Microtubules composed of α4A undergo curved growth mainly mediated by its core structure

Dear Editor,

Microtubules consisting of α/β-tubulin dimers exhibit various shapes in different cell stages and cell types, which are important for their diverse roles in eukaryotic cells. Both α- and β-tubulin comprise multiple genes in vertebrates, for example, mice have at least seven α-tubulin and eight β-tubulin genes. The distribution and expression of these isotypes vary widely among different tissues and developmental stages. Tubulin α1A is generally expressed in post-mitotic neurons and exhibits a decrease in postnatal and adult stages (Yue et al., 2014), while tubulin α4A is highly expressed in brain and heart during later stages of development (Yue et al., 2014). *In vitro* studies have shown that purified recombinant α/β3 microtubules display a higher catastrophe frequency than α/β2B microtubules (Pamula et al., 2016), and GMPCPP-α1B/β2B microtubules are more stable and have more protofilaments than GMPCPP-α1B/β3 (Ti et al., 2018), suggesting that some tubulin isotypes affect microtubule properties. However, the effects of specific α-tubulin isotype on microtubule properties remain largely unknown.

Our recent work on α1A/β2A and α1C/β2A microtubules reveals that microtubule polymerization properties are affected by specific α-tubulin isotypes (Diao et al., 2021). To further understand the effect of α-tubulin isotypes on microtubule properties, we expressed and purified α1A/β2A, α1C/β2A, and α4A/β2A dimers from insect cells (Supplementary Figure S1A and B). *In vitro* microtubule reconstitution assay with total internal reflection fluorescent (TIRF) microscopy (Supplementary Figure S1C) showed that all these α-tubulin isotype and β2A dimers were able to polymerize into microtubules in the presence of GMPCPP-stabilized microtubule seeds at 7.5 μM tubulin (Figure 1A). Surprisingly, during microtubule growth, we observed that α1A/β2A and α1C/β2A microtubules underwent straight growth while α4A/β2A microtubules underwent curved growth (Figure 1A and Video S1). Microtubules

present various morphology in cells, including straight, curved, and bundled microtubules. However, previous studies about microtubules assembled *in vitro* report that, no matter whether the tubulins are mixed isotypes purified from different species or single isotype purified from insect cells, microtubules usually grow in a relative straight way unless they encounter obstacles resulting in bending or catastrophe (Pamula et al., 2016; Aher et al., 2018). Since α4A/β2A microtubules grew with random-oriented bending, we measured the curvature (1/radius) of these microtubules in an obvious bent state that was defined as the curvature equal to or greater than 0.02 μm^{-1} (Figure 1B). Quantitative data showed that the curvature of α4A/β2A microtubules was 0.0989 ± 0.03 μm^{-1}, which was significantly larger than that of α1A/β2A and α1C/β2A microtubules (Figure 1C). Moreover, we generated kymographs to quantify the parameters of microtubule dynamics (Supplementary Figure S1C–E). The plus-end growth rate of α4A/β2A microtubules was similar to that of α1A/β2A microtubules and significantly lower than that of α1C/β2A microtubules. However, the catastrophe frequency of α4A/β2A and α1C/β2A microtubules was similar and significantly lower than that of α1A/β2A microtubules. The maximum length of α4A/β2A microtubules was lower than that of α1C/β2A microtubules, but longer than that of α1A/β2A microtubules (Supplementary Figure S1E). Taken together, these results indicate that microtubules composed of different α-tubulin isotypes including α1A, α1C, and α4A possess distinct properties, and especially, α4A/β2A microtubules display curved growth.

To explore the molecular basis of the curved growth of α4A/β2A microtubules, we compared the amino-acid sequences of α1A and α4A, and found 19 different amino acids between these two isotypes, among which twelve located within the core structure and the rest seven were in the C-terminal tail (Figure 1D). To identify whether the most variable C-terminal tail of α-tubulin accounted for the bending property of α4A/β2A microtubules, we generated and purified chimeric α-tubulin constructs with the swapped C-terminal tail between α1A and α4A (Supplementary Figure S1F). *In vitro* microtubule reconstitution assay showed that microtubules

composed of α1A with swapped α4A C-terminal tail [α1A(α4A-C)/β2A] still underwent straight growth, similar to that of α1A/β2A microtubules; and microtubules composed of α4A with the swapped C-terminal tail of α1A [α4A(α1A-C)/β2A] also exhibit curved growth, similar to that of α4A/β2A microtubules (Figure 1E and Video S2). Quantitative data showed that the curvature of α4A(α1A-C)/β2A microtubules was significantly larger than that of α1A(α4A-C)/β2A microtubules (Figure 1F). Therefore, these results suggest that the curved growth of α4A/β2A microtubules is not determined by the most variable C-terminal tail of α-tubulin.

