanjad–Sakati syndrome [11] in humans, and a progressive motor neuropathy in the mouse [12]. TBCB, on the other hand, has been implicated in human

cancer [13], neurodevelopmental malformations [14], schizophrenia [15], and neurodegenerative processes [16].

TBCB shares with TBCE two similar domains, a CAP-Gly domain at the N-terminus, and a UBL domain at the C-terminus, but while TBCB it is not able to interact with or dissociate the tubulin heterodimer by itself, TBCE is, per se, effective in promoting this dissociation. Nonetheless, TBCE interacts with TBCB, originating the TBCE–TBCB complex, which displays a more efficient stoichiometric tubulin dissociation activity than TBCE alone. Upon dissociation, TBCB, TBCE, and α-tubulin form a stable ternary complex. The disassembly of this ternary complex results in either TBCB and α-tubulin, and free TBCE, or TBCE and α-tubulin, and free TBCB. Free β-tubulin subunits might be recyclable in the presence of TBCA or TBCD [9].

The function of the CAP-Gly domains of both cofactors is still unknown. This domain is a protein-interaction module that typically plays a role controlling microtubule end dynamics in end-binding proteins (EBs), which can track along microtubule ends [17-19]. In addition to EBs, an increasing number of proteins that control microtubule organization and dynamics, known as microtubule plus-end-tracking proteins (+TIPs), have been identified. These proteins connect to the microtubule plus ends through an interaction with members of the EB family [17-19], the only known protein family that can track microtubule ends autonomously. Recently, a long list of +TIPs candidates has been published by Yu et al. (2011) [20], but neither TBCB nor TBCE has been included.

In this work, we have used a multidisciplinary approach to study the molecular mechanism of TBCB's regulation of microtubule dynamics. For this purpose, we have cloned the human *Tbcb* gene and characterized mutant versions of its product, having established that the last three amino-acid residues of this protein are crucial for TBCB autoinhibition. In fact, the overexpression of the mutated form of TBCB lacking the DEI/M-COO^- motif, similar to the EEY/F-COO^- element in EB1 and related proteins, produces a massive microtubule destruction in vivo. Using extensive biophysical and biochemical approaches, we unmasked the molecular mechanism by which TBCB controls microtubule depolymerization by means of EB1. In addition, we show for the first time that TBCB interacts directly with cytosolic chaperonin containing TCP-1 (CCT) during the folding process of α-tubulin. All the results obtained from this work led us to propose three different models to explain the autoinhibition of TBCB, its role in tubulin folding as a CCT cofactor, and the mechanism by which the deregulation of TBCB activity induces the microtubule catastrophe in living cells.

Materials and Methods

Human TBCB gene cloning

The human *tbcb* coding sequence was amplified by PCR from a testis cDNA sample (BD Biosciences, USA) using a pair of primers designed with the appropriate restriction enzyme recognition sites at their ends: forward primer 5′ GTG AAG CTT CAT ATG GAG GTG ACG GGG GTG 3′; reverse primer 5′ CGC GGA TCC TCA TAT CTC GTC

CAA CCC 3′. The amplified coding sequence was then inserted in the *Hind*III and *Bam*HI sites of the mammalian expression vector pcDNA3.1 (Invitrogen, Life Technologies, USA) to generate the pcDNA3.1-TBCB recombinant plasmid. Human TBCB was cloned into the pEYFP vector from Clontech (Clontech Laboratories, USA). TBCBΔ3 and TBCBΔ9 cDNA fragments were produced by PCR. The resulting fragments were cloned into pET29c and sequenced (EMD Millipore Bioscience Novagen, USA).

TBCA and TBCE protein purification and characterization

Human TBCE cDNA wild-type (accession number **U61232**) and human TBCA [21] were His-tagged at the C-terminus and cloned into the pRJ-pFastBac vector [8] for recombinant baculovirus production using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Life Technologies, USA). These were then used to infect commercially obtained Sf9 insect cells to produce recombinant TBCE, which was purified following protocols already described elsewhere with minor modifications [8,9]. TBCAHis was purified from a 50 mL culture of Sf9 cells infected with baculoviruses carrying the human TBCAHis cDNA cloned. Cells were pelleted by centrifugation, washed, and stored frozen at –70°C. Pellets were resuspended in 7.5 mL of 0.5 mM Tris buffer (pH 8) containing protease inhibitors and sonicated three times during 20 seconds at 4°C. Extract was spun at 60,000 ×*g* in a Ti 50.2 ultracentrifuge rotor (Beckman-Coulter) for 30 min at 4°C. Supernatant was supplemented with a buffer stock to produce final concentrations of 50 mM Tris-buffer (pH 8) and 500 mM NaCl and 10 mM imidazole and loaded into a 1 mL His-Trap column (GE Healthcare). Fractions containing TBCA–His were pooled and concentrated by ultrafiltration using an Amicon Ultra 10K filter (Millipore, USA). Protein concentrate was applied to a high-resolution gel-filtration column (Superdex-75 HR, GE Healthcare, USA), equilibrated and eluted with 20 mM Bis-Tris buffer (pH 7) containing 100 mM KCl, 1 mM DTT and 0.5 mM PMSF at 0.4 mL/min.

Nonclassical two-dimensional electrophoresis

In the first dimension, the protein complexes were fractionated by charge and shape, and then were denatured and their molecular composition determined in the second dimension. The samples were loaded onto a native 0.75 mm thick minigel (7 cm × 8 cm) as described [22, 23]. After two hours of electrophoresis, a single running lane containing the native electrophoresed sample was excised with a blade on glass, loaded onto a preparative 1.5 mm thick SDS-minigel (7 cm × 8 cm), and fixed to the gel with 0.5% agarose prepared in 1× SDS loading buffer. Denaturing electrophoresis was performed for three hours at 10 mA constant current, after which the gel was stained with Coomassie Blue G-250. In a similar manner, bands of interest were excised with a blade on glass, dried in a Speed-Vac concentrator (Thermo Fisher Scientific, USA) and rehydrated with loading 1× SDS loading buffer, heated at 90°C for 2 min, and loaded onto a regular 8.5% SDS minigel. Electrophoresis was performed as described above.

Antisera production, immunocytochemistry, and cell cultures

Affinity-purified primary antibodies were produced against purified human TBCB recombinant protein. Rabbit sera were affinity purified as described previously [24]. For immunocytochemistry, the antibodies used were anti-α- and anti-β-tubulin (B512 and Tub2.1, respectively) and anti-acetylated tubulin from Sigma-Aldrich. The anti-glutamylated tubulin antibody (GT335) was a gift from Dr. Janke (CNRS, Montpellier, France). Secondary antibodies were Alexa-Fluor-488-conjugated goat anti-rabbit IgG and goat-anti-mouse IgG, Alexa-Fluor-647-conjugated goat anti-mouse IgG (Molecular Probes, Invitrogen), Cy3-conjugated goat anti-mouse IgG and goat anti-mouse IgG_1, and Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). For some experiments, microtubules were depolymerized with 2 μM nocodazole and cold (4°C) treatments for 30 min.

Microtubule depolymerization experiments

Bovine brain tubulin was purified as described [25]. Purified tubulin was incubated at 35°C for 20 minutes, and as a control, TBCB or ovalbumin was added for another 20 minutes in buffer A (MES 100 mM pH 6.7, EGTA 1 mM, and $MgCl_2$ 1 mM) with 2 mM GTP and 30% glycerol. The pellet and supernatant were separated after centrifugation at 45,000 ×*g* for 1 hour at 30°C through a 50% sucrose cushion containing 1 mM GTP.

Fractionation by gel filtration of complexes formed between cofactors TBCB and TBCE and TBCB

Purified tubulin cofactors and complexes formed in reactions conducted at 30°C for 30 minutes were fractionated in a Superdex 200 PC 3.2/30 gel-filtration precision column using an Ettan LC (GE Healthcare) at room temperature. The elution buffer contained 0.1 M MES (pH 6.7), 1 mM $MgCl_2$, 1 mM EGTA, and 25 mM KCl. Fractions of 25 μL were eluted at 40 μL/min and were analyzed by SDS–PAGE.

Confocal microscopy, cell counts, and statistical analysis

Transitory transfection experiments were performed using Lipofectamine Plus reagent (Life Technologies) or the FuGene 6 reagent (Roche) following the manufacturer's instructions. The GFP:EB1 construct was kindly supplied by Dr. Akhmanova (Utrecht University, The Netherlands). Cell counts in Figure 1C were performed at 30 h post transfection using a 63× Zeiss oil immersion objective starting from a random field and scanning horizontally from that point. Values presented in the histogram of Figure 1C were obtained by double immunofluorescence with anti-α-tubulin/TBCB. Values in Figure 5D were obtained by double immunofluorescence with anti-α-tubulin/TBCB combined with Hoechst 33258 and GFP labeling in cotransfection experiments. Cell counts were performed on confocal microscopy projection images using a Nikon A1R LSM confocal microscope equipped with an argon (488 nm) laser, two HeNe (564 and 633 nm) lasers, and a diode (405 nm) laser. Only cells with no microtubules, which

presented healthy-looking nuclei, as assessed by Hoechst staining, were considered. In colocalization experiments, images were scanned sequentially to avoid fluorescent channel emission cross talk / bleed through. A *t* test was performed on data obtained from two different coverslips of at least three different experiments. Statistical analysis of data and graphing were performed using SigmaPlot 8.0 software (Systat Software, Richmond, CA). Histograms represent mean values and standard error bars.

Tubulin dimer dissociating assay and nondenaturing electrophoresis

Aliquots of purified brain tubulin were mixed with different amounts of purified TBCE in 15 μL reactions containing 50 mM MES (pH 6.7), 1 mM $MgCl_2$, and 1 mM GTP in the absence and presence of a stoichiometric excess of TBCB or TBCBΔ3, and incubated for 30 min at 30°C. The reaction mixtures were diluted with a sucrose-containing native loading buffer and loaded onto a 6% nondenaturing polyacrylamide gel [22, 23]. Native gels were stained directly with Coomassie Brilliant Blue.

