

## **Tau and MAP6 establish labile and stable domains on microtubules**

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## Summary

We previously documented that individual microtubules in the axons of cultured juvenile rodent neurons consist of a labile domain and a stable domain, and that experimental depletion of tau results in selective shortening and partial stabilization of the labile domain. After confirming these findings in adult axons, we sought to understand the underlying mechanism that accounts for the formation and maintenance of these microtubule domains. We found that fluorescent tau and MAP6 ectopically expressed in RFL-6 fibroblasts predominantly segregate on different microtubules or different domains on the same microtubule, with the tau-rich ones becoming more labile than in control cells and the MAP6-rich ones being more stable than in control cells. These and other experimental findings, which we studied further using computational modeling with tunable parameters, indicate that these two MAPs do not merely bind to pre-existing stable and labile domains but actually create stable and labile domains on microtubules.

## Introduction

Decades of work on primary cultures of vertebrate neurons have revealed characteristics of the axonal microtubule array that are fundamental to defining the structural properties of the axon as well as orchestrating axonal transport <sup>1-3</sup>. Best known about axonal microtubules is that almost all of them are oriented plus-end-out <sup>4-6</sup>, with individual microtubules composed of a stable domain toward the minus end of the microtubule and a labile domain toward its plus end <sup>7-9</sup>. The labile domain is many times more dynamic than the stable domain and consists of tubulin subunits that are notably less post-translationally modified than those that comprise the stable domain. In addition, axonal microtubules are known to be rich in tau, a microtubule-associated protein (MAP) long believed to stabilize axonal microtubules <sup>10</sup>.

However, a recent study from our laboratory on cultures of juvenile rat neurons belies this long held dogma<sup>11</sup>. In that study, we demonstrated that tau is enriched on the labile domain of axonal microtubules while another MAP, namely MAP6, is enriched on the stable domain (see also,<sup>12</sup>, with tau outcompeting MAP6 to enable axonal microtubules to assemble and maintain long labile domains. Such a scenario offers a novel explanation for why the brain is so rich in tau, which is not to stabilize microtubules but rather to ensure that axons contain a robust labile microtubule fraction<sup>13</sup>.

Based on the results of those studies, we posited a mechanism whereby differences in the microtubule-binding properties of tau and MAP6 account for their capacity to form stable and labile microtubule domains on axonal microtubules<sup>11,13</sup>. Our hypothesis stems from the idea of lattice gating, wherein the binding of one MAP to the microtubule changes its lattice to promote binding of more of the same MAP<sup>14,15</sup>. We posited that this, together with tau having a much faster microtubule-binding on/off rate than MAP6<sup>16</sup>, could account for the existence on axonal microtubules of long labile domains, portions of which can be stabilized when MAP6 binding overwhelms tau binding. Of course, axons contain many other MAPs, some of which could participate similarly to MAP6 and others similarly to tau, but further investigating these two MAPs is warranted based on proof-of-principle, as well as their documented involvement in disease and injury of the nervous system<sup>17-19</sup>.

A potential caution in how we have interpreted our results to date is that not all results on juvenile neurons can be extrapolated to adult neurons. Adult neurons are known for having especially stable microtubules due in part to MAP6 but also due to other factors that are relatively deficient in juvenile neurons, such as post-translational polyamination of the microtubules<sup>20</sup>. Here, we began our studies by ascertaining whether axons in the adult rodent brain, like those of cultured juvenile rodent neurons, contains a significant labile microtubule fraction that is lost upon tau depletion. From there, we evaluated the capacity

of tau and MAP6 to form labile and stable domains on microtubules when ectopically expressed in simple fibroblasts. Finally, we used computational modeling to ascertain the parameters necessary for the properties of these two MAPs to account for the formation and maintenance of these two microtubule domains.

## Results

Adult axons contain a significant labile microtubule fraction that depends on tau. An abundance of work indicates that adult brain axons are rich in stable microtubules, some of which are more stable than those in cultured juvenile neurons<sup>3</sup>. However, it remains unclear whether axons of adult neurons contain a robust labile microtubule fraction, and if so, whether tau plays a similar role in adult neurons as in juvenile neurons to enable that labile microtubule fraction to exist. To address this issue, we first exposed living adult mouse brain sections from adult mice to nocodazole for times ranging from 15 minutes to 90 minutes and quantified the microtubule mass remaining in axon-rich regions via quantitative immunofluorescence of  $\beta$ -III tubulin staining (Figure 1A). As with the published work on juvenile neuronal cultures, there was a rapid diminution after 15-30 minutes of around half the microtubule mass, followed by little or no detectable diminution over the next 90 minutes, the remaining timeframe of the experiment. These results show that adult axons contain a robust labile microtubule fraction that is surprisingly similar to that of cultured juvenile neurons in both the proportion of the total microtubule mass and in the rate of disassembly.

