CHAPTER

A TIRF microscopy assay to decode how tau regulates EB's tracking at microtubule ends

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CHAPTER OUTLINE

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Abstract

Tau is a major microtubule-associated protein (MAP) mainly expressed in the brain. Tau binds the lattice of microtubules and favors their elongation and bundling. Recent studies have shown that tau is also a partner of end-binding proteins (EBs) in neurons. EBs belong to the protein family of the plus-end tracking proteins that preferentially associate with the growing plus-ends of microtubules and control microtubule end behavior and anchorage to intracellular organelles. Reconstituted cell-free systems using purified proteins are required to understand the precise mechanisms by which tau influences EB localization on microtubules and how the concerted activity of these two MAPs modulates microtubule dynamics. We developed an in vitro assay combining TIRF microscopy and site-directed mutagenesis to dissect the interaction of tau with EBs and to study how this interaction affects microtubule dynamics. Here, we describe the detailed procedures to purify proteins (tubulin, tau, and EBs), prepare the samples for TIRF microscopy, and analyze microtubule dynamics, and EB binding at microtubule ends in the presence of tau.

1 INTRODUCTION

Microtubules result from the assembly of α–β tubulin heterodimers (Amos & Schlieper, 2005). They are major components of the eukaryotic cytoskeleton and are essential for cellular architecture, mitosis, and intracellular transport (Desai & Mitchison, 1997). Microtubule ends constantly alternate between phases of polymerization and depolymerization, a molecular process known as dynamic instability (Mitchison & Kirschner, 1984). Transitions from growth to shortening are defined as catastrophes and reverse events as rescues. In living cells, a panel of microtubule-associated proteins (MAPs) interacts with free tubulin and/or microtubules to regulate their properties. Tau is a structural MAP mainly expressed in brain. Tau promotes microtubule polymerization and is responsible for microtubule stabilization and bundling in axons. Tau is involved in the development and maintenance of the nervous system and the deregulation of its function is associated with

Alzheimer's disease and neurodegenerative pathologies such as frontotemporal dementia (FTDP17) (Ingram & Spillantini, 2002). Beyond its microtubule-stabilizing properties, tau is also a regulator of actin both in vitro and in vivo (Frandemiche et al., 2014; He et al., 2009). Tau acts as a direct linker of dynamic microtubules and actin filaments, enabling the coorganization of the two networks in purified cell-free systems (Elie et al., 2015). In neurons, tau is present as several isoforms. The longest isoform is composed of 441 residues: an N-terminal projection domain (1–198) including two short inserts (N), and a C-terminal domain (198–441) containing four cytoskeleton-binding repeats (R). Besides its direct interaction with microtubules and actin, tau has been recently described as a partner of EBs in neurons thereby regulating their subcellular localization (Sayas et al., 2015). EBs belong to the family of plus-end tracking proteins (+TIPs), namely MAPs that preferentially associate with the growing plus-ends of microtubules (Galjart, 2010). They form a complex interaction network at microtubule plus-ends where they regulate microtubule dynamics and microtubule association with distinct subcellular targets. Among the +TIPS, end-binding-1 (EB1) is an archetype. It autonomously end-tracks growing microtubules where it recruits other +TIPs, ensuring tight control of microtubule end properties (Bieling, Telley, Hentrich, Piehler, & Surrey, 2010; Dixit et al., 2009; Honnappa et al., 2009; Zanic, Stear, Hyman, & Howard, 2009; Zimniak, Stengl, Mechtler, & Westermann, 2009). EB1 and its homologues (EB2, EB3 in mammals; Mal3, Bim1 in yeast) are highly conserved proteins. They are composed of an N-terminal calponin–homology domain required for microtubule binding, and a C-terminal dimerization domain (De Groot et al., 2010; Hayashi & Ikura, 2003; Honnappa, John, Kostrewa, Winkler, & Steinmetz, 2005; Slep et al., 2005) that encompasses the interaction site of EBs with multiple partners (Akhmanova & Steinmetz, 2010). Recent data have pointed out that the presence of EB proteins on microtubules is modulated by structural MAPs (like tau, MAP1B, or MAP2) primarily characterized for their binding along the microtubule lattice (Kapitein et al., 2011; Sayas & Avila, 2014; Sayas et al., 2015; Velot et al., 2015).

In that context, we developed an in vitro assay to dissect the interaction of tau with EBs and to study how this interaction affects microtubule dynamics (Ramirez-Rios et al., 2016). This reconstituted system is based on TIRF microscopy, which is particularly appropriate to study the behavior of +TIPs and various MAPs in the presence of microtubules (for example, Bieling et al., 2010; Telley, Bieling, & Surrey, 2011). The binding of GFP-fusion–EBs on growing microtubule can be precisely monitored by fluorescence measurements leading to data: (a) on the relative quantities of GFP–EBs bound to microtubules in the absence or in the presence of tau and (b) on the combined influence of EBs and tau on microtubule dynamic parameters. In addition, only low concentrations of proteins (on the nanomolar scale) are required to perform those assays, diminishing potential artifacts that could result from highly concentrated samples like abnormal binding rates or extensive nucleation events. Another strength of this method is to perform comparative studies between wild-type and mutant proteins. For instance, the use of deletion mutants is powerful to determine the protein regions essential to regulate microtubule dynamics

or involved in the interaction between biological partners. This approach is also relevant to study how regulating elements, such as phosphorylation, could affect EB/tau interaction and microtubule dynamics. The study of such a regulation is extremely complex in cells as phosphorylation can depend on several enzymes and be difficult to control and rationalize in vivo. The tau literature emphasizes that the deregulation of tau's phosphorylation state is related to subsequent pathologies such as Alzheimer's disease. In vitro reconstituted approaches using purified proteins allow targeting the phosphorylation sites of tau one by one or in combination and testing them systematically in relation with EB end-tracking properties and microtubule dynamics.