Affinity chromatography

Purified TBCBΔ3 was coupled specifically at its amino terminus using EDC-NHS coupling chemistry in a Hi-trap NHS-activated HP column (GE Healthcare). This column gave us complete control over the experimental conditions (extract preparation, column loading, bound partner elution, time, and temperature). Thus, HEK293 cell extracts were prepared, sonicated to fragment microtubules, and loaded into the column at 4°C, to avoid protein degradation. We used a slow loading rate, to allow binding of the interactors to the column. 300 mg of HEK293 were resuspended and subject to a hypotonic shock in Tris buffer 20 mM (pH 7.3) and PMSF 0.5 mM (buffer H). Subsequently, cells were sonicated three times during 30 s at 130 watts at 4°C. Protein extract (1 mL at 18 mg/mL) obtained from human HEK293 cells was applied to the NHS column equilibrated in buffer H. The column was washed with 10 mL of the same buffer, and specifically interacting proteins (detail) were eluted with a NaCl gradient (red line).

Results

Auto-inhibition of TBCB

TBCB is encoded by a unique gene in the human genome. This protein is composed of two functional structural domains connected by a coiled-coil segment (Fig 1A). At the N-terminus, TBCB contains a ubiquitin-like domain. This domain is spherical (PDB ID, 1V6E), behaves as a monomer of about 14 kDa and is a ubiquitous protein interaction domain present in many unrelated proteins. The C-terminal domain is a CAP-Gly characteristic of +TIPs proteins. This domain is also globular, with a three-layer β–β structure (three antiparallel β-sheets), as represented by the *C. elegans* F53f4.3 protein CAP-Gly domain (Fig 1A, PDB: 1TOV, [26]). The unique α-helix is preceded by a disordered stretch of 17 residues, and the last 6–7 amino acid residues protrude from the globular domain. CAP-Gly domains serve as recognition domains for EEY/F-COO^-

peptides [27]. This sequence assumes an extended conformation, and the side chain of the terminal tyrosine packs with several hydrophobic amino acid residues in the CAP-Gly domain [28]. The crystal structure of the CAP-Gly of TBCB (*C. elegans*) revealed that this domain consists of 84 amino acid residues, and although it does not form a dimer in vitro, the conserved groove, involved in the interaction with EEY/F-COO^- elements characteristic of EB, CLIP-170, and α-tubulin, holds the C-terminal peptide of the neighboring molecule in the asymmetric unit of the crystal [26].

The structural prediction [29] for the human TBCB's last nine amino acid residues (Fig 1a) is that of a disordered peptide, protruding from the globular domain and thus being able to interact with a CAP-Gly domain groove. Indeed, theoretical models [28, 30] have shown putative interactions between the $p150^{Glued}$ CAP-Gly domain and the C-terminal peptide of EB1 and TBCB.

Taking into account the structural features of the TBCB C-terminal domain, we decided to go further in understanding the TBCB and TBCB C-terminus interaction and to determine whether the C-terminal region would also affect the tubulin binding ability of TBCB. These ideas led us to propose the hypothesis of an autoinhibitory mechanism where the TBCB C-terminal extension folds over the globular part of its own CAP-Gly domain and structurally blocks the conserved groove involved in the interaction with EEY/F-COO^- elements characteristic of EBs, CLIP-170, and α-tubulin. For this purpose, we have cloned the human *Tbcb* gene and constructed two *Tbcb* mutants lacking the last three (TBCBΔ3) and last nine (TBCBΔ9) amino acid residues, predicted to be unstructured. These truncated proteins were overexpressed in HeLa cells and visualized using new polyclonal anti-TBCB antibodies (see Material and Methods).

Previously, we showed that the overexpression of either TBCE or TBCD in human cell lines leads to the sequestration of free α- and β-tubulin respectively, leading to massive microtubule depolymerization [6-8]. On the other hand, murine TBCB overexpression only leads to a moderate microtubule depolymerization effect, probably because of the limiting concentrations under TBCB overexpression conditions, of endogenous TBCE required for the binding and dissociation of the tubulin heterodimer [9].

Unpredictably, overexpression of the TBCBΔ3 mutant in HeLa cells induced a massive microtubule destruction effect, comparable only to that observed upon TBCE overexpression (Fig 1b; [9]). Quantification of the microtubule destruction effect revealed that at 30 h post transfection, over 60% of the TBCBΔ3 positive cells exhibited no detectable microtubules, while only less than 20% of the overexpressing cells had an apparently unaffected microtubular cytoskeleton. Similar results were obtained for the TBCBΔ9 mutant. These findings strongly support the proposed idea that TBCB is self-inhibited by its C-terminus and that the removal of only the last three amino-acid residues from this domain is sufficient to activate TBCB.

These results prompted us to investigate whether TBCBΔ3 was able to depolymerize microtubules assembled in vitro. For this purpose, we purified brain tubulin and the untagged TBCBΔ9 and TBCBΔ3 proteins (Fig S1a). Stoichiometric amounts of purified TBCBΔ3 or ovalbumin (negative control) were incubated with GTP and polymerized purified tubulin. The incubation mix was then centrifuged, and both the soluble and insoluble fractions were analyzed by SDS-PAGE to determine the amounts of tubulin and

TBCBΔ3 proteins in the two fractions (Fig S1b). These experiments revealed that TBCBΔ3 was essentially present in the supernatant fractions. Moreover, similar amounts of tubulin were found in the supernatant and pellet fractions in the presence or absence of TBCBΔ3, which were also similar to those found when ovalbumin was used (Fig S1b). These results lead to the conclusion that TBCBΔ3 is not able to depolymerize microtubules in vitro, presumably because of the lack of a factor mediating its in vivo effect.

The proposed model for TBCB's autoinhibition implies the interaction of the C-terminal tail of this cofactor with its own CAP-Gly domain. To test this hypothesis, we performed quantitative binding assays by using fluorescence polarization of fluorescein labeled peptides (Table 1; see also Supplementary Material and Supplementary Table 1). The equilibrium dissociation constants show that the C-terminal nonapeptide (peptide 1) presents a higher affinity for TBCB (12 μM) than that displayed by the same peptide lacking the last three residues (peptide 2), which was six times lower. Similarly, the C-terminal deleted proteins TBCBΔ3 and TBCBΔ9 showed a sixfold reduction in the affinity for the complete C-terminal peptide of TBCB (peptide 1). Besides, the binding affinities exhibited by the C-terminal deleted peptide 2 for the deleted TBCB forms are significantly lower than for the full-length protein. Together these results strongly support the idea that the last three amino acid residues of the TBCB C-terminus are involved in the binding to TBCB, thus reinforcing our model of TBCB autoinhibition.

Finally, CAP-Gly domains have a high affinity for the C-terminal tail of α-tubulin (i.e., CLIP-170 CAP-Gly domain 2; [31]). In order to corroborate our model, we have also quantified the binding affinity of TBCB to two different α-tubulin peptides (Tables 1 and S1; peptide 3 and peptide 4). We designed a peptide (GEGEEEGEEY) corresponding to the C-terminus of α-tubulin isotypes 1 and 2, containing the last tyrosine residue, known to be critical for binding to CAP-Gly domains [31]. As expected, all three polypeptides (TBCB, TBCBΔ3, and TBCBΔ9) displayed a K_D value higher than that exhibited by the TBCB-peptide (EEDYGLDEI). In the case of wild-type TBCB and α-tubulin (GEGEEEGEEY), the K_D is about four times higher (43 μM) than that obtained for the TBCB peptide (EEDYGLDEI).

Altogether, the results indicate that TBCB does not interact with tubulin dimers and demonstrate that the TBCB CAP-Gly domain is functionally different from other CAP-Gly domains. Finally, they confirm that TBCB is autoinhibited by the last three amino acid residues of its C-terminus.

The C-terminal acidic tail of TBCB is responsible for TBCE interaction

Previous work from our group has shown that when incubated together, purified mammalian TBCB and TBCE produce a new peak in gel filtration analysis chromatograms that correspond approximately to the sum of the molecular masses of individual TBCB and TBCE [9]. Further analysis of this peak revealed the presence of both cofactors suggesting that purified TBCB forms a binary complex with TBCE ([9] and Fig 2a, left panel). Bearing in mind these results and that the last three amino acid residues of the TBCB C-terminus are critical for its activity, we have investigated

whether the TBCBΔ3 or TBCBΔ9 truncated proteins are also able to dimerize with TBCE. Interestingly, when purified TBCE is incubated with purified TBCBΔ3 (Fig 2a, right panel) no additional peaks are detected in gel filtration chromatograms. Indeed, chromatograms only revealed peaks corresponding to the species present when the single purified proteins were analyzed. This observation was confirmed by SDS-PAGE analysis of the corresponding fractions (Fig 2a). These results strongly suggest that none of the TBCB C-terminus deleted mutants is able to interact with TBCE and that the last three amino acid residues of TBCB are essential for the TBCE recognition.

TBCE and TBCB, the tubulin heterodimer dissociation machine

Because the TBCE and TBCB interaction is required for an efficient tubulin heterodimer dissociation activity, the results above predict that the last three amino acid residues of TBCB are also critical for this process. Therefore, we have investigated the tubulin heterodimer dissociation activity of TBCE in the presence of either full-length TBCB or TBCBΔ3 and have quantified the different molecular species produced by native-PAGE. When tubulin heterodimers are incubated with TBCE in the presence of TBCB, an extra band (Fig 2b), which probably corresponds to the ternary complex composed of TBCB, TBCE, and α-tubulin, is detected in native gels. To confirm the composition of this band (Fig 2b, second dimension), we have analyzed the sample where TBCE was incubated with TBCB and α-tubulin using a nonclassical two-dimensional PAGE method [23]. Thus the incubation sample was analyzed in a native gel for the first dimension (Fig 2b). Then, to resolve the complex's composition, the respective lane of the native gel was directly applied into a denaturing gel for the second dimension. This analysis confirmed that the new extra band corresponds to the ternary complex (Fig 2b and 2d). Interestingly, in similar incubations where TBCB was replaced by TBCBΔ3, the ternary complex is absent, and instead an extra band that migrates a little bit less than that corresponding to tubulin heterodimers was found. To determine the molecular composition of this new band, we performed a second-dimension analysis similar to that described above (Fig 2c and 2d). This analysis showed that this band corresponds to the binary complex containing α-tubulin and TBCBΔ3. Therefore, TBCBΔ3 is able to interact with α-tubulin but not with TBCE.