To ascertain whether this labile fraction is selectively maintained by tau, we used contemporary antisense oligonucleotides (ASOs) to lower tau levels in the adult mouse brain. 4 weeks after intracerebroventricular (ICV) injection of tau ASOs or control ASOs, we found via western blotting tau levels to be substantially reduced throughout the brain and spinal cord (60% reduction in hippocampus, 70% in motor cortex, 85% in spinal cord, 65% in midbrain, 83% in cerebellum, and 71% in brain stem) (Figure 1B). Immunohistochemistry (IHC) revealed in axon-rich regions there was a 43% diminution of  $\beta$ -III tubulin staining, and this corresponded to a 45% loss of tyrosinated tubulin staining (Figure 1C). Interestingly, there was no loss of acetylated tubulin staining, suggesting that a significantly higher proportion of the tubulin that remained was acetylated compared to control. Given that acetylated tubulin is enriched in the stable microtubule fraction while tyrosinated tubulin is enriched in the labile microtubule fraction, these results

are also remarkably similar to those previously obtained on the axons of juvenile cultured neurons <sup>11</sup>. Specifically, a bit less than half of the microtubule mass is labile, and tau is needed for that labile fraction to exist. When tau is depleted, more of the microtubule mass is stable than in control axons, despite the fact that there is less total microtubule mass, indicating that in the absence of tau, even the small amount of remaining labile microtubule mass becomes less labile (i.e., somewhat more stable, but not as stable as the stable fraction).

Ectopic expression of tau and MAP6 in RFL-6 fibroblasts. As a result of alternative splicing, there are multiple developmentally expressed tau isoforms containing different numbers of repeats (3 or 4 related to exon 10) of the microtubule-binding domain, as well as the presence or absence of exons 2 and 3. 3R tau is expressed throughout life in humans whereas 4R tau becomes expressed only later in development, with the additional microtubule-binding domain of 4R presumably enabling it to bind with greater affinity to the microtubule lattice <sup>21</sup>. In mice, very little 3R tau is expressed in adults, with almost all of the tau expressed as 4R. We used separate DNA constructs for 3R and 4R DNA tau, each conjugated to mCherry. MAP6, originally called stable tubule only peptide (STOP), consists of two isoforms expressed in neurons, namely neuronal MAP6 (MAP6-N) and embryonic MAP6 (MAP6-E) <sup>22,23</sup>. MAP6-E lacks the C-terminal repeat domain of MAP6-N. We used separate DNA constructs for MAP6-N and MAP6-E, each conjugated to EGFP. In a small number of experiments, we varied the fluorescent tags to ensure that the results were not due to the different properties of the tags, and we also used a mutant MAP6-N construct that lacked a domain necessary for membrane interactions. DNA constructs for EGFP-tagged alpha tubulin served as controls.

Constructs (shown schematically in figure 2) were transfected into RFL-6 cells (flat fibroblasts of rat origin, which are useful for experiments of this kind; see Materials and Methods) using Lipofectamine 2000, either one at a time or in MAP6/tau combinations, and imaging was conducted the following day (18 hours later).

The transfection regimen was optimized through trial-and-error, as assessed by immunofluorescence microscopy, for levels of expression similar to endogenous levels in the axons of cultured neurons, as opposed to the high overexpression of these proteins used in most previous studies in which these MAPs were ectopically expressed in non-neuronal cells <sup>24-26</sup>.

Observations on cells individually expressing MAP6 or tau. Previous studies established that high levels of tau ectopically expressed in non-neuronal cells cause microtubules to become tightly bundled, often in procristalline fashion, and very stable to nocodazole-induced depolymerization (see for example <sup>25</sup>. MAP6 overexpression also results in microtubule stabilization in studies of this kind, but in some experiments required cells to be exposed to cold temperature for the MAP6 to associate with and thereby stabilize microtubules <sup>27</sup>. In the present studies, all of the tau and most of the MAP6 fluorescence appeared as microtubule associated, with some of the MAP6 fluorescence appearing as particles (Figure 3A). The MAP6-rich particles displayed movement along the microtubules but did not colocalize with markers for mitochondria or endoplasmic reticulum (mitotracker or ER-tracker; data not shown), which we explored because previous studies showed that MAP6 can associate with these organelles <sup>28</sup>. However, when we used the mutant MAP6 construct without the membrane-interacting N-terminal 3 lysine, the fluorescence was entirely microtubule associated, which is consistent with the particles being membranous vesicles of unknown identity (Figure 3A).