2 PURIFICATION OF TUBULIN

2.1 COMPOSITION OF BUFFERS

1. AB buffer: 0.1 M Pipes, 0.5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA, pH 6.8 with KOH.[a]
2. CB buffer: 50 mM pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8.[a]
3. BRB80 buffer: 80 mM pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8.[a]
4. Na-HEPES buffer: 100 mM Na-HEPES, pH 8.6 (HCl).

2.2 REAGENTS

1. ATTO-565 NHS ESTER—ATTO-Tech, AD 565-35
2. ATTO-488 NHS ESTER—ATTO-Tech, AD 488-31
3. Na-HEPES—Sigma Aldrich, H3784
4. Glycerol—Sigma Aldrich, G5516
5. GTP—Sigma Aldrich, G8877
6. ATP disodium salt—Sigma Aldrich, A2383
7. β-Mercaptoethanol—Sigma Aldrich, M6250
8. DMSO—Sigma Aldrich, D5879
9. Na-HEPES—Sigma Aldrich, H3784
10. PIPES—Sigma Aldrich, P6757
11. $MgCl_2$—Sigma Aldrich, 2670
12. KCl—Sigma Aldrich, P9541
13. EGTA—Sigma Aldrich, E3889
14. L-glutamic acid potassium salt—Sigma Aldrich, G1501
15. Fractogel EMD SO3 Resin—MERCK, 1.16882-0100

[a]Final pH is adjusted with KOH.

2.3 METHOD

2.3.1 Step 1: Purification of tubulin from mammalian brains

Neuronal tubulin is purified by successive cycles of polymerization and depolymerization. This protocol is adapted from the original method described by Shelanski, Gaskin, and Cantor (1973).

- *Grinding of the brains*: Brains should come from freshly killed animals (bovine or porcine). If this is not the case, tubulin yield and quality will be poor. The brains are plunged into a cold saline solution (1.5% (w/v) NaCl) for transport from the slaughterhouse. The protocol below is described for three cow brains. In the cold room, brain stems and meninges are removed as much as possible with kitchen cleaning paper. The brains are weighted (usually $\approx$ 700 g at this step), then homogenized with cold AB buffer[b] (1 mL of AB buffer per gram of brain) supplemented with 1 mM ATP and 0.1% (w/v) β-mercaptoethanol using a cold blender. Four cycles of 20 s each are necessary: the first cycle at low speed, the second cycle at moderate speed, and the third and fourth cycles at high speed. The homogenate is first centrifuged at low speed (e.g., 15,000 rpm for 20 min in a Beckman rotor JLA16.250 at 4°C) to remove large debris. Second, the resulting supernatant is centrifuged at 100,000 × *g*, 4°C, 90 min.
- *First polymerization/depolymerization reaction*: The tubulin present in the supernatant is polymerized by adding a half-volume of prewarmed anhydrous glycerol (supernatant/glycerol ratio of 2/1 (v/v), 0.2 mM GTP, 1.5 mM ATP, 3 mM $MgCl_2$). The mixture is placed in a water bath, 37°C, 60 min. During polymerization, bubbles appear and the solution becomes viscous. The solution is then centrifuged (100,000 × *g*, 90 min, 35°C) in a prewarmed rotor (Beckman centrifuge, rotor Ti-45). The supernatants are removed and the pellets are solubilized in a few milliliters of cold AB buffer (around 1–2 mL per pellet, ~4 pellets per starting brain). The pellets are homogenized in a cold Dounce tissue grinder. The tubulin concentration is determined at A_{280} nm (with 0.1 A_{280} corresponding to 1 μM or 0.1 mg/mL tubulin) and is adjusted to 250 μM with cold AB buffer. The solution is kept on ice, 30 min to complete the depolymerization process, and then is centrifuged at 140,000 × *g*, 30 min, 4°C.
- *Second polymerization/depolymerization reaction*: The supernatant is supplemented by a half-volume of prewarmed glycerol, 1 mM GTP, 0.5 mM ATP, and 4 mM $MgCl_2$. The mixture is warmed up to 37°C, incubated for 60 min in a water bath, and then centrifuged at 100,000 × *g*, 90 min, 35°C. The pellets are solubilized with cold AB buffer as previously described. After 30 min on ice, the solution is centrifuged at 4°C, 140,000 × *g*, 30 min to remove residual aggregates. As microtubules absorb at 350 nm, A_{350} should be close to 0 at the end of the depolymerization step. Final concentration is measured at 280 nm. It should be around 20 mg/mL, i.e., low enough to ensure optimal depolymerization but

[b]Buffer composition is detailed in Material section later.

suitable for the next purification step. At the end of this step, the solution consists of tubulin and associated proteins like MAPs. Samples (about 300 mg of proteins) are flash frozen in liquid nitrogen and stored at −80°C (no more than 6 months).