TBCE and TBCD were characterized as the tubulin cofactors that are able to dissociate the tubulin heterodimer [6-8]. Although it was stated that TBCB cannot dissociate the tubulin heterodimer, we decided to investigate whether TBCB or TBCBΔ3 or both also have this ability. For this purpose, we decided to repeat the dissociation experiments by performing them in the presence of TBCA to improve the visualization of the tubulin heterodimer dissociation. It was previously shown that this cofactor, capturing the β-tubulin subunits emerging from tubulin dimers dissociated by TBCE, forms a binary complex detectable in native gels [8]. As observed in Fig 2d, when tubulin heterodimers are incubated with TBCE in the presence of TBCB and TBCA, dissociation is complete, forming the ternary complex (TBCB, TBCE, and α-tubulin) described above, which comigrates with TBCA and the binary TBCA and β-tubulin complex. On the other hand, when TBCE and tubulin heterodimers are incubated with TBCBΔ3 instead of TBCB, the

ternary complex is not detected, but binary complexes containing TBCBΔ3 and α-tubulin, and TBCA and β-tubulin are clearly visible. In contrast, neither TBCB nor TBCBΔ3 by itself gives rise to the formation of TBCA and β-tubulin complexes, demonstrating that they do not have the ability to dissociate the tubulin heterodimer in the absence of TBCE (Fig 2d).

To understand better the role of these cofactors in tubulin heterodimer disruption, we decided to perform a time course of the dissociation activities of TBCE alone and in the presence of stoichiometric amounts of TBCB or TBCBΔ3. We observed that while TBCE alone dissociates 60% of the tubulin heterodimers in 30 minutes (Fig 2e), TBCE in the presence of TBCB is able to dissociate about 90% in less than 30 seconds (the minimal time required to mix the incubation components and load it into the gel). In contrast, in the same period, but in the presence of TBCBΔ3, only 25% of tubulin heterodimers are dissociated by TBCE. Although in the presence of TBCE there is a clear difference between the dissociating activities of the TBCB and the TBCBΔ3 (90% to 25%), the small increase in the dissociation activity percentage in the presence of TBCBΔ3 compared with that observed for TBCE alone (9%) might be because TBCBΔ3 can form a binary complex with α-tubulin that would facilitate the dissociation activity of TBCE (Fig 2f and 2g).

Together, our data clearly show that the last three residues in TBCB are not required for the formation of the binary complex with α-tubulin. However, they are not only implicated in TBCB autoinhibition but also essential for the interaction of TBCB with TBCE and therefore required for the assembly of an efficient tubulin heterodimer dissociation machine.

Moreover, based on these results, we put forward the hypothesis that TBCE would interact with the C-terminus of TBCB, which would lead to the derepression of this cofactor triggering a microtubule catastrophe. However, the observation that neither TBCBΔ3 nor TBCBΔ9 (results not shown) is able to interact with TBCE is still puzzling because their overexpression resulted in massive microtubule depolymerization in HeLa cells (see Fig 1b and 1c).

In solution, TBCB is a monomer as revealed by biophysical studies and cross-linking experiments

The possible TBCB autoinhibition is supported by the interaction observed between the C-terminal peptide molecules in the crystal structure of the CAP-Gly domain of *C. elegans* TBCB [26]. To obtain clues regarding the behavior of native TBCB that could help us to elucidate the molecular mechanism underlying its autorepressed activity, we used different experimental approaches to investigate whether TBCB is a monomer or a dimer in solution. For this purpose, we first determined the circular dichroism (CD) spectra of the TBCB, TBCBΔ3, and TBCBΔ9 proteins (Supplementary Material). The CD spectra of the three proteins (Fig S2a, left panels) was characterized by the presence of two minima at 208 and 217 nm, which are indicative of a mixed population of α-helical (20%, $[\theta]_{222/208} = 0.87$) and β-strand (30%) conformations. The absence of a minimum value at 222 nm (typical of all α proteins) and a zero crossing (typical of a α-

helices and β-strands) suggest the additional presence of disordered (random coils and turns) structures (50%).
Subsequently, we decided to study the state of aggregation of these proteins by dynamic light scattering, but obtained inconclusive results (DLS, [32]; see Supplementary Material). By using a gel filtration analysis, we have previously characterized the molecular components of the different complexes formed between TBCB, TBCE, and tubulin [9]. Based on the elution volumes, the estimated molecular mass of TBCB was 30%–40% larger (40 kDa) than that predicted from its amino acid sequence (27 kDa). Curiously, this value for TBCB coincided with the molecular mass estimated from its mobility on SDS-PAGE. However, the apparent molecular mass of the complexes formed between TBCB and TBCE, and between TBCB and TBCE and α-tubulin, suggested that they were the result of the sum of the molecular masses of the monomeric subunits. These apparent discrepancies in the values of TBCB molecular masses were maintained upon DLS estimations of the molecular mass of TBCB (69 kDa), TBCBΔ3 (53 kDa), and TBCBΔ9 (34 kDa; Fig S2b). In order to clarify these observations, we decided to investigate the state of oligomerization of the three proteins by cross-linking experiments (Fig 3a) and analytical ultracentrifugation (Fig 3b). We used glutaraldehyde as a general protein cross-linker and a protein (EB1) with a very low K_D for dimer formation, as a positive control. Interestingly, the results revealed that purified TBCB, as well as TBCBΔ3, migrates in SDS-PAGE as a single band with an apparent molecular mass of about 38–40 kDa. Nevertheless, when these two proteins are incubated with glutaraldehyde (0.05%), the band corresponding to 40 kDa, although still visible in trace amounts, is substituted by a new band corresponding to species with a molecular mass of about 27 kDa (Fig 3a). This molecular mass is in agreement with the theoretical molecular mass of these two proteins. On the other hand, the EB1 control protein, which normally migrates as a single band of 32–34 kDa after glutaraldehyde treatment, migrates as a band of 64 kDa, which is consistent with its size in gel filtration experiments (Fig S2d). These results were confirmed by an analytical ultracentrifugation analysis that was performed at 20°C at 160,000 ×*g* (Fig 3b). Indeed, the analytical ultracentrifugation showed that the molecular mass of TBCB was ~25.5 kDa, corresponding to the size of the monomer (27 kDa). Taken together, all these results led us to conclude that TBCB behaves as a monomer and to suggest that its self-inhibition occurs within the same molecule and not between two or more TBCB molecules.

TBCB localizes to the centrosome and mitotic spindle microtubules

TBCB has been shown to colocalize with Pak1 protein on newly polymerized microtubules [13]. Because the overexpression of TBCB and TBCBΔ3 leads to microtubule depolymerization, we decided to investigate the subcellular distribution of the overexpressed YFP:TBCB protein in HeLa cells throughout the cell cycle.
As observed for the wild-type endogenous protein [9], YFP:TBCB is mostly a soluble cytoplasmic protein in interphase cells (Fig 4a). A prominent spot of YFP:TBCB is often localized at the centrosome (double spot) and at the base of the primary cilium (Fig 4B). Indeed, the distribution of YFP:TBCB during mitosis revealed two clear YFP:TBCB spots at prophase (Fig 4a, top middle). Interestingly, as mitosis progressed toward

metaphase, TBCB was also localized to spindle microtubules (Fig 4a, top right). This localization was also observed for overexpressed wild-type untagged TBCB by immunostaining and is in accordance with previous analyses performed for endogenous TBCB in human and mouse cells [9,13,33]. Furthermore, with this new analysis, we observed that during anaphase A, YFP:TBCB becomes more visible as thin filaments bridging the midzone, and by anaphase B most of this cofactor had progressively disappeared from the centrosome and was concentrated on the midbody microtubules (Fig 4a, bottom). At the end of telophase, TBCB was apparently absent from the centrosome, concentrating in a unique spot at the midbody. These localization results show evidence that TBCB can bind to microtubules. However, we know from all the results described above that TBCB cannot recognize tubulin heterodimers, suggesting this binding to be indirect, occurring through the interaction of TBCB with a microtubule binding protein.

TBCBΔ3 interacts with EB1 and the cytosolic chaperonin CCT

That in vivo TBCB is able to localize at mitotic spindle microtubules and to promote microtubule destabilization, whereas in vitro it is not able to depolymerize or even to interact with microtubules, strongly suggests that in vivo TBCB should have an interactor(s) that mediates its functions toward microtubules. This prompted us to search for TBCB molecular interactors. In a first approach, we have performed different experiments such as yeast two-hybrid and immunoprecipitation techniques, but we were unsuccessful in the identification of any TBCB interactor. This could be ascribed to the fact that we have not used specific conditions required to avoid disruption of weak interactions. To overcome these problems, we constructed an affinity column with bound recombinant untagged TBCBΔ3, the derepressed version of TBCB. Bound proteins or complexes were specifically eluted with a salt gradient that produced a double peak between 100 and 200 mM NaCl, and the corresponding fractions were analyzed by SDS-PAGE.

The different bands (Nos. 1–6, Fig 5b) detected in SDS-PAGE gels were subjected to trypsin digestion followed by mass fingerprinting analysis. Notably, this analysis revealed that band No. 1 corresponds to human EB1. The sequence coverage was 83% corresponding to MARE 1 (UniProt accession C1BKD9, Fig S3A). The analysis showed that band Nos. 3–5 correspond to the eight distinct human CCT subunits required to assemble CCT completely. All the CCT-subunits were identified with a sequence coverage higher than 45%, and for most of the cases, the coverage was about 70% (Fig S4a). The presence of complete CCT hetero-oligomeric particles in the eluted fractions was also confirmed by conventional electron microscopy. Notably, CCT is a group II chaperonin mostly committed to the folding of actins and monomeric α- and β-tubulin [34]. Therefore, this is the first evidence demonstrating that a tubulin folding cofactor is able to bind directly to CCT, an interaction that may be relevant for the tubulin folding process.