Relative to control, little, if any, increase in microtubule bundling was observed with either tau or MAP6 at these expression levels. Curiously, MAP6-rich microtubules displayed a great deal of lateral mobility of the microtubules (an average of 63 lateral motion events per cell) as well as curving and curling of the microtubules that presumably arose due to their mobility, whereas the tau-rich microtubules were, by comparison, far straighter and displayed lesser lateral mobility (an average of 21 lateral motion events per

cell) (Figure 3B and Figure 3C-D). In cells expressing EGFP-tubulin (with no ectopic expression of either tau or MAP6), the microtubules displayed some curving, but the number of curves was significantly less compared to MAP6-expressing cells, although the microtubules displayed similar lateral mobility as the MAP6-rich microtubules (an average of 43 lateral motion events per cell) (Figure 3C-D and Movie 1-3). (We use the term curl to indicate a closed curve, but the microtubule density were too complex to quantify curls).

MAP6 and tau, when expressed together, segregate on microtubules. Next, we co-expressed the fluorescently tagged tau and MAP6 in RFL-6 cells. For the most part, tau and MAP6 showed very little overlap in their distribution on microtubules. In most regions of the cell, the microtubules were too long and the arrays too complex to ascertain whether the tau-rich microtubules and MAP6-rich microtubules were separate microtubules or domains on the same microtubules. However, in sparser regions of the cells, it was clear that at least some of the individual microtubules consisted of tau-rich and MAP6-rich domains on the same microtubule. Some microtubules consisted of both MAPs in close proximity to one another but even on these microtubules, there was very little or no overlap between the two. We suspect that these microtubules represent those in transition from tau-rich to MAP6-rich; i.e, those undergoing stabilization. Quantification shows only 24.1% [Mander's Overlap coefficient (Costes Automated Threshold),  $R=0.241$ ] of the microtubule array associating with both MAPs. These various results were obtained in the case of both nMAP6 (Figure 4A-D) and eMAP6 (Figure 4E-H).

As with the cells expressing tau or MAP6 alone, the tau-rich microtubules (or segments of microtubules) were generally straighter with very little lateral mobility while the MAP6-rich microtubules (or segments of microtubules) were often curved and curled with a great deal of lateral mobility.



To explore the influence of each MAP on the stability properties of the microtubules, after expression of one fluorescent MAP or the other (or EGFP-tubulin), using the ratio of detyrosinated tubulin to total  $\alpha$ -tubulin as a readout for microtubule stability, the expression of tau overall made the microtubule array less stable than the EGFP-tubulin-expressing cells while MAP6 expression overall made the microtubule array more stable than the EGFP-tubulin-expressing cells (Figure 5).

RFL-6 cells co-expressing both MAPs treated with nocodazole displayed a selective loss over time of the tau-rich microtubules. Tau-rich microtubules began to depolymerize within minutes of adding the drug, with well over half of the microtubule mass depolymerized within 15 minutes, and nearly all the microtubule mass depolymerized by two hours in the drug. By contrast, less than 20% of the microtubule mass was depolymerized, even after two hours, in the case of the MAP6-rich microtubules (Figure 6A). Segregation onto separate domains of individual microtubules was even more striking after nocodazole treatment due to decreased density and complexity of the microtubule array. At 1 hour, it becomes clear that tau-rich microtubules had MAP6-rich internal domains that curved (indicated by arrowhead) (Figure 6A). After 2 hours of nocodazole treatment cells co-expressing MAP6 and tau were fixed and stained for detyrosinated alpha tubulin as well as general alpha tubulin to confirm that the stable microtubules were MAP6-rich. After 2 hours of nocodazole treatment, all of the remaining microtubules were strongly immuno-positive for detyrosinated tubulin and were mostly decorated by MAP6 (Figure 6B).

These results confirm the capacity of MAP6 to act as a stabilizer of microtubules at physiological levels, even in the absence of cold treatment in this particular cell type, whereas tau does not confer any detectable stabilization to the microtubules at this expression level. In fact, tau binding makes the microtubule less stable than they would otherwise be.