2.3.2 Step 2: Purification of MAP-free tubulin

- *Ion exchange chromatography*: A protein sample prepared in Step 1 is quickly unfrozen in a water bath at 32°C and then kept on ice. The protein solution is diluted to 15–18 mg/mL with 0.1 mM GTP/CB buffer. The full volume is slowly injected (0.5 mL/min) to 200 mL Fractogel EMD SO_3 resin packed into a chromatography column previously equilibrated with 0.1 mM GTP/CB buffer. As tubulin is negatively charged at pH 6.8, it does not bind the resin and is eluted in the flow-through volume (1 mL/min). Fractions with absorbance at 280 nm greater than 0.9 are pooled.
- *Polymerization/depolymerization reaction*: The tubulin solution is then submitted to a polymerization cycle in BRB80, 5 mM $MgCl_2$, 1 mM GTP, 33% (v/v) glycerol, about 60 min at 37°C. Polymerization is achieved when the solution turns whitish. The solution is then gently poured over a cushion consisting of 60% glycerol (v/v) in BRB80 buffer at 35°C and then centrifuged at 35,000 rpm, 35°C, 90 min (Beckman rotor Ti-45). In the optimal conditions, the glycerol cushion and the tubulin solution are used at equal volume. The supernatants are discarded and the pellets are quickly washed with 200–300 µL of warm BRB80 buffer. A small volume of cold BRB80 (100–200 µL) is added to each pellet and the mixture is transferred into a Dounce tissue grinder. The microtubules are disassembled in cold BRB80 buffer and the homogenized solution is kept on ice during 30 min. When the solution is transparent and fluid, the protein concentration is checked at 280 nm. The concentration should be around 40 mg/mL. The aggregated tubulin is removed by centrifugation 15 min, 4°C, 70,000 rpm (Beckman rotor TLA-100.3). The supernatant is recovered and the final concentration of tubulin is estimated at 280 nm (1:100 dilution in BRB80 buffer). The solution is aliquoted (5–10 µL aliquots), flash frozen, and stored in liquid nitrogen.
- *Self-assembly activity*: The self-assembly activity of tubulin is checked by mixing 70 µM tubulin, 1 mM GTP in BRB80 buffer, and monitoring microtubule polymerization at 350 nm, 37°C, 30 min. The result should match a sigmoid function (Fig. 1A).

2.3.3 Preparation of labeled tubulin

Preparation of fluorescent tubulin is based on Hyman et al. (1991).

- *Ion exchange chromatography*: A protein sample prepared in Step 1 is quickly unfrozen in a water bath at 32°C. The protein solution is diluted to 15–18 mg/mL with 0.1 mM GTP/CB buffer. The full volume is slowly injected (0.5 mL/min) to 200 mL Fractogel EMD SO_3 resin packed into a chromatography column previously equilibrated with 0.1 mM GTP/CB buffer. As tubulin is negatively

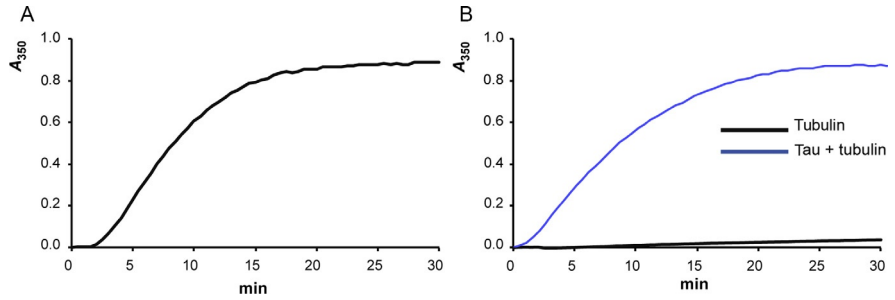

FIG. 1

Self-assembly of tubulin alone or in the presence of tau monitored by spectrophotometry at 350 nm. (A) *Purified tubulin*: 70 μM tubulin, BRB80, 1 mM GTP, 37°C. (B) *Polymerization activity of tau*: *blue line*—2 μM tau, 20 μM tubulin, BRB80, 50 mM KCl, 1 mM GTP, 37°C. *Control: black line*—20 μM tubulin, BRB80, 50 mM KCl, 1 mM GTP, 37°C.

charged at pH 6.8, it does not bind the resin and is eluted in the flow-through volume (1 mL/min). Fractions with absorbance at 280 nm greater than 0.9 are pooled.

- *First polymerization/depolymerization reaction*: The tubulin solution is then subjected to a first polymerization cycle in BRB80 buffer, 5 mM $MgCl_2$, 1 mM GTP, 33% (v/v) glycerol, about 1 h at 37°C. The solution is gently poured over a cushion (warmed up to 35°C) consisting of 60% (v/v) glycerol in Na-HEPES buffer, and then centrifuged at 35,000 rpm, 35°C, 90 min (Beckman rotor Ti-45). In the optimal conditions, the glycerol cushion and the tubulin solution are used at equal volume. The supernatants are discarded and the pellets are quickly washed with 200–300 μL warm Na-HEPES/40% (v/v) glycerol solution.
- *Labeling reaction*: Each pellet is dissolved in 100 μL Na-HEPES/40% glycerol buffer and maintained at 35°C. All the pellets are pooled. From this step, all reaction mixtures are protected from the light to avoid photobleaching of the fluorophore dye. To generate red tubulin, the ATTO-565 fluorophore (100 mM in DMSO) is added to the polymerized tubulin according to a 1:40 fluorophore: tubulin volume ratio and mixed for 6 min at 35°C. The labeling reaction is then stopped by adding two volumes of freshly made 100 mM K-glutamate solution. At this stage, it is important to control labeling, as excessive incubation with the fluorophore can affect the tubulin properties and leads to inactive protein and aggregation. We recommend keeping the labeling fluorophore/tubulin molar ratio between 0.5 and 1.
- *Depolymerization of the labeled microtubules*: The solution of labeled microtubules is gently poured over a BRB80/60% (v/v) glycerol cushion and then centrifuged at 70,000 rpm (Beckman rotor TLA-100.3), 35°C, 30 min. The supernatants are discarded and the pellets are quickly washed with BRB80 buffer (prewarmed up to 35°C). The labeled microtubules are disassembled in cold BRB80 and the solution is kept on ice, then centrifuged for 15 min at 4°C,

40,000 rpm (Beckman rotor TLA-100). The pellet corresponding to aggregated tubulin is discarded and the supernatant is recovered. Tubulin concentration is estimated by absorbance at 280 nm (1:100 dilution in BRB80 buffer) and is adjusted to 10–15 mg/mL in BRB80 buffer, 1 mM GTP, 5 mM $MgCl_2$, 30% (v/v) glycerol. The tubulin is assembled by incubation for 30 min at 37°C.