In addition to these interactors, we also found that band No. 2 corresponds to α- and β-tubulin (Fig 5b). In fact, five α-tubulin isotypes and seven β-tubulin isotypes (Fig S4b) were unequivocally identified with a sequence coverage for all isotypes higher than 20%

and generally with a value of about 50%–60% (Fig S4b).These results also show that the HEK293 cell line expresses all known tubulin isotypes. Because TBCBΔ3 does not bind tubulin heterodimers or microtubules in vitro and only binds to α-tubulin, but not to β-tubulin monomers, these results strongly suggest that TBCBΔ3 would bind microtubules through a partner and that this partner must be EB1.

EB1 overexpression prevents TBCBΔ3 microtubule destruction

The finding that EB1 is a TBCB interactor makes this protein the most attractive candidate for explaining how TBCB is able to regulate microtubule dynamics. Because EB1 is known to stabilize the plus ends of microtubules, its interaction with TBCB would explain how TBCB is able to promote a microtubule catastrophe when overexpressed. In this context, we may expect that TBCB has the ability to sequester EB1 from microtubule plus ends. If these hypotheses were true, the overexpression of EB1 would be sufficient to rescue the observed phenotype of microtubule depolymerization when TBCB and TBCBΔ3 are overexpressed. This would also provide evidence of the interaction of EB1 with TBCB in vivo. To examine this model, we have cotransfected HeLa cells with wild-type TBCB and GFP:EB1 or TBCBΔ3 and GFP:EB1. As predicted, the obtained results show that microtubule destruction resulting from simultaneous overexpression of TBCBΔ3 + GFP:EB1 or TBCB and GFP:EB1 was substantially less accentuated than that observed in cells only overexpressing TBCBΔ3 or TBCB, respectively (Fig 6a). We also observed that the typical GFP:EB1 comets, resulting in the localization of this protein at growing microtubule plus ends, were no longer observable (Fig 6a, bottom), suggesting that excess TBCBΔ3 was interacting with EB1 modifying its intracellular distribution.

To confirm further whether TBCBΔ3 interacts with EB1 in this system, we next quantified and compared the microtubule destruction effect at specific time points against the background of overexpressing GFP:EB1+TBCBΔ3 versus overexpressing GFP:EB1+TBCB. Therefore, cotransfected cells and controls were fixed at different time points. Triple labeling experiments revealed that 10 times as many cells preserved their microtubule cytoskeleton when cotransfected with both genes (Fig 6b). Hence, this system suggests that the TBCBΔ3 depolymerization effect is virtually blocked by overexpressed EB1 and thus confirms that TBCBΔ3 and EB1 interact in vivo.

Discussion

In this work, we found that the TBCB CAP-Gly domain is autoinhibited by interaction with the last three residues of its C-terminus. Our data also show that these last three residues of TBCB are required for TBCE recognition, interaction and tubulin heterodimer dissociation. Therefore, we have proposed a molecular mechanism explaining how TBCB and TBCE form a binary complex that efficiently recognizes and dissociates the tubulin heterodimer.

Biophysical studies revealed that a TBCB protein lacking the last three amino acid residues (TBCBΔ3) behaves as the wild-type protein with a similar CD spectrum and

response to unfolding by heat. Cross-linking experiments and analytical ultracentrifugation, in contrast to dynamic light scattering, show that TBCB behaves as a monomer of 25.5 kDa. The TBCBΔ9 protein, which lacks the last nine amino acid residues, presents a similar CD spectrum and the same unfolding temperature as TBCB and TBCBΔ3. This truncated version of the TBCB protein shows a completely different behavior under heat denaturing conditions, but the values from the dynamic light scattering also suggest that it is a monomer (Fig S2). When overexpressed in human cells, TBCB is able to induce microtubule loss [9]. Although initially this could be ascribable to its interaction with endogenous TBCE, we found that the mutant lacking the last three amino acid residues and unable to interact with TBCE depolymerizes microtubules in vivo with a higher efficiency. This suggests that the C-terminal region is an autoinhibitory sequence and that the mechanism of microtubule depolymerization by TBCBΔ3 is TBCE independent (Fig 7a). Microtubule destruction was accompanied by an intense tubulin background in the cells when detected with both anti-α- and anti-β-tubulin antibodies, suggesting the presence of soluble tubulin heterodimers in the cytosol of the cells. This cytoplasmic background is very unusual in TBCE or TBCD overexpressing cells where these two cofactors sequester either α- or β-tubulin respectively upon tubulin heterodimer dissociation, which leads to microtubule depolymerization and microtubule network collapse.

To gain insights into the mechanism by which TBCB causes microtubule depolymerization, we have studied whether or not TBCB was able to depolymerize microtubules assembled in vitro. We demonstrated that TBCB was not a microtubule depolymerizing enzyme in itself (Fig S1b), which led us to propose the hypothesis of the existence of a TBCB partner that would be implicated in the TBCB microtubule depolymerization mechanism. Specifically, we proposed that TBCBΔ3 was derepressed on its presumed ability to bind to a partner through which it would promote microtubule depolymerization. For this reason, we decided to construct an affinity column containing the derepressed version of TBCB. The reasoning behind the use of the derepressed TBCB mutant was to increase the possibility of identifying partners that would not be easy to discover with the wild-type repressed protein.

Therefore, we have performed TBCBΔ3 affinity binding studies by constructing an affinity column with this polypeptide bound to a matrix and incubating it with a soluble human cell protein extract. Our results show that this derepressed TBCB protein was able to interact with Hsp90, CCT, and EB1. Indeed, the amount of Hsp90 that appears to be bound to the affinity column containing TBCBΔ3 was low compared with EB1 and CCT. Consequently, and although this interaction has a putative role, we did not continue studying this interaction. The same was true for CCT, although we can envisage an important role for the CCT-TBCB interaction in the process of CCT-mediated α-tubulin folding.

Model of TBCB-mediated α-tubulin folding bound to CCT

The establishment that CCT is one of the interactors of TBCB, as revealed by the use of the affinity column (Fig 5) adds new data to the model of how proper tubulin folding and dimerization may occur in vivo. This is the first time that a tubulin folding cofactor was

found to associate with this chaperonin. The possibility of TBCB being a CCT substrate was excluded because TBCB interacts with CCT while binding α-tubulin [9]. This strongly suggests that the interaction of TBCB with CCT might contribute for the proper tubulin folding and dimerization. The interaction of TBCB with CCT is also supported by the fact that after incubation of different amounts of TBCB with purified bovine CCT, an extra band with a higher molecular mass than those corresponding to CCT or TBCB was recognized by anti-TBCB antiserum (our unpublished results). This band migrates at the same position as the extra band containing CCT, α-tubulin, and TBCB previously observed to occur in in vitro translation assays [9]. Together, these results led us to propose a model in which TBCB would recognize α-tubulin bound to CCT (Fig 7b). It has been shown in vitro and in vivo that free α- or β-tubulin will aggregate in the absence of a partner (the other tubulin partner or tubulin cofactors, [4,5]). Therefore, our proposed mechanism predicts that α-tubulin would be released from CCT bound to TBCB ensuring that the α-tubulin monomer would never aggregate. Later, the monomeric tubulin subunit would be transferred to TBCE for dimer assembly and incorporation into growing microtubules or would be transferred to the degradative pathway involving the proteasome if not properly folded [35].

Model of TBCB-mediated microtubule depolymerization

Previous studies have shown that TBCB is regulated by phosphorylation being a substrate of Pak1 as revealed in a yeast two-hybrid screen. Pak1 directly phosphorylates TBCB, and both proteins colocalize on newly polymerized microtubules [13]. We have also shown that the YFP:TBCB protein colocalizes with microtubules of the mitotic spindle, and as mitosis progresses, the staining gradually increases in microtubules of the spindle midzone where active polymerizing microtubules are present. Moreover, TBCB depletion in neuronal cells by siRNA induces axonal extension and growth cone detachment suggesting a role for TBCB at microtubule tips [33]. Recently, TBCB was identified as a target for nitrogen-containing bisphosphonates (N-BP), drugs extensively used in the treatment of bone diseases. In fact, TBCB is upregulated in mammalian cells after N-BP treatment inducing the loss of microtubule architecture in sites of active microtubule assembly, such as neuronal protrusions [36]. This effect might be explained by the observation that overexpression of TBCB in growth cones leads to microtubule depolymerization [33].