Tau on/off rate is rapid, whereas MAP6 on/off rate is slow. Previous studies of the on/off rate of tau and MAP6 association with axonal microtubules indicated that tau has an average turnover time of 15-18.6 seconds, whereas MAP6 showed a significantly slower turnover time<sup>29-31</sup>. To compare them against one another in the same experimental regimen, we used FRAP. In this assay, the on/off rate of association of the MAP to the microtubule thereby correlates with the rate of fluorescence recovery after photobleaching. The data are somewhat complicated by the fact that MAP6-rich microtubules show much greater lateral mobility than tau-rich microtubules, which disproportionally contributes to the recovery rate of MAP6. In addition, rate of diffusion of free MAP6 was greater than for free tau, as indicated by studies in which cells were treated with nocodazole for two hours to generate microtubule-deficient regions in which the diffusion of free tau or MAP6 could be assessed. Other complications include the fact that the tau-rich microtubules underwent dynamics faster than the MAP6-rich microtubules and the fact that some additional bleaching that occurred during the acquisition of the images. With these caveats in mind, we identified the diffusion ( $T_1$ ) and binding ( $T_2$ ) rates of MAP6 and tau. Generally consistent with previous data on these two MAPs, we found the  $T_1=1.4$  for tau and  $T_1=6.5$  for MAP6. We found  $T_2=5.2$  seconds for tau, which is 14 times faster than that of MAP6, which we found to be 73.6 seconds (see Figure 7 and its legend for more details).

Computational modeling. To further explore how tau and MAP6 separate into distinct domains along the microtubule, we introduce a minimal stochastic computational model of tau and MAP6 binding/unbinding (Figure 8A). Molecular details of the system are deliberately simplified to provide proof-of-principle that domain separation is an emergent pattern that can form through localized binding/unbinding of two protein populations, without either population having an *a priori* preferred binding location along the

microtubule. We treat the microtubule as a quasi-one-dimensional array of discretized binding sites, and we allow tau and MAP6 proteins to stochastically bind and unbind at random locations along the microtubule. When tau is bound near the plus end, we allow the microtubule to grow and shrink through a stochastic dynamic instability process, whereas the microtubule length is stable in the absence of tau near the plus end.

Simulations of this minimal model demonstrate that domain separation of tau and MAP6 emerges over time, in a manner that is sensitive to the relative on/off association rates of the two protein populations (Figure 8B and Movie 4). If tau binds and unbinds more rapidly than MAP6, in agreement with our experimental measurements, our simulations show that tau has the initial advantage in populating the microtubule, and the presence of tau near the plus end, in turn, promotes rapid microtubule growth. As the microtubule grows longer, the slower-binding MAP6 gradually gains a foothold along the length of the microtubule, forming long-lasting MAP6 clusters punctuated by frequent turnover of tau. Meanwhile, microtubule growth leads to a re-set of the “clock” near the plus end, with the newly polymerized segments of microtubule more likely to be bound by tau. In this way, separate domains emerge in which tau density is highest near the plus end, and MAP6 is mostly absent from the plus end and appears along the length (Figures 8 and 9). We predict a high degree of microtubule-to-microtubule variation in these distributions due to the stochastic nature of protein binding events, which agrees with experimental observations of high variability in tau and MAP6 distributions (Figure 4, Figure 9).

Next, we characterized how simulated tau and MAP6 distributions depend on the ratio of their binding rates ( $\tau_{ON}/m_{ON}$ ) (Figure 9). As expected, high ratios of  $\tau_{ON}/m_{ON}$  produce long microtubules (Figure 9A) with high tau density near the plus end (Figure 9B). Perhaps less intuitively, increasing the  $\tau_{ON}/m_{ON}$  ratio also increases the overall density of MAP6 (Figure 9C), because higher tau density at the plus end promotes

microtubule growth, which in turn allows more opportunities for the slower-binding MAP6 to accumulate along the length of the microtubule. For all values of  $\tau_{ON}/m_{ON}$  examined, we note that tau density is highest near the plus end of the microtubule (Figure 9B), while MAP6 density is highest away from the plus end (Figure 9C). This is a key confirmation of the hypothesis that tau and MAP6 domain separation can arise from unbiased binding of two protein populations.