- *Depolymerization reaction and storage*: The solution of labeled microtubules is transferred over a BRB80/60% (v/v) glycerol cushion and then centrifuged at 70,000 rpm (Beckman rotor TLA-100.3), 35°C, 30 min. The supernatants are discarded and the pellets are quickly washed with warm BRB80 buffer. The microtubules are disassembled in cold BRB80 and the solution is kept on ice for 30 min then centrifuged for 10 min, 4°C at 70,000 rpm (Beckman rotor TLA-100). The supernatant is kept and submitted to a second centrifugation step (10 min, 4°C, 70,000 rpm, Beckman rotor TLA-100) to remove the residual aggregated tubulin. Small aliquots (2 μL) are prepared, flash frozen, and stored in liquid nitrogen.
- *Concentration of labeled tubulin and rate of labeling*: They are estimated in BRB80 buffer according to the following formula:

$$\text{Tubulin concentration} = \left(A_{280} - \left(A_{564} \times \text{CF}^{\text{atto}-565}\right)\right)/{\epsilon_{280}}^{\text{tubulin}}.$$

$$\text{Labeling rate} = \left(A_{564}/{\epsilon_{564}}^{\text{atto}-565}\right)/\text{tubulin concentration}.$$

[c] $\text{CF}^{\text{atto-565}} = \epsilon_{280}/{\epsilon_{564}}^{\text{atto-565}} = 0.12$; ${\epsilon_{564}}^{\text{atto-565}} = 120{,}000\,\text{M}^{-1}\,\text{cm}^{-1}$; ${\epsilon_{280}}^{\text{tubulin}} = 115{,}000\,\text{M}^{-1}\,\text{cm}^{-1}$.

3 EXPRESSION AND PURIFICATION OF RECOMBINANT TAU AND EB PROTEINS

3.1 COMPOSITION OF BUFFERS

- Purification of tau
  1. Lysis buffer: 40 mM Tris–HCl, 500 mM NaCl, 0.1% Triton X-100, protease inhibitors, pH 7.
  2. Washing buffer: 20 mM Tris–HCl, 500 mM NaCl, 10 mM Imidazole, 0.1% Triton X-100, pH 7.
  3. Elution buffer: 20 mM Tris–HCl, 500 mM NaCl, 200 mM Imidazole, pH 7.
  4. BRB80 buffer: 80 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8.[d]
  5. K-BRB80 buffer: 80 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, 50 mM KCl pH 6.8.[d]

[c] The correction factor (CF) and the extinction coefficient (ε) need to be adjusted according to the selected fluorophore (refer to the manufacturer's catalog).
[d] Final pH is adjusted with KOH.

- Purification of EB1-GFP, GFP-EB3EB3-NL-LZ-GFP
  1. Lysis buffer: 40 mM Tris–HCl, 500 mM NaCl, protease inhibitors, pH 7.
  2. Washing buffer: 20 mM Tris–HCl, 200 mM NaCl, 10 mM Imidazole, pH 7.
  3. Elution buffer: 20 mM Tris–HCl, 200 mM NaCl, 200 mM Imidazole, pH 7.
  4. K-BRB80 buffer: 80 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, 50 mM KCl pH 6.8.[d]
  5. Tris/NaCl buffer: 20 mM Tris–HCl, 150 mM NaCl, pH 7.

3.2 REAGENTS

1. *Escherichia coli* strain BL21 (DE3) pLysS—ThermoFisher Scientific, C6060
2. *E. coli* strain BL21 AI cells—ThermoFischer Scientific, C6070
3. Plasmid pDEST17 DNA—ThermoFischer Scientific, 11803012
4. pET28a DNA—NOVAGEN, 69864
5. LB Broth Miller—Euromedex, 0103
6. Stock solution: 100 g/mL ampicillin—Euromedex, EU0400-D
7. Stock solution: 35 g/mL kanamycin—ThermoFisher Scientific, 15160047
8. Stock solution: 20% (w/v) L-arabinose—Sigma Aldrich, A3256
9. Stock solution: 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG)—ROCHE, 10724815001
10. EDTA-free Protease inhibitor—ROCHE, 11836170001
11. 20,000 units/mL DNase-II in water—Sigma Aldrich, D4138
12. Triton X-100—Sigma-Aldrich, T8787
13. Tris–HCl—Sigma Aldrich, T3253
14. NaCl—Sigma Aldrich, S5886
15. Na-HEPES—Sigma Aldrich, H3784
16. PIPES—Sigma Aldrich, P6757
17. $MgCl_2$—Sigma Aldrich, 2670
18. EGTA—Sigma Aldrich, E3889
19. KCl—Sigma Aldrich, P9541
20. KOH—Sigma Aldrich, 484016
21. Imidazole—Sigma Aldrich, I0250
22. Bradford reactive—Sigma Aldrich, B6916
23. Talon metal affinity resin—Ozyme-Clontech, 635502
24. Superdex 200 gel filtration column—GE-Healthcare, 17-5175-01
25. Desalting PD-10 column—GE Healthcare, 28-9180-08

3.3 METHODS

3.3.1 Purification of 1N4R-tau

Highly purified tau preparations are essential to study tau effects on the regulation of microtubule dynamics in vitro.

A N-terminal 6× His-tagged wild-type 1N4R tau gene (GENBANK accession number: NM_001123067.2) subcloned in the pDEST17 vector and kindly provided

by Dr. Nicolas Sergeant (Lille, France) is transformed into strain BL21 AI *E. coli*. Two flasks containing 500 mL of LB medium are inoculated with 10 mL of an overnight bacterial culture and the appropriate antibiotic (100 μg/mL ampicillin). The culture is kept under constant shaking at 37°C until the A_{600} reaches approximately 0.5–0.6. Tau expression is induced by adding 0.2% (w/v) L-arabinose and the culture is kept overnight at 18°C. We recommend to keep the induction temperature lower than 20°C and not to freeze the cell solution after induction in order to minimize protein degradation.