EBs proteins are dimeric proteins formed by two functional domains [37]. The N-terminal domain mediates microtubule binding, while the C-terminal domain has a coiled coil responsible for dimerization and an unstructured tail (Fig 7c). Several models have been proposed to explain EB binding to the plus end of microtubules. Crystal structures of the C-terminal domain of EB1 and the CAP-Gly domains of the dynactin subunit $p150^{Glued}$ led Hayashi and coworkers [38] to postulate that EB1 is autoinhibited and that this conformation is unhampered by binding of a CAP-Gly-containing protein ($p150^{Glued}$, Fig 7c). Although TBCB and TBCE contain a CAP-Gly domain, these proteins were never described as localizing at microtubule tips as other +TIPs proteins. TBCE was also a good candidate for such a role, but we could not see interaction with EB1 in vitro. While the model proposed by Hayashi and coworkers [38] is well supported by the

different structures solved (Fig 7), the mechanism by which EBs bind to microtubules remains unclear. Although the removal of the EB tail blocked, as predicted, binding to partners [39], it had no effect in vivo on microtubule plus-end accumulation. Also, removal of the C-terminal tail of EBs does not alter the global conformation of the protein [40], which does not support the model proposed by Hayashi and coworkers [38]. In addition, Buey and colleagues (40) suggested that the negative charge of the domain is responsible for the specificity of the EBs to the microtubule tip. Despite the different models that try to explain the preference of EB proteins for the growing microtubule plus end, it seems that EBs recognize an inaccessible region of tubulin in the GTP bound form [41]. It is thought that the binding of EBs to microtubule tips is dynamic, being characterized by rounds of binding and unbinding [19]. Although we detected an interaction of TBCB with EB1, in vivo and using our polyclonal antibodies against human or murine TBCB, we never found typical EB-comets. TBCB is a CAP-Gly-containing protein, but when we performed double immunolabeling with GFP-EB1 and TBCB we could not colocalize TBCB to the microtubule tips that were clearly seen for EB1. For this reason, it was surprising to find that EB1 was one of the major interactors of TBCB. Thus, we decided to study the in vitro interaction of TBCB with EB1 using purified untagged proteins. Unexpectedly, we found no interaction under the conditions tested (Fig S2d). We could not rule out the possibility that specific posttranslational modifications in the EB1 protein were required for TBCB binding. This would not be the case for TBCB because the protein used in the affinity column was purified from *E. coli* and could not have posttranslational modifications. The detailed mass spectrometric analysis of the EB1 polypeptide showed an acetylation at alanine-2 (Fig S3a). This cotranslational acetylation occurrence takes place only in eukaryotes. This usual modification probably does not add functional diversity to the EB1 polypeptide, supporting the notion that TBCB and EB1 do not interact unless EB1 is derepressed. We isolated microtubule fragments containing different α- and β-tubulin polypeptides (Fig S4b) bound to TBCB. In our experiments ([9] and this work), TBCB did not interact along the microtubule, although we cannot rule out an interaction with microtubule ends. That we could isolate microtubule fragments with bound EB1 provided us with strong evidence that TBCBΔ3 binds and sequesters EB1 from microtubule ends leading to microtubule depolymerization [42].

We have established that the C-terminal peptide of TBCB is required for binding to TBCE and for efficient tubulin heterodimer dissociation (Fig 2). TBCB also recognizes EB1 at the plus end of the microtubule, and our results suggest that this interaction takes place when EB1 is also derepressed, probably after interaction with another +TIP protein or after binding to microtubules.

Finally, we have also demonstrated that cotransfection with EB1 prevents TBCBΔ3 microtubule destruction and that cells recover their normal phenotype, confirming that TBCBΔ3 and EB1 interact in vivo. This is more than sufficient to justify microtubule destabilization, but, as TBCB forms an active heterodimer with TBCE in tubulin dissociation, we suggest that this is also the mechanism by which these proteins regulate microtubule dynamics (Fig 7c).

In this way, TBCB participates in microtubule dynamics, and as shown here, the deregulation of TBCB activity induces a microtubule catastrophe in living cells.

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Conflict of interest

None of the authors have a financial interest related to this work.

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Legends to figures

Figure 1. TBCB is an autoinhibitory protein

a) Schematic drawing of human TBCB depicting the three characterized (UBL, coiled-coil and CAP-Gly) domains. The UBL domain (green) corresponds to PDB ID, 1V6E (UBL of murine TBCB), and the CAP-Gly domain (blue) to PDB ID, 1TOV (CAP-Gly domain of F53f4.3, [26]. In light blue are the corresponding residues that form the conserved groove in $p150^{Glued}$, interacting with the C-terminal peptide of α-tubulin [30, 31]. The last nine residues, which are present in the solved domain of F53f4.3, are shown in red. All structures were drawn using Pymol software (http://www.pymol.org).
b) Confocal microscopy projection image of TBCBΔ3 overexpression on HeLa cells. TBCBΔ3 (green) produces conspicuous microtubule destruction (white arrow). Moderate TBCBΔ3 levels also severely affect the microtubule cytoskeleton. A high cytoplasmic tubulin background is observed in these two cells.
c) Statistical analysis of the percentages of cells containing normal, abnormal or absent microtubules in TBCE, TBCB, and TBCBΔ3 overexpressing HeLa cells. A highly significant increase in cells containing a completely destroyed microtubular cytoskeleton is observed when the TBCBΔ3 mutant (asterisk) is overexpressed compared with wild-type TBCB. See also Figure S1.

Figure 2. Biochemical studies of TBCBΔ3

a) TBCBΔ3, in contrast to complete TBCB, does not form a binary complex with TBCE. Plots of A_{280} absorbance against elution volume from the size-exclusion chromatography experiments. The elution profiles of TBCB, TBCBΔ3, TBCE, and the interaction of these proteins were analyzed by gel filtration through a Superdex 200 PC 3.2/30 column (GE-Healthcare). Three plots are shown: TBCB and TBCBΔ3 alone (blue), TBCE alone (red), and the combination of TBCB and TBCE or TBCBΔ3 and TBCE (green). Fractions were

subjected to SDS-PAGE. The final concentrations used were 18 μM for TBCE and 15 μM for TBCB and TBCBΔ3.
b) and c) Nonclassical two-dimensional native SDS-PAGE of complexes formed in tubulin dissociation experiments. Aliquots of TBCB (b) or TBCBΔ3 (c) and TBCE and tubulin heterodimers were incubated at 30°C for 30 min and loaded onto a 6% native minigel (1D). C, control of tubulin heterodimers; C1, TBCBΔ3; C2, tubulin dissociation in the presence of TBCE and TBCBΔ3 for 30 min. Bands containing this first dimension were excised from the gel and loaded onto an 8.5% SDS-polyacrylamide gel as described in Experimental procedures (2D). After electrophoresis, the gels were stained with Coomassie Blue. The second dimension (2D) shows the molecular compositions of the ternary complex in panel b (TBCE, TBCB, and α-tubulin) and of the binary complex (TBCBΔ3 and α-tubulin) in panel c.
d) TBCB and TBCBΔ3 do not dissociate the tubulin heterodimer. Aliquots containing TBCB or TBCBΔ3 were incubated at 30°C for 30 min with tubulin heterodimers in the presence and absence of TBCE and in the presence of TBCA. The drawing on the right shows the migration of the different proteins and complexes.
e) TBCE slowly dissociates the tubulin heterodimer. Time-course of tubulin dissociation in the presence of stoichiometric amounts of TBCE. C, control of tubulin heterodimers incubated for 30 min. Samples were frozen in dry ice until they were loaded into the native gel.
f) TBCE/TBCB, the tubulin dimer dissociation machine. Time-course of tubulin dissociation in the presence of TBCE and stoichiometric amounts of TBCB or TBCBΔ3. C1, TBCB; C2, TBCBΔ3; C3, control of tubulin heterodimers; C4, tubulin dissociation in the presence of TBCE for 30 min. After times indicated, samples were frozen on dry ice until they were loaded into the native gel. The drawing on the right shows the migration of the different proteins and complexes. Final concentrations used were 3 μM for tubulin, 2.5 μM for TBCE, and 2.5 μM for TBCB and TBCBΔ3.
g) Quantification of tubulin dissociation by TBCE/TBCB and TBCE/TBCBΔ3. Quantification of the proportions of tubulin dissociation in the experiment shown in e and f (time 0) using ImageJ software. Data were analyzed using SigmaPlot software. Experiments were performed in triplicate, and the graph reports the mean ± the standard deviation.

Figure 3. Biophysical studies of TBCB and TBCBΔ3

a) Cross-linking of TBCB and TBCBΔ3 with glutaraldehyde. TBCB at 1 μM and TBCBΔ3 at 1.5 μM were analyzed in SDS-PAGE in the presence and absence of glutaraldehyde (at 0.05%). EB1 at 1.5 μM was used as control (dimeric protein).
b) Analytical ultracentrifugation of TBCB. Sedimentation velocity experiments performed at 160,000 × *g* and 20°C were analyzed to yield an estimated molecular mass of 25.5 kDa, in agreement with the molecular mass of the theoretical monomer (27 kDa). See also Figure S2.

Figure 4. YFP:TBCB is associated to the centrosome and mitotic microtubules

A) Confocal microscopy image of YFP:TBCB localization in interphase (top, left) and mitotic HeLa cells. YFP:TBCB is mostly cytoplasmic and concentrates at the centrosomes of interphase and prophase HeLa cells (top, center, arrows). In anaphase A, YFP:TBCB is clearly associated with spindle microtubules, also decorating microtubules bridging the midzone (bottom left, arrow). A midbody localization pattern is more obvious during anaphase B and telophase (bottom images, arrow), where there is no longer a centrosomal signal.

B) Relationship of the YFP:TBCB signal with respect to the primary cilium in G1 (left) and G2 HeLa cells (right). Glutamylated tubulin labeling the primary cilium is recognized by the GT335 antibody (red).

Figure 5. Microtubules bound EB1, Hsp90, and CCT are interactors of TBCB

a) Search for TBCB partners using affinity chromatography. Purified TBCBΔ3 was purified and coupled specifically to a Hi-Trap NHS-activated HP column (GE-Healthcare). Human HEK293 protein extract (1 mL at 18 mg/ml) was applied to the column. Bound proteins were eluted using an NaCl gradient (red line). Absorbance intensity at 280 nm (Y-axis) is plotted against the collected volume (X-axis).

b) SDS-PAGE analysis of the fractions eluted from the NHS column. Lane 1: Molecular mass marker; lane 2: aliquot of the cell extract (Control); Lane 3: FT, unbound proteins eluted in the void volume (flow-through); lanes 4–15: fractions eluted with 100–200 mM of NaCl.

Figure 6. EB1 prevents TBCBΔ3 microtubule destruction.

a) Triple-labeled confocal microscopy projection images of TBCBΔ3 overexpression on HeLa cells. TBCBΔ3 (green) produces conspicuous microtubule destruction (filled arrow). Moderate TBCBΔ3 levels also severely affect the microtubule cytoskeleton (empty arrow).

b) Statistical analysis of the proportions of cells containing normal, abnormal, or absent microtubules in TBCE, TBCB, and TBCBΔ3 overexpressing HeLa cells 24 and 48 hours after transfection. A highly significant increase in cells containing a completely destroyed microtubular cytoskeleton is observed when the TBCBΔ3 mutant is overexpressed compared with the wild-type construct. See also Figures S3 and S4.