To further characterize this emergent pattern formation, we define distribution asymmetry ( $DA$ ) for each protein as the ratio of the fraction of sites occupied by the protein near the plus-end vs. along the length (Figure 9D). A distribution asymmetry of  $DA = 1.0$  indicates that a protein is equally likely to be found near the plus end as along the length of the microtubule, whereas  $DA > 1.0$  indicates that the protein has higher density near the plus end, and  $DA < 1.0$  indicates higher density along the length. For all values of  $\frac{\tau_{ON}}{m_{ON}}$ , we find steady-state values of  $DA > 1.0$  for tau and  $DA < 1.0$  for MAP6, demonstrating that the two protein populations organize into distinct spatial domains. While the distribution asymmetry for tau is highest when the proteins have the same binding rate ( $\frac{\tau_{ON}}{m_{ON}} = 1$ ), in this case the microtubules remain short and exhibit very little binding of MAP6 along the length. When tau binding is on the order of ten times that of MAP6 ( $\frac{\tau_{ON}}{m_{ON}} = 10$ ), in agreement with experimentally observed binding kinetics, tau and MAP6 are both bound to the microtubule, with tau enriched near the plus end ( $DA \approx 1.5$ ) and MAP6 predominantly localized along the length ( $DA \approx 0.1$ ).

## Discussion

Because juvenile and adult neurons express a variety of MAPs with different properties, the impact of changes in one MAP cannot be rigorously interpreted without considering the others<sup>32</sup>. Recent studies from our laboratory gave example to this point, showing that experimental depletion of tau from cultured

juvenile neurons results in net microtubule loss from the axon but with greater levels of MAP6 bound to the microtubules that remain<sup>13</sup>. Interestingly, the lost microtubule mass was entirely of the labile fraction, not the stable fraction, thus belying the popular dogma of tau as a stabilizer of axonal microtubules. Based on these and other results, we concluded that tau binds to the labile domains of axonal microtubules to promote their elongation but also to outcompete the binding of *bona fide* stabilizers such as MAP6. Without tau, the labile domains do not assemble as avidly and become more stable as MAP6 binds to them. The question remained, however, as to whether these results only pertain to development. Our present studies on adult mouse brain confirm that the situation in adult axons is surprisingly similar to juvenile neurons, with a robust labile microtubule fraction that depends on tau for its existence. In fact, it makes sense that adult neurons express such high levels of tau to preserve the labile microtubule fraction because the factors that stabilize the stable fraction are notably greater in adult neurons compared to juvenile neurons<sup>3,20</sup>.

To better understand how tau and MAP6 compete with one another to yield stable and labile domains on individual microtubules, we took a reductionist approach by expressing them individually or together in RFL-6 rat fibroblasts. When ectopically expressed in these cells at levels roughly physiological to their levels in the axon, MAP6 stabilizes the microtubules but tau does not, and when expressed together, the two MAPs predominantly segregate onto separate microtubule fractions, with tau on the labile fraction and MAP6 on the stable fraction. Compared to the microtubules in control cells, the tau-associated microtubules are less stable, and the MAP6-associated microtubules are more stable. Thus, it is not that these two MAPs selectively bind to pre-existing stable or labile microtubules in the cells but rather that these two MAPs endow the microtubules to which they bind with their stability properties. In some cases, there was sufficient clarity in the micrographs to visualize tau-rich and MAP6-rich domains on individual microtubules, indicating that the microtubule-binding properties of these two MAPs are sufficient to account for the domain structure of axonal microtubules. Our computational model demonstrates that the

observed tau-rich and MAP6-rich domains, and resulting stability properties of microtubules, can be fully explained by the binding/unbinding dynamics of these two proteins.

Tau and MAP6 have dissimilar microtubule-binding domains and thus a simple competition for the same sites on the microtubule is unlikely. A contemporary hypothesis for how the tau/MAP6 competition is called lattice gating, which posits that the binding of one protein to the microtubule alters its lattice to make it more or less amenable to the binding of more of the same protein and/or other proteins<sup>33,34</sup>. For example, once tau starts binding, there is a cooperativity that causes more tau to bind, which is why tau appears as clusters or islands on the microtubule as its binding ensues<sup>14,35</sup>. In addition, tau forms envelopes around the microtubule that compress it in ways that change its binding properties<sup>15</sup>.

On the basis of our FRAP results, and reinforced by our computational model, our thinking is a tortoise-hare model in which tau binds and unbinds very quickly to the microtubule in a cooperative fashion, thus outcompeting MAP6, which binds and unbinds much more slowly. If the microtubule is highly dynamic, the hare always wins because the microtubule (by virtue of rapid intermittent assembly and disassembly) keeps rebooting the race to the start. However, if the microtubule is more long-lived, for example by virtue of its length, then MAP6, the tortoise, can begin to get a foothold and its own cooperative binding becomes competitive with tau and can even outcompete tau. This would not necessarily require stabilizers other than MAP6 because an especially long microtubule would undergo rescue before it completely depolymerizes, thus enabling the region toward the minus end to be long-lived by virtue of rescue events.