The bacteria are harvested by centrifugation at 4000 × *g*, 4°C, 20 min. The resulting pellet is solubilized in 30 mL lysis buffer. Cells are lysed by three cycles of freezing/unfreezing in liquid nitrogen and incubation in a warm water bath (30–35°C) followed by three cycles of sonication. The resulting lysate is incubated with DNAse (100 μL at 7.3 mg/mL) and 1 mM $MgCl_2$, 10 min, on ice.

The first purification step is based on the thermostability of tau. The bacterial lysate is heated at 75°C during 10 min, precipitating most of the proteins but not tau. The precipitated proteins are eliminated by centrifugation (25,000 × *g*, 40 min at 4°C) and the supernatant is incubated with cobalt resin preequilibrated in the washing buffer for 40 min at 4°C (6 mL of resin suspension for 1 L of bacterial culture). The resin is transferred into an empty column, washed with three volumes of washing buffer, and the protein is eluted with 10 mL elution buffer by steps of 0.5 mL. Protein concentration is measured by Bradford. The most concentrated fractions are pooled and concentrated using a concentration device (cutoff 30 kDa) to get a final concentration of ∼10 mg/mL. To eliminate residual contaminants, about 250 μL of this protein solution is injected into a size exclusion column (Superdex 200) previously equilibrated with BRB80 buffer. Tau fractions are pooled, concentrated with a concentration device (cutoff 30 kDa) to 200 μM (around 10 mg/mL). The concentrated tau protein solution is aliquoted, flash frozen in liquid nitrogen, and stored at −80°C until use. The purity of the protein is determined with a 10% SDS-PAGE gel. We favor the Bradford method to estimate the tau concentration.

Mutant forms of tau used in Ramirez-Rios et al. (2016) (e.g., phosphomimetic S262E-tau and deletion mutants) are purified according to the same protocol.

Tau's activity is checked by mixing 20 μM tubulin, 2 μM tau, K-BRB80 buffer, 1 mM GTP, and monitoring microtubule polymerization at 350 nm, 37°C, 50 min (Fig. 1B). Tau's efficiency to polymerize tubulin is compared to a control solution without tau. At this concentration (i.e., close to the critical concentration for tubulin polymerization), self-assembly of tubulin alone is not detected by spectrophotometry at 350 nm.

3.3.2 Purification of recombinant human end-binding proteins

EB1-GFP, GFP-EB3, and GFP-EB3-NL-LZ (EB3 mutant lacking the C-terminal region) genes were subcloned into the pET28a vector and the proteins were purified as described (Buey et al., 2011). The constructs contain a N-terminal (GFP-EB3) or C-terminal (EB1-GFP and GFP-EB3-NL-LZ) 6 × His tag. These plasmids are transformed into strain BL21 (DE3) pLyS *E. coli*. 500 mL of LB medium are inoculated

with 10 mL of an overnight bacterial culture and the appropriate antibiotic (35 μg/mL kanamycin). The culture is kept under constant shaking at 37°C until the A_{600} reaches approximately 0.5. Protein expression is induced by adding 1 mM IPTG at 20°C overnight. The bacteria are harvested by centrifugation at $4000 \times g$, 4°C, 20 min. The resulting pellet is solubilized in 30 mL lysis buffer. Cells are lysed by three cycles of freezing/unfreezing in liquid nitrogen and a water bath (30–35°C). The resulting lysate is incubated with DNAse (100 μL at 7.3 mg/mL) and 1 mM $MgCl_2$, 10 min, on ice.

The mixture is centrifuged 30 min, 4°C at 30,000 rpm (Beckman rotor Ti-70).

The supernatant is incubated with cobalt resin preequilibrated in washing buffer for 1 h at 4°C (6 mL of resin suspension for 1 L of bacterial culture). After incubation, the resin is washed with three volumes of washing buffer and the protein is eluted with 10 mL of elution buffer by steps of 0.5 mL. As EB proteins are very sensitive to salt concentration, an intermediate desalting step is necessary before the injection into the size exclusion column. EB containing fractions are pooled, concentrated with a concentration device (cutoff 30 kDa) and loaded into a 1 mL PD-10 desalting column previously preequilibrated with K-BRB80 buffer for EB1-GFP and Tris/NaCl buffer for GFP-EB3 and GFP-EB3-NL-LZ. A fraction of EB proteins precipitate in the column at this step of desalting. After desalting, EB1-GFP, GFP-EB3, and GFP-EB3-NL-LZ proteins are further purified by gel filtration. The Superdex 200 column is equilibrated either with K-BRB80 buffer for EB1-GFP or Tris/NaCl buffer for GFP-EB3 and GFP-EB3-NL-LZ. Peak fractions are pooled, concentrated, flash frozen in liquid nitrogen, and stored at −80°C. EB1-GFP can be stored in K-BRB80 buffer however GFP-EB3 and GFP-EB3-NL-LZ proteins precipitated in this buffer and therefore are stored in Tris/NaCl buffer. The concentration of EBs is estimated by the Bradford method and refers to monomer of EBs.

4 SET-UP AND ANALYSIS OF TIRF MICROSCOPY ASSAYS

4.1 BUFFERS AND STOCK SOLUTIONS

1. mPEG-silane: 1 mg/mL PEG-silane in 96% ethanol, 0.2% (v/v) HCL. Warm up the solution at 50°C and stir the PEG-silane solution to dissolve the powder correctly. Store in the dark at room temperature.
2. Biotin-PEG-silane: 1 mg/mL Biotin-PEG-silane in 96% ethanol, 0.2% (v/v) HCL. Store in the dark at room temperature.
3. PLL-PEG: Store the powder at −20°C. Make aliquots of 10 mg approximately under Atmospheric Argon.

   Stock solution: 1 mg/mL in 10 mM filtered HEPES buffer, pH 7.4, and stored at −20°C.
4. BSA: 10% (w/v) BSA in PBS. Filter the solution (filter of 0.2 μm) and stored at −20°C.
5. Neutravidin: 1 mg/mL in water—stored at −20°C.