Figure 7. Models of TBCB auto-inhibition, interaction with CCT and with EB1 at the microtubule end

(a) The C-terminal region of TBCB functions as an autoinhibitory peptide when bound to the CAP-Gly domain of the protein.

The three domains of TBCB are depicted. The N-terminus contains the UBL (Fig 1, PDB ID, 1V6E, green).The central domain is a coiled-coil domain (CC in yellow), and the C-

terminal domain contains the CAP-Gly domain (PDB ID, 1V6E, blue/purple).The acidic tail of CAP-Gly domain is shown in red and orange. We propose that the C-terminal tail of TBCB is responsible for the autoinhibition of the protein (red peptide) through the interaction with the CAP-Gly domain (blue), specifically with the highly conserved hydrophobic cavity present in the CAP-Gly domain (light blue). In contrast, if the C-terminus region does not interact with the CAP-Gly domain (orange peptide), the protein is derepressed. The hypothetical models of TBCBΔ3 and TBCBΔ9 showing the structure of the protein lacking the last three or nine amino acids are shown.

b) Model of TBCB-mediated α-tubulin folding bound to CCT. In a first step, prefolding transfers α-tubulin polypeptides (yellow) to CCT (PDB ID, 2XSM, grey) [34]. After the binary complex is formed with CCT, the α-tubulin, which is in quasi-native state, interacts with TBCB that would be attached to the chaperonin to follow quickly with the postchaperonin folding pathway necessary for the incorporation of properly folded tubulin dimer (αβ tubulin) to the microtubule avoiding the transit of the monomer through the cytoplasm or degradation by the proteasome pathway [35].

c) Model of TBCB-mediated microtubule dynamics. Microtubules polymerize by addition of GTP-tubulin dimers (PDB ID, 1TUB, yellow/blue). After they are incorporated into the microtubule, the GTP bound to the β-tubulin subunit is hydrolyzed to GDP (yellow/light blue). EB1 (orange) is an intrinsic +TIP protein that specifically decorates growing microtubule plus ends. EB1 can bind to other +TIP proteins, such as CLIP-170 and $p150^{Glued}$, through a CAP-Gly domain (PDB ID, 2HL5, pink) and may displace its theoretical C-terminal inhibitory tail. Although EBs show an elongated conformation in solution [40], TBCB does not interact with EB1 in vitro supporting an autoinhibition model similar to that proposed by Hayashi and coworkers in 2005 [38]. In any case, the elongated EB1 protein would bind specifically to the microtubule tip and not the lattice [40], and the C-terminal tail would be oriented away from the MT tip or masked by a CAP-Gly-containing protein. The motif DEI/M-COO^{-} present in TBCB is responsible for its autoinhibition but when derepressed induces microtubule depolymerization. Once EB1 is on the microtubule, it would be recognized by TBCB inducing EB1 detachment. In addition, TBCB might be the target for TBCE, inducing tubulin dimer dissociation. As a result of these interactions, microtubules would be directed to a catastrophe.

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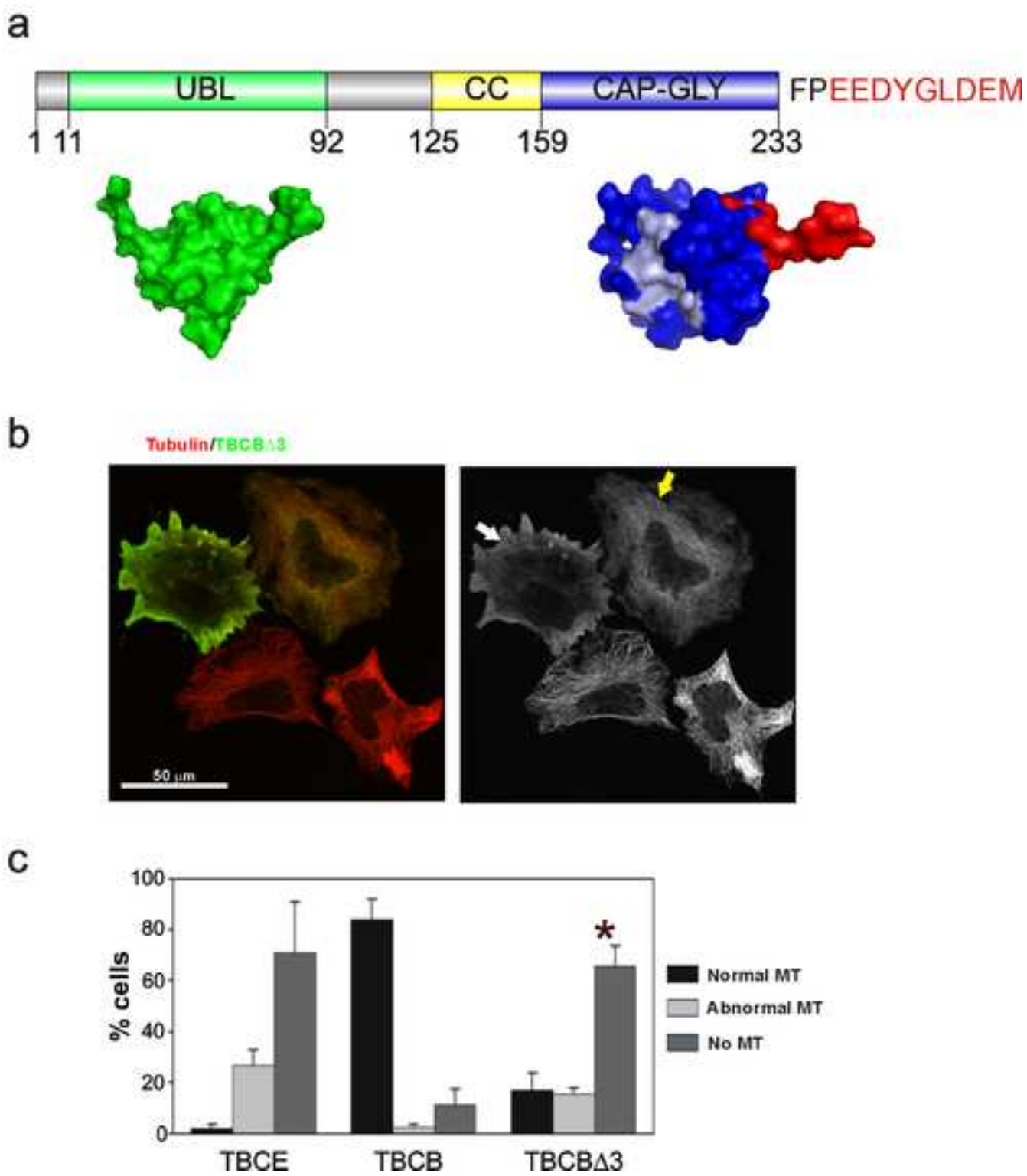
Figure 1



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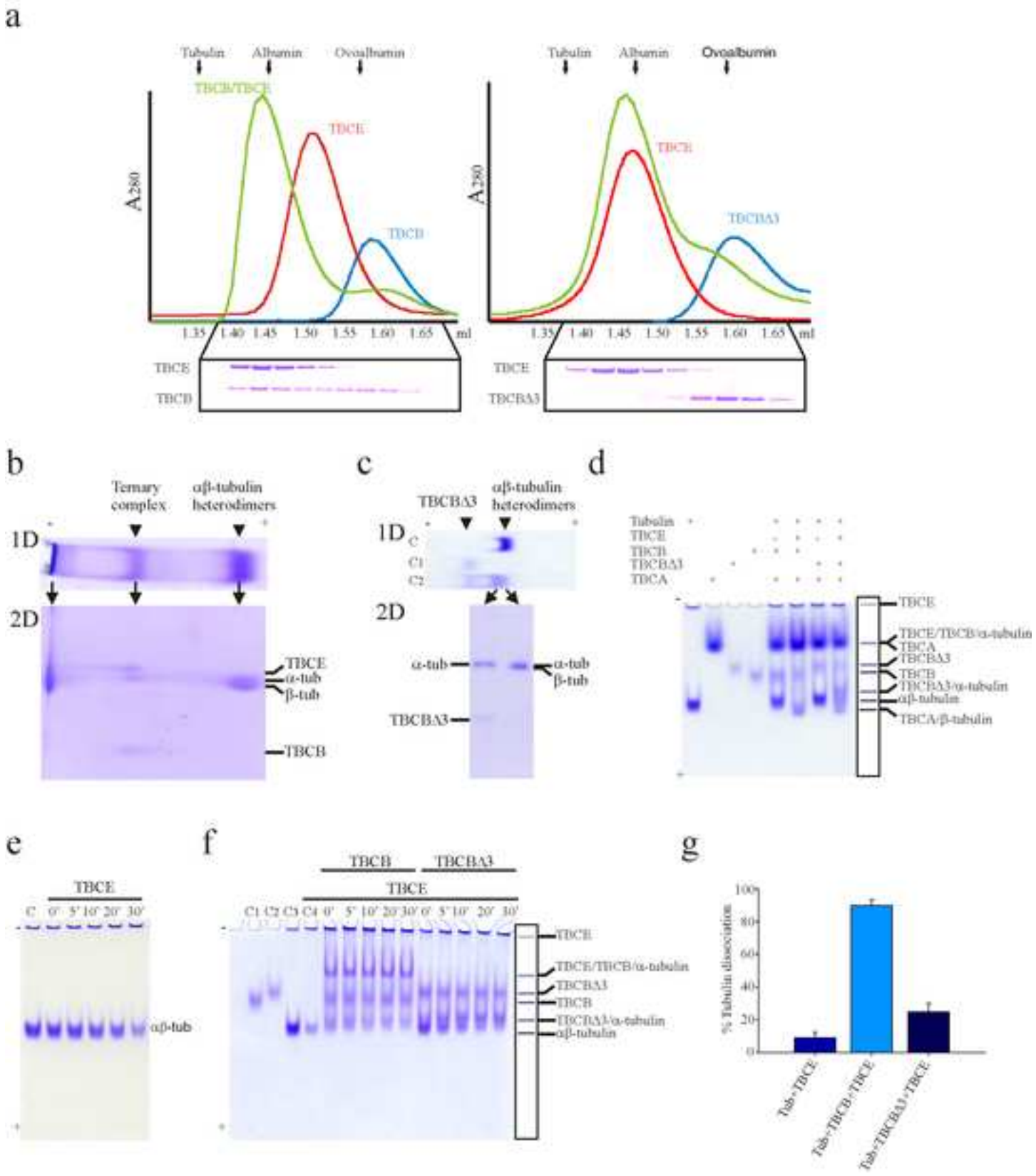