We then analyzed the remaining different twelve amino acids within the core structure between α1A and α4A. Since both α1A/β2A and α1C/β2A formed straight microtubules (Figure 1A), we excluded the two distinct amino acids between α1A and α1C in the core region, including Gly232 (Ser232 in α1C) and Thr287 (Ser287 in α1C) in α1A, and both of them resided on tubulin surface (Figure 1D), where α4A (Ser232 and Thr287) was identical either to α1C or to α1A (Supplementary Figure S2). Moreover, because of no charged amino acid among these twelve distinct residues, we finally selected three amino acids of α4A as candidates, including Val7 and Met16 in the internal structure and Ser126 between two adjacent protofilaments (Figure 1D).

To investigate whether these three amino acids controlled the curved growth of α4A/β2A microtubules, we purified α4A with point mutation of three amino acids (Supplementary Figure S2F), and surprisingly found that α4A(V7I, M16I, S126A)/β2A [α4A(3M)/β2A] microtubules grew in a straight manner (Figure 1G and Video S3). Further, we purified α4A with point mutation of two amino acids or one amino acid (Supplementary Figure S2F), and found that both α4A(V7I, M16I)/β2A [α4A(2M)/β2A] and α4A(M16I)/β2A began to assemble into straight microtubules, while α4A(V7I)/β2A kept the curved growth of microtubules (Figure 1G and Video S3). Meanwhile, quantitative data showed that the curvature of α4A(3M)/β2A, α4A(2M)/β2A, and α4A(M16I)/β2A microtubules was dramatically lower than that of α4A(V7I)/β2A microtubules (Figure 1H). Moreover, *in vitro* microtubule reconstitution assay with the corresponding α1A(I16M) mutant showed that

α1A(I16M)/β2A microtubules tended to slightly bend (Figure 1G and Video S3). Quantitative data showed that the curvature of α1A(I16M)/β2A microtubules was larger than those of α4A(3M)/β2A, α4A(2M)/β2A, and α4A(M16I)/β2A microtubules, but significantly smaller than that of α4A(V7I)/β2A microtubules (Figure 1H), implying that the I16M mutation of α1A can only partially produce the microtubule bending phenotype. Thus, the Met16 is necessary but not sufficient for the microtubule bending behavior, some other amino acids are also required to join with the curved growth of α4A.

In cells, a variety of microtubule-associated proteins (MAPs) are involved in regulating microtubule functions. Tau is an abundant neuronal MAP that can enhance microtubule stability and regulate microtubule dynamics. A recent study has shown that Tau forms "cohesive envelopes" on microtubules, which acts as selectively permeable barriers for other MAPs and affect microtubule structure, such as inhibiting kinesin motility and straightening curved microtubules (Siahaan et al., 2022). Then, we wanted to know whether Tau could affect the curved growth of α4A/β2A microtubules. *In vitro* microtubule reconstitution assay with α4A/β2A in two different concentrations of Tau (Figure S1G), showed that in the relatively low concentration of Tau (10 nM), α4A/β2A microtubules still underwent curved growth and spontaneously formed hooks at their plus-ends, while in the relatively high concentration of Tau (100 nM) that was usually used in the *in vitro* studies (Prezel et al., 2018; Siahaan et al., 2019) and also close to but slightly lower than the physiological condition (0.5–2 μM) in neuronal cells (Wegmann et al., 2018), the curved growth of α4A/β2A microtubules was almost totally prevented (Figure 1I and K). As a control, α1A/β2A microtubules kept growing in a straight manner no matter in the presence of low or high concentration of Tau (Figure 1I). Quantitative data showed that the curvature of α4A/β2A microtubules in the high concentration of Tau was significantly smaller than that in the low concentration of Tau, and similar to that of α1A/β2A microtubules in the low or high concentration of Tau (Figure 1J). Taken together, these results suggest that the high concentration of Tau is able to straighten the curved growth of α4A/β2A microtubules.