6. GTP: 20 mM in water—stored at −20°C.
7. DTT: 200 mM in water—stored at −20°C.
8. KCl: 500 mM in water—stored at −20°C.
9. Glucose: 450 mg/mL in BRB80—stored at −80°C.
10. BRB80 5X: 400 mM PIPES, 5 mM EGTA, 5 mM $MgCl_2$, pH 6.8,[e] stored at −20°C.
11. Methylcellulose 4000: 1% (w/v) in water—fresh solution. The powder is dissolved in deionized and filtered water prewarmed at 60°C and gently agitated until complete dissolution. The solution is stored at 4°C in the dark (no more than 1 week).
12. Antifading solution: 3.5 mg/mL Catalase +25 mg/mL Glucose oxidase in BRB80. Filter the solution, freeze aliquots immediately in liquid nitrogen, and store at −80°C.
13. KOH 1 M in water—fresh solution.
14. Hellmanex 2% (v/v) in water—fresh solution.

4.2 REAGENTS

1. Double-face precut tape (70 µm thick, 3 mm wide)—LIMA company (France)
2. High vacuum silicone grease—Sigma Aldrich, Z273554
3. Acetone 100%—VWR, 20066.321
4. Ethanol 96%—VWR, 20823362
5. Hellmanex III—Sigma Aldrich, Z805939-1EA
6. KOH—Sigma Aldrich, 484016
7. mPEG-silane, 30K—Creative PEGWorks, PSB-2014
8. Biotin-PEG-silane, MW 3400—Laysan BIO, BIOTIN-PEG-SIL
9. Neutravidin—ThermoFisher Scientific, 31000
10. PBS tablets—Sigma Aldrich, P4417
11. PLL-PEG—Jenkem, PLL20K-G35-PEG2K
12. Catalase—Sigma Aldrich, C9322
13. Glucose oxidase—Sigma Aldrich, G6766
14. GMPCPP—Jena Bioscience, NU-405S
15. GTP—Sigma Aldrich, G8877
16. DTT—Sigma Aldrich, D0632
17. Bovine Serum Albumin—Sigma Aldrich, A7030

4.3 SPECIFIC EQUIPMENT

1. Microscope slides Menzel-Gläser, SUPERFROST 76 mm × 26 mm—ThermoFischer Scientific, 12134682

[e]Final pH is adjusted with KOH.

2. Coverslips Menzel-Gläser (18 mm × 18 mm)—ThermoFischer Scientific, 11798681
3. Minicentrifuge with slide drying cassettes Galaxy mini array—VWR, 93000-204
4. Glass staining dishes—VWR, MARI4200004
5. Polypropylene slide/coverslip holder—Dutscher, 391058

4.4 METHODS

4.4.1 Preparation of fluorescent microtubule seeds

Microtubule seeds were polymerized by incubating 5 μM biotin-labeled tubulin (Hyman et al., 1991) and 5 μM ATTO-565 labeled tubulin in the presence of 1 mM GMPCPP in 100 μL BRB80 for 1 h at 37°C. The GMPCPP stock solution (10 mM) is kept at −20°C and is freshly unfrozen before the experiment. After 1 h incubation, the mixture is centrifuged at 45,000 rpm, 5 min, 35°C (Beckman rotor TLA-100). The pellet is quickly washed with 100 μL warm BRB80 buffer and solubilized in 100 μL BRB80, 1 mM GMPCPP buffer. Aliquots are prepared, flash frozen, and stored in liquid nitrogen. Before each use, aliquots are quickly unfrozen. They are kept at 32°C throughout the experiment. Most of the time, the seed solution needs to be diluted. A fresh diluted solution should be prepared for each experiment.

4.4.2 Preparation of tubulin, tau, and GFP-EBs

Aliquots of concentrated tubulin mix corresponding to unlabeled tubulin and fluorescent tubulin are prepared. Depending on the fluorophore labeling rate, the fluorescent tubulin represents 10%–25% of the total tubulin concentration. As the labeled tubulin tends to aggregate, the tubulin mix is centrifuged at 70,000 rpm (Beckman rotor TLA-100) 4°C, 10 min. The concentration of the soluble fraction is calculated according to the formula described earlier. Small aliquots of tubulin mix are flash frozen in liquid nitrogen. Each aliquot corresponds to one experimental condition.

Tau and GFP-EB proteins are unfrozen and diluted in cold BRB80 and K-BRB80 buffers, respectively. To eliminate aggregates, the diluted solutions are centrifuged at 70,000 rpm (Beckman rotor TLA-100), 10 min, 4°C. Concentrations of soluble GFP-EBs and tau are determined by Bradford assay. Aliquots are stored on ice for 1 h maximum.

4.4.3 Assembly of flow chambers

Glass slides are cleaned by successive chemical treatments as previously described (Portran et al., 2013). This whole cleaning procedure includes six steps: (1) incubation for 30 min in acetone at room temperature under agitation on an orbital shaker (80 rpm); (2) sonication for 30 min in acetone; (3) incubation for 15 min in ethanol 96% under agitation (80 rpm); (4) individual wash of each glass slide and coverslip 10 times in deionized filtered water; (5) incubation for 2 h in a diluted detergent solution (2% (v/v) Hellmanex) under agitation (80 rpm); and (6) individual wash of each coverslip and slide 10 times in deionized filtered water.

In our case, we activate the surface coverslips and microscope slides by KOH. Both are incubated for 15 min in 1 M KOH and sonicated by repeated cycles (4 min sonication followed by 1 min pause, repeated 5 times) at room temperature. The coverslips and glass slides are then washed in deionized filtered water, dried using filtered airflow or a bench centrifuge equipped with slide drying cassettes, and incubated overnight under agitation (80 rpm) in the dark in silane-PEG-biotin and silane-PEG solutions, respectively. We recommend using horizontal shaking and to avoid incubation times longer than 18 h. Each silanized coverslip and slide is washed in 96% ethanol 10 times, and then in deionized filtered water 10 times. They are dried immediately by centrifugation or filtered airflow. After this treatment, the silanized material is kept at 4°C in a dry, dark place for a few days (we recommend to store the material at 4°C for 1 week maximum).