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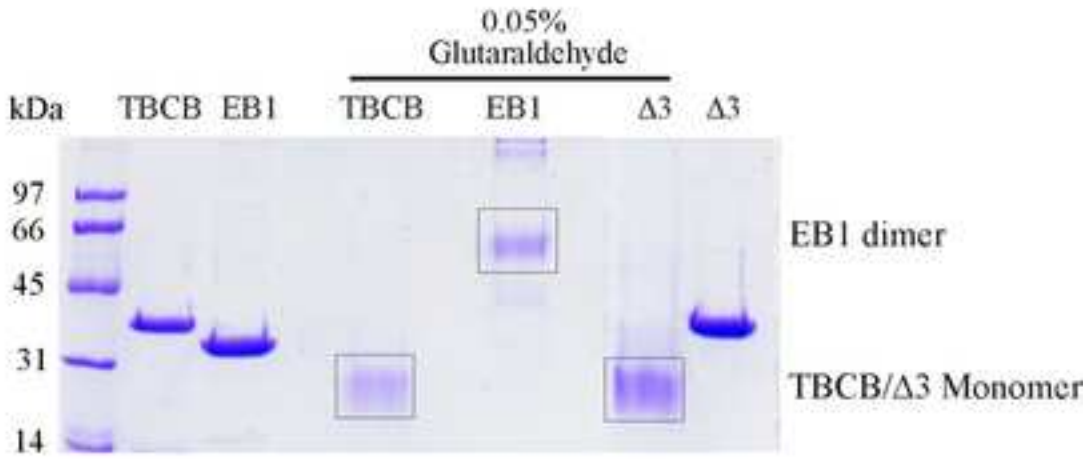
a



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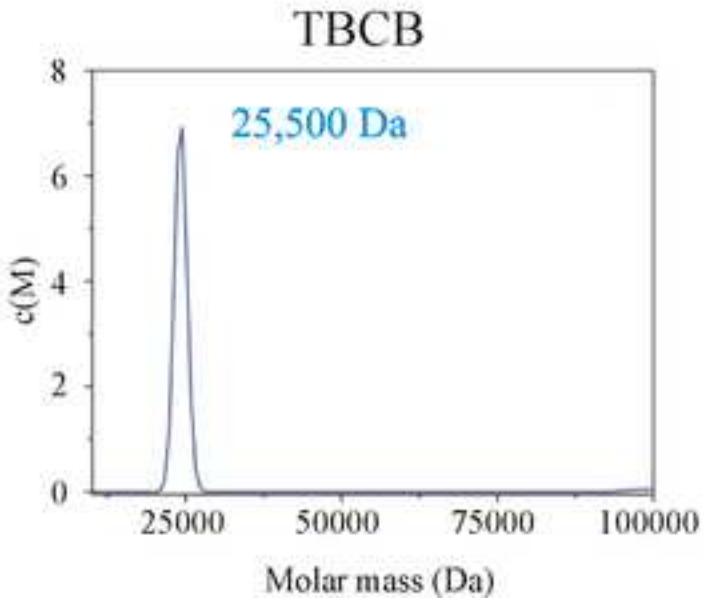

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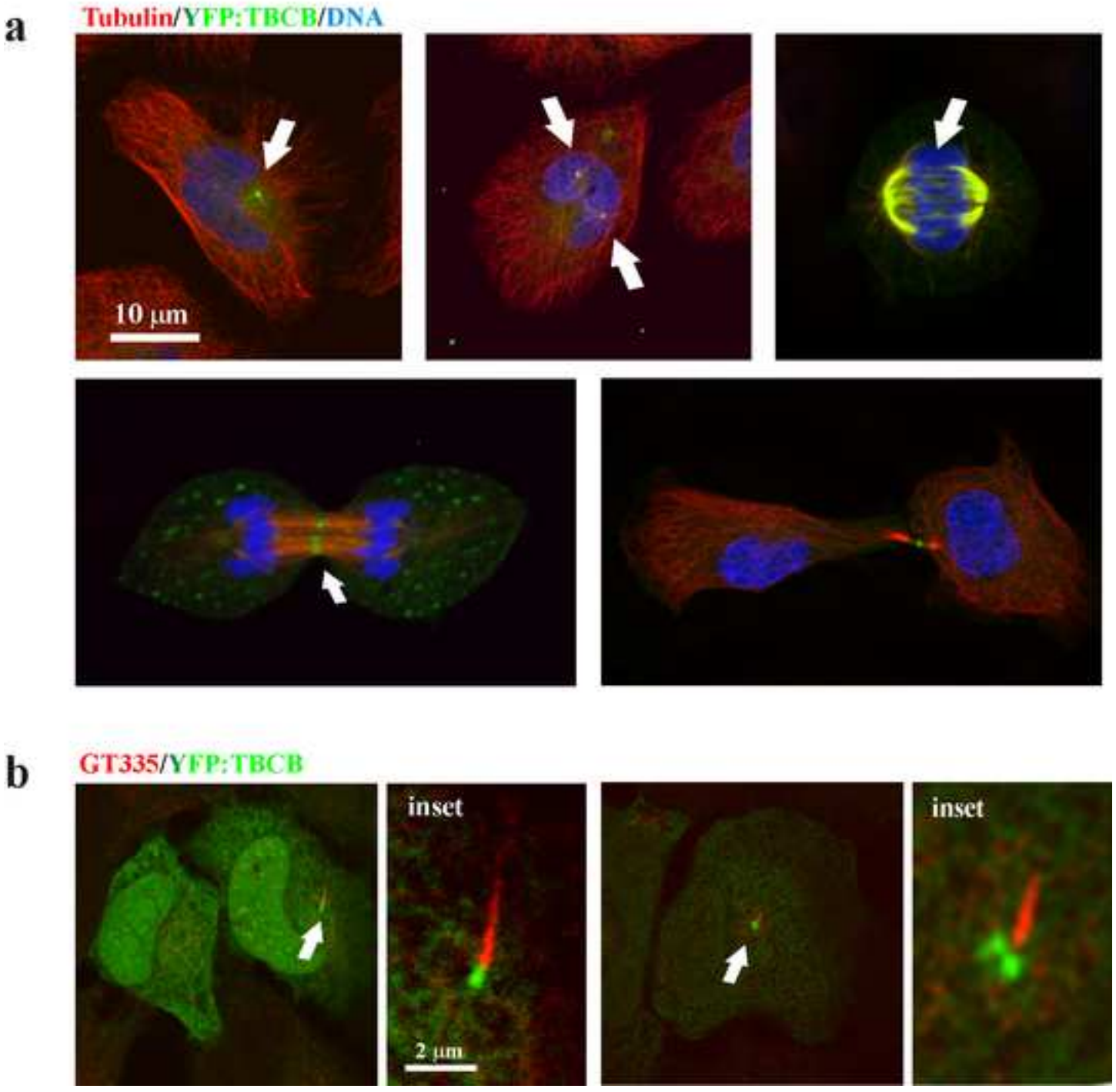

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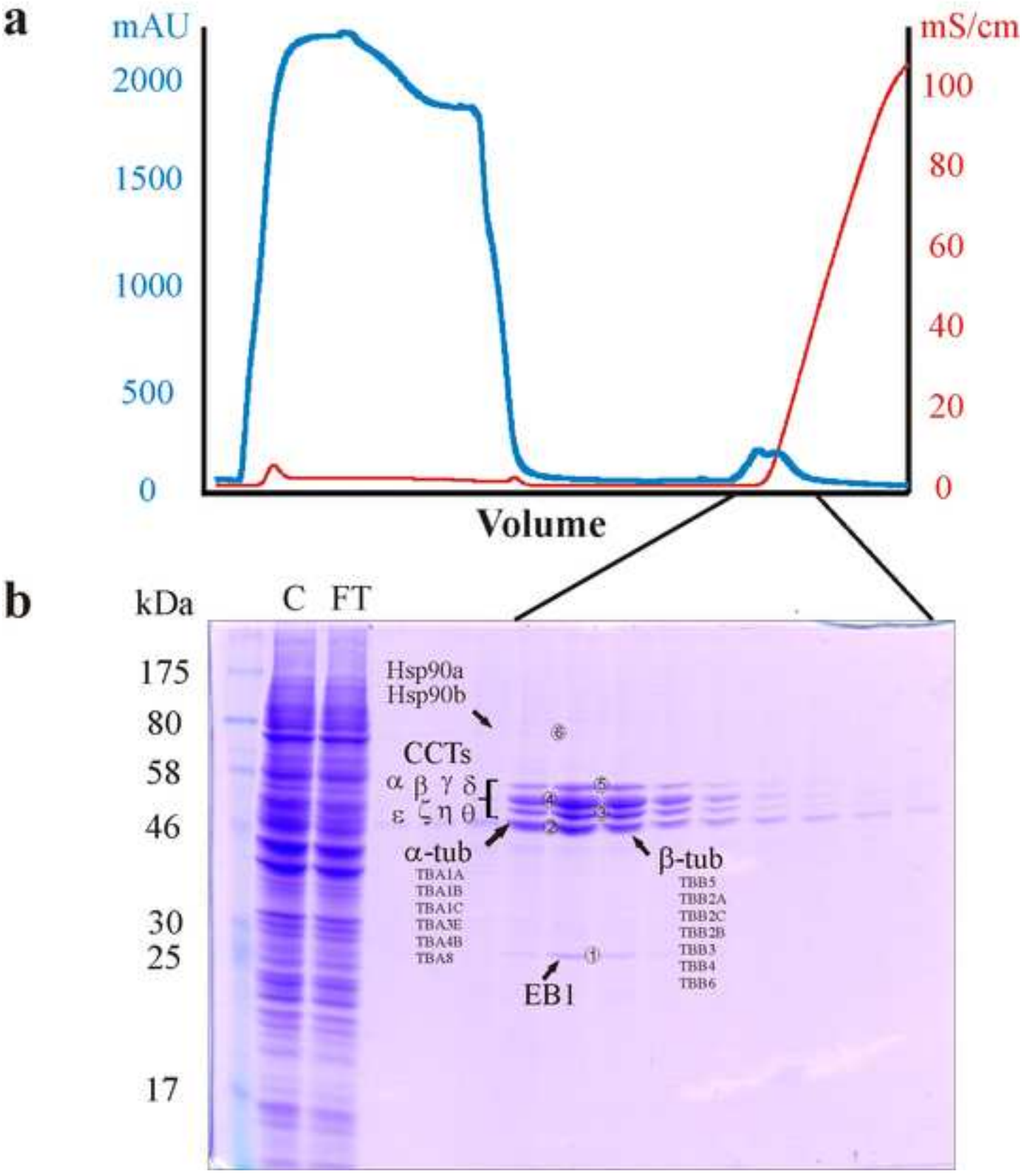