The deformation of microtubules in cells has been revealed due to various mechanical forces, including microtubule polymerization against stationary obstacles, acto-myosin contractility, and the interaction with molecular motors such as dynein or kinesin (Bicek et al., 2009). In the present study, we identified that microtubules composed of α4A/β2A undergo special curved growth, which was mainly determined by the less hydrophobic Met16 of α4A in corresponding to isoleucine in other α-tubulin isotypes, providing a new insight into the mechanism of microtubule deformation that special tubulin isotype tunes microtubule morphology. A recent study showed that tubulin α4A was essential for platelet biogenesis by maintaining the number and arrangement of microtubule coils in the platelet marginal band (Strassel et al., 2019), the discovery of α4A/β2A microtubules with curved growth provides new evidence for α4A in maintaining the extreme bending microtubules in the platelet marginal band. Moreover, a previous study has shown that tubulin α1A plays an essential and noncompensated role in neuronal saltatory migration (Belvindrah et al., 2017), indicating that different tubulin isotypes have specific functions. However, the distribution of most tubulin isotypes in cells remains largely unclear, mainly due to the lack of specific antibodies to distinguish these highly conserved isotypes. Whether α1A, α1C, or α4A have special subcellular localization, such as in the branching sites of neuronal axon or dendrite, and play unique roles, remains to be further explored. In addition, we found that the curved α4A/β2A microtubules can be straightened by high concentration of Tau, which is highly expressed in neuron and enriched in axon, to some extent, ensuring convenient long-distance axon transportation.

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Figure 1 α4A/β2A microtubules display unique curved feature mediated by the Met16 of α4A and is able to be straightened by high concentration of Tau. (**A**) Representative time-lapse TIRF images of microtubules formed by α1A/β2A, α1C/β2A, or α4A/β2A (green, ~4% HiLyte-488-labeled porcine brain tubulin was

added to visualize microtubules) in the presence of GMPCPP-stabilized microtubule seeds (red) at 7.5 μM tubulin. Arrowheads indicated the curved microtubules. Scale bar, 10 μm. (**B**) Magnified images showing the shapes of α1A/β2A, α1C/β2A, and α4A/β2A microtubules, and schematic showing the parameters of microtubule curvature. Scale bar, 3 μm. (**C**) Histograms showing the curvature of α1A/β2A (*n* = 42 microtubules), α1C/β2A (*n* = 40 microtubules), α4A/β2A microtubules (*n* = 115 microtubules). (**D**) Schematic showing the amino acid sequence alignment of mouse α1A and α4A (left), and schematic presentation of the different amino acids between α1A and α4A within the core structure of α-tubulin in the Cryo-EM model of GMPCPP-microtubule (PDB ID: 3JAT) viewed from the lumen side (right). I7V, I16M, and A126S are shown with red spheres; G232 and T287 are shown with blue spheres; others are shown with green spheres. (**E**) Representative TIRF images of microtubules (green) formed by α1A(α4A-C)/β2A and α4A(α1A-C)/β2A in the presence of GMPCPP-stabilized microtubule seeds (red) at 7.5 μM tubulin. Arrowheads indicated the curved microtubules. Scale bar, 10 μm. (**F**) Histogram showing the curvature of α1A(α4A-C)/β2A (*n* = 40 microtubules) and α4A(α1A-C)/β2A microtubules (*n* = 40 microtubules). (**G**) Representative TIRF images of microtubules (green) formed by α4A and α1A mutants including α4A(3M)/β2A, α4A(2M)/β2A, α4A(V7I)/β2A, α4A(M16I)/β2A, and α1A(I16M)/β2A in the presence of GMPCPP-stabilized microtubule seeds (red) at 7.5 μM tubulin. Arrowheads indicated the curved microtubules. Scale bar, 10 μm. (**H**) Histogram showing the curvature of α4A(3M)/β2A (*n* = 44 microtubules), α4A(2M)/β2A (*n* = 46 microtubules), α4A(V7I)/β2A (*n* = 37 microtubules), α4A(M16I)/β2A (*n* = 40 microtubules), and α1A(I16M)/β2A microtubules (*n* = 38 microtubules). (**I**) Representative TIRF images of microtubules (red, ~4% rhodamine-labeled porcine brain tubulin was added to visualize microtubules) formed by α4A/β2A or α1A/β2A from GMPCPP-stabilized microtubule seeds (red) at 5 μM tubulin in the presence of 10 nM or 100 nM His-Tau-GFP (Green). Arrowheads indicated the microtubule hooks. Scale bar, 10 μm. (**J**) Histogram showing the curvature of α4A/β2A in the presence of 10 nM (*n* = 32 microtubules) or 100 nM His-Tau-GFP (*n* = 30 microtubules), and

α1A/β2A microtubules in the presence of 10 nM (n = 32 microtubules) or 100 nM His-Tau-GFP (n = 30 microtubules). (**K**) Magnified time-lapse TIRF images showing the process of α4A/β2A microtubule hook formation in the presence of 10 nM His-Tau-GFP and α4A/β2A microtubule hook straightening in the presence of 100 nM His-Tau-GFP. Arrowheads indicated the microtubule plus-ends. Scale bar, 3 μm. All values were obtained from at least three independent experiments. The data are presented as mean ± SEM and analyzed using one-way ANOVA or Student's t-test. $^{**}P < 0.01$ and $^{***}P < 0.001$.