Of course, things are more complex in the axon. MAPs with *bona fide* microtubule stabilizing properties, such as MAP7<sup>36</sup>, could give MAP6 an assist, just as others with different properties, such as MAP1b<sup>37</sup> could give tau an assist. Tau's association with the microtubule is sensitive to its phosphorylation<sup>38</sup>, while MAP6's

association with the microtubule is sensitive to its palmitoylation status<sup>30</sup>. Thus, a signaling pathway that leads to a local increase or decrease in microtubule stability could achieve such an effect via these modifications of tau or MAP6. In addition, stable and labile microtubule fractions can vary in how stable or labile they are, just as they can vary in their ratio of tau to MAP6: the more tau, the more labile, the more MAP6, the more stable. Additionally, the differences in the curvature/mobility of the microtubules rich in each of these two MAPs suggest that the domain structure of the microtubule created by tau and MAP6 is not only relevant to the microtubule's stability properties but also to how they interact with molecular motor proteins.

Finally, it is worthwhile to speculate that our results may contribute to a better understanding of how dysfunction of these MAPs contributes to neurological disorders. Tau dysfunction has been linked to a wide array of neurodegenerative diseases<sup>17,19</sup>, while MAP6 dysfunction has been linked to neurodevelopmental disorders such as autism and schizophrenia<sup>18,39</sup>. Our results suggest that phenotypes resulting from tau or MAP6 dysfunction may be explained, at least in part, by how each of these microtubule-associated proteins (MAPs) reacts to alterations in the other. Exploring this idea will be a goal for the future.

**Limitations of the study.** Most of the experimental work was conducted in simple fibroblasts as reductionist approach to understand how two neuronal proteins interact with and change the properties of microtubules relevant to axons. However, fibroblasts are not neurons and hence the study is limited by the fact that a vast array of factors present in neurons are not present in these fibroblasts.

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**Author contributions.** KK conducted all fibroblast culture experiments and modified the DNA constructs as needed. He also conducted the antisense oligonucleotide experiments on adult mouse brain with the supervision and participation of DVW and WH, and he acquired and analyzed the data and created the figures for these portions of the work. XS and LQ conducted the nocodazole study on adult mouse brain, analyzed the data and created the relevant figure. EMC and ES conducted the computational modeling and created the relevant figures. PWB designed the project, helped with data analysis and interpretation, and wrote the manuscript along with KK and EMC.

**Availability of data and materials.** The data that support the findings discussed here are available from the corresponding author upon reasonable request.

**Declaration of interests.** The authors have declared that no conflict of interest exists.

**Ethical approval.** All animal procedures employed during this study were approved by the Institutional Animal Care and Use Committee of Drexel University and are consistent with AAALAC guidelines.



## Figure Legends

**Figure 1: Axons of adult mouse brain contain a robust labile microtubule fraction as well as a robust stable microtubule fraction and depletion of tau from adult brain causes selective loss of the labile microtubule fraction from axons.** (A) Living adult mouse brain slices were obtained by standard procedures and then exposed in tissue culture medium for various periods of time to either nocodazole or DMSO vehicle, followed by immunostaining for  $\beta$ III-tubulin and quantification of fluorescence intensity in axon-rich regions. The results demonstrate the presence of labile (steep slope portion of graph) and stable (flat slope portion of graph) microtubule fractions. Panels A-D show control and three different time points in drug. Panel E shows the quantification. Data are displayed relative to control. Scale bar is 50 microns. (B) Western blot analysis of tau levels in different brain regions and spinal cord of non-injected, tau or control ASO injected 10-12-week-old mice. GAPDH was used as loading control. Some bands are non-specific. Specific bands used for quantification are indicated by red dotted lines. Big tau is indicated by red arrow. (C) IHC for  $\beta$ III-tubulin tubulin, acetylated tubulin and tyrosinated tubulin on hippocampus of tau ASO or control ASO injected mice. Quantification of protein levels were assessed by measuring fluorescence intensities for  $\beta$ III-tubulin, acetylated tubulin, or tyrosinated tubulin in axon-rich regions of the brain sections. P values are obtained from Student's t-test. \* $p < 0.05$ , \*\*  $p < 0.01$ . Data are expressed as mean  $\pm$  SEM. Scale bar is 50 microns.