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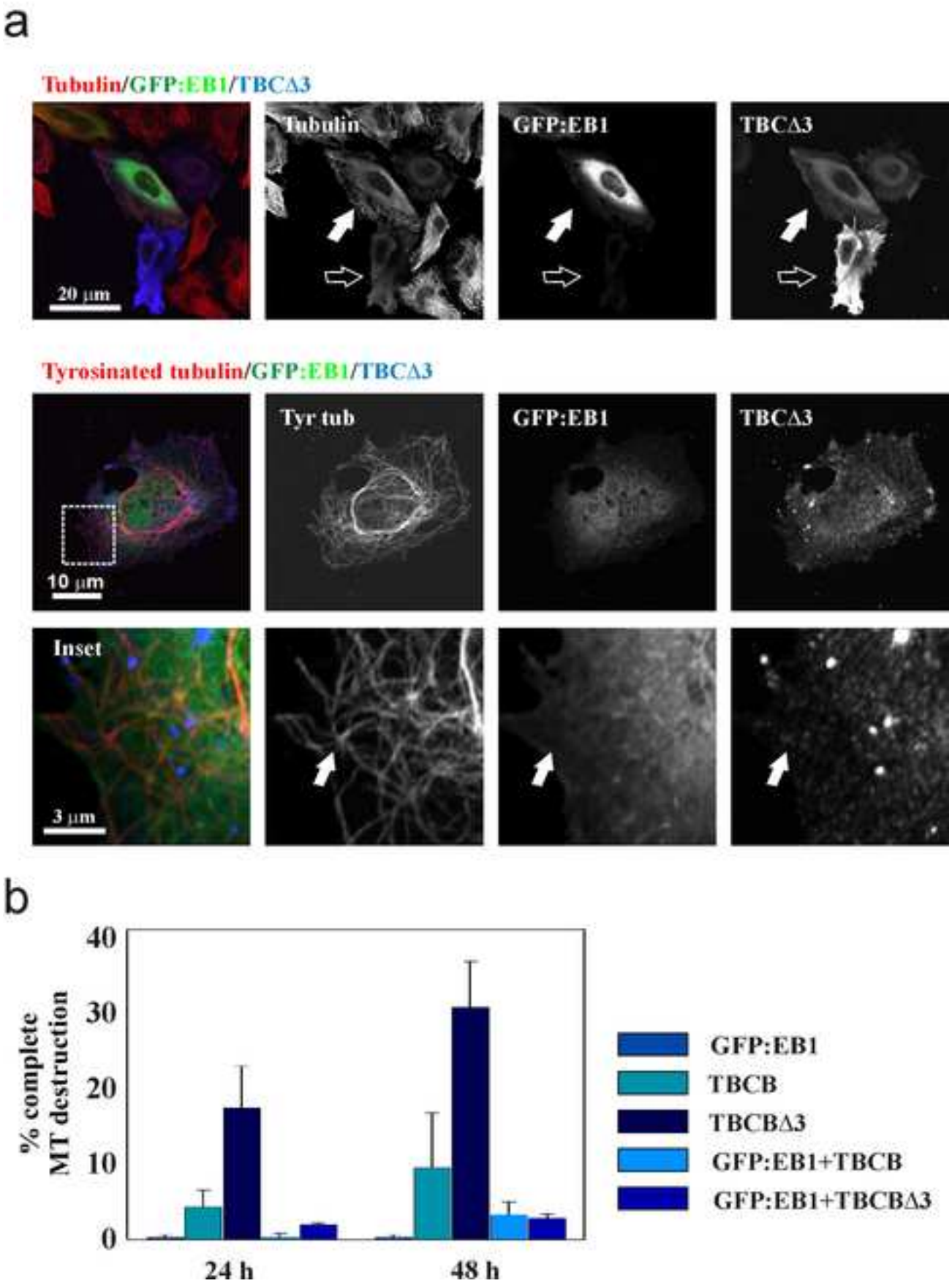

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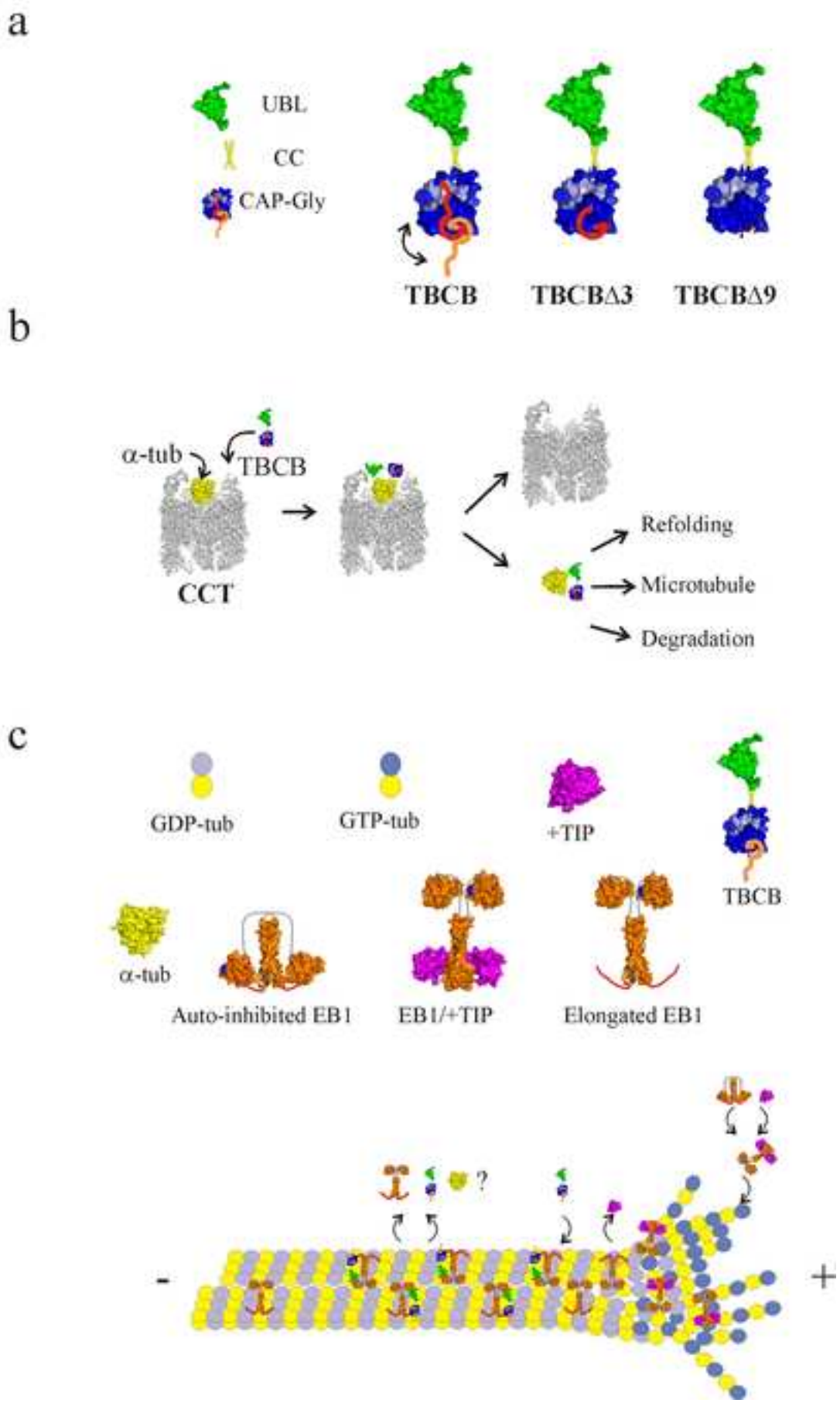

Table 1. Determination of the equilibrium dissociation constants for the binding of the fluorescein labeled TBCB and α-tubulin peptides to TBCB, TBCBΔ3, and TBCBΔ9 by fluorescence polarization (FP). FP binding assays of TBCB with FL-peptides were repeated up to a protein concentration of 100 μM (FigS5). See also Figure S5.

Peptide	Protein	Kd, μM
EEDYGLDEI (TBCB)	TBCB	12 ± 1
"	TBCBΔ3	74 ± 3
"	TBCBΔ9	79 ± 4
EEDYGL (TBCBΔ3)	TBCB	71 ± 3
"	TBCBΔ3	178 ± 15
"	TBCBΔ9	249 ± 30
GEGEEEGEEY(α1/2-tub)	TBCB	43 ± 2
"	TBCBΔ3	113 ± 7
"	TBCBΔ9	145 ± 9
GEGEEEGEE(α1/2-tubΔY)	TBCB	140 ± 4
"	TBCBΔ3	312 ± 20
"	TBCBΔ9	472 ± 69

Supplementary Material

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