The chambers are built by placing a coverslip over the top of a microscope slide using two pieces of double-tape precut adhesive tape. In this study, the volume of the flow chambers is about 15 μL. It is important to have a homogeneous set of chambers to be able to compare the different datasets.

The flow chamber is perfused with 30 μL neutravidin solution (25 μg/mL in 1% (w/v) BSA/BRB80). After 5 min, the chamber is filled with 50 μL PLL-g-PEG (0.1 mg/mL in 10 mM HEPES, pH 7.4) and washed immediately with 300 μL 1% (w/v) BSA/BRB80. Fluorescent microtubule seeds are diluted 1000–5000 times in warm BRB80. 50 μL of the diluted seed solution is perfused into the chamber. After 5–15 min, the chamber is washed with 100 μL 1% (w/v) BSA/BRB80. Meanwhile the reaction mixture can be prepared.

4.4.4 Reaction mixture

The reaction mixture is composed of 4 mM DTT, 1% (w/v) BSA, 50 mM KCl, 1 mM GTP, 1 mg/mL glucose, 70 μg/mL catalase, 580 μg/mL glucose oxidase, 0.1% (w/v) methylcellulose (4000 centipoises—to be prepared 1 day before performing the experiment) in BRB80 and the proteins added at the end (tubulin, EBs, tau).

In our study, we perfused 30 μL reaction mix in the chamber. We first perform the control conditions with tubulin alone. The concentration of tubulin is adjusted according to the polymerization efficiency of tubulin. For example, the microtubule elongation rate is a good reference parameter, easily quantifiable. In our study, we are working with 12–14 μM tubulin, which corresponds to ~1.3 μm/min of microtubule elongation speed at 32°C. Then tau and/or EBs can be added to the reaction mixture. Tau is known to stabilize microtubule polymerization by preventing catastrophes and promoting rescues (for detailed parameters of microtubule dynamics, see Ramirez-Rios et al., 2016). We also observed that high concentrations of tau increase self-assembly of microtubules inducing extensive nucleation events. By contrast high concentrations of GFP-EB1 inhibit microtubule growth by strongly promoting catastrophe frequency. For this reason, we fixed the maximal concentrations of tau and EB1-GFP to 75 nM. As GFP-EB3 and GFP-EB3-NL-LZ tend to bind to the microtubule lattice, we decreased their concentrations to 10 nM (Buey et al., 2011).

Once the reaction mixture is perfused, the chamber is sealed with silicon grease and put on the microscope lens. Temperature at the level of the lens is controlled and maintained at 32°C.

4.4.5 Microscope setup

Imaging is performed on an inverted Eclipse Ti (Nikon) microscope with an Apochromat 60X1.49N.A oil immersion objective (Nikon), equipped with an Ilas2 TIRF system (Roper Scientific), a cooled charged-coupled device camera (EMCCD Evolve 512, Photometrics), and a temperature stage controller (Linkam Scientific). The whole setup is controlled by the MetaMorph 7.7.5 software (Molecular Devices). Dual-color time-lapse imaging for localization of EB proteins on dynamic microtubules is performed at one frame each 2 s with an 80 ms exposure time, for 30 min. For excitation, we used 488 and 561 nm lasers.

4.4.6 Data analysis

The image analysis is achieved with ImageJ software—version 1.43u (W. Rasband, NIH, USA) and a homemade toolset (KymoTool—available upon request to eric.denarier@univ-grenoble-alpes.fr).

The two channels monitoring tubulin growth ($\lambda = 561$ nm) or GFP-EB comets ($\lambda = 488$ nm) are separated from the initial dual-view movie. The kymographs are drawn by tracing a 5-pixel thick segmented line (to fit the curvature of the microtubules) over a microtubule on the timelapse maximal projection of the microtubule movie. The selection is reported on the movie of EB comets and is straightened to draw each line of the kymograph. The new stack is resliced and a maximal intensity projection is performed resulting in a kymograph of the comet along the microtubule over time (Figs. 2 and 3). To determine GFP-EB intensity variations at

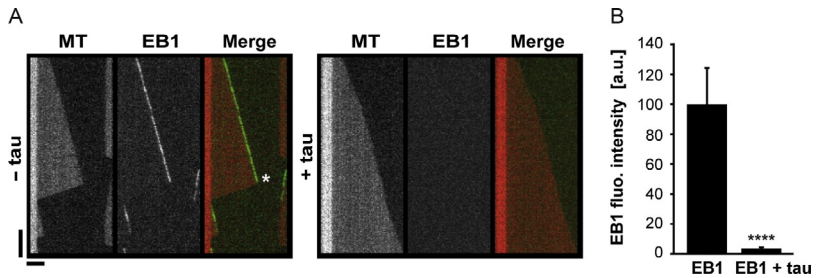

FIG. 2

TIRF kymographs illustrating the inhibitory effect of tau on EB1 localization at microtubule ends. (A) Kymographs of microtubules assembled with EB1-GFP (75 nM) or equimolar concentration of EB1-GFP and tau (75 nM). The *white stars* indicate catastrophe events. *Horizontal and vertical scale bars*, 5 μm and 60 s. MT, microtubule. (B) Quantification of EB1-GFP fluorescence intensity at microtubule ends in the absence or in the presence of tau. $P < 0.0001$ (Mann–Whitney U-test). a.u., arbitrary unit.

Adapted from Ramirez-Rios, S., Denarier, E., Prezel, E., Vinit, A., Stoppin-Mellet, V., Devred, F., et al. (2016). Molecular Biology of the Cell, 27, *2924–2934.*

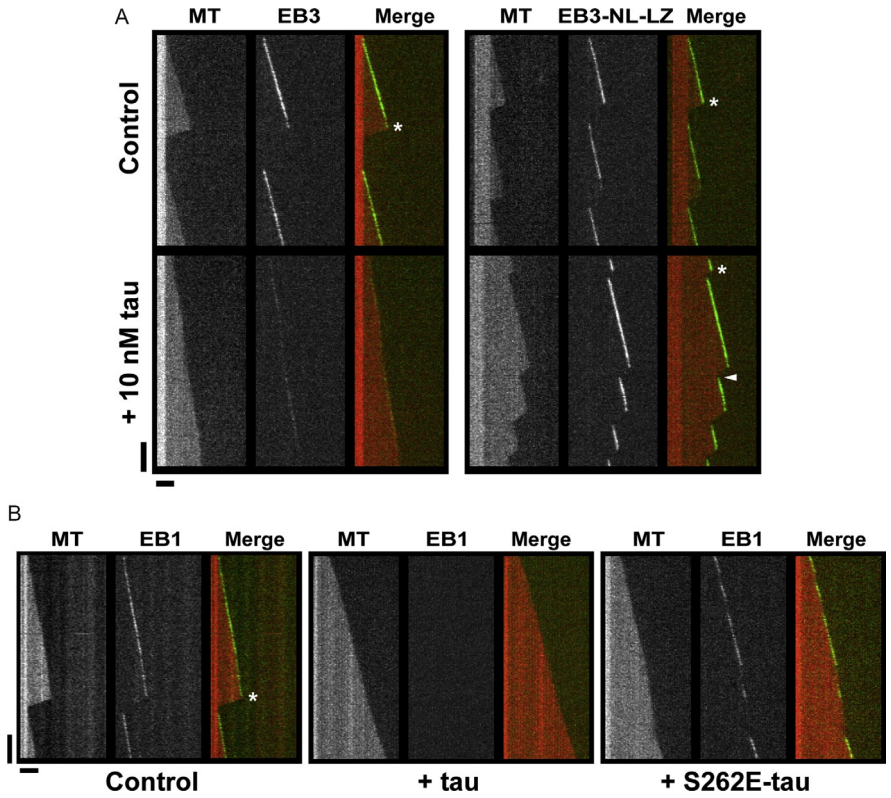

FIG. 3

TIRF kymographs illustrating the effects of EB or tau mutations on tau/EBs interplay. (A) Kymographs of microtubules assembled in the presence of wild-type GFP-EB3 or GFP-EB3-NL-LZ mutant (10 nM) with or without equimolar concentrations of tau (10 nM). (B) Kymographs of microtubules assembled in the presence of EB1-GFP (75 nM), equimolar concentrations of EB1-GFP and tau (75 nM) or equimolar concentrations of EB1-GFP and S262E-tau (75 nM). *Horizontal and vertical scale bars*, 5 μm and 60 s. *White stars and arrowheads* indicate catastrophe and rescue events, respectively. MT, microtubule.

Adapted from Ramirez-Rios, S., Denarier, E., Prezel, E., Vinit, A., Stoppin-Mellet, V., Devred, F., et al. (2016). Molecular Biology of the Cell, 27, *2924–2934.*

microtubule ends, the total fluorescence intensity at microtubule growing ends is measured in the green channel over a region-of-interest (ROI) defined by a 5-pixel thick line on the kymograph. The maximum values across the five pixels are reported in a table. To estimate the fluorescence from the local background, the same ROI is moved 10 pixels away from the microtubule end inside the background region. The average value across the five pixels is reported in the previous table. The fluorescence of GFP-EBs is obtained by subtracting the contribution of the

background to the total fluorescence (Maurer, Bieling, Cope, Hoenger, & Surrey, 2011). Signals with a negative value are removed from the calculation. The signal is then averaged along the ROI to get the fluorescence intensity of each comet. Comparisons of fluorescence intensities with and without tau proteins are calculated from experiments recorded with the same acquisition set-up and laser intensity.

The parameters of microtubule dynamics are estimated from the same kymographs. Microtubule growth and shrinkage rates are determined by measuring the slopes of growth and shrinkage phases, respectively. The catastrophe and rescue frequencies are calculated by dividing the number of catastrophe and rescue events by the total time spent in growth and shrinkage, respectively (for more details on microtubule dynamic parameters in the presence of EBs, tau, or tau/EBs combination, see Ramirez-Rios et al., 2016).

Figs. 2 and 3 show two examples of the influence of tau on EB1 (Fig. 2) and EB3s (Fig. 3A) localization at microtubule ends. In both cases, tau inhibits EB's end-tracking properties, inducing a strong decrease in EB's fluorescence intensity at microtubule ends. This inhibition is associated with changes in microtubule dynamics: EBs alone promote catastrophe events (Figs. 2 and 3), whereas combination of equimolar concentrations of EBs, and tau inhibits catastrophes and induces the apparition of persistently growing microtubules. Deletion of the C-terminal domain of EB3, which comprises the interaction site with multiple MAPs (Akhmanova & Steinmetz, 2010) and restores EB3s binding at microtubule ends (Fig. 3A). In this case, microtubules are dynamics and exhibit catastrophes and rescues due to the simultaneous destabilizing activity of the GFP-EB3-NL-LZ mutant and stabilizing activity of tau, respectively (Fig. 3A). The phosphomimetic mutation, S262E, located within tau's microtubule-binding repeats allows the recovery of EB1s binding at microtubule ends (Fig. 3B), suggesting that phosphorylation modulates the inhibitory effect of tau on EB's behavior.

5 CONCLUSION

We present a method that combines TIRF microscopy and site-directed mutagenesis to study how the interplay between tau and EBs modulate both EB's behavior at microtubule ends and microtubule dynamic properties. This method can be applied to study many variants of tau such as pseudo phosphomutants, FTDP17 disease-like mutants, or short tau fragments similar to those found in Alzheimer's disease and related dementia, providing new insights into the possible dysregulation of tau/EBs crosstalk in these pathologies.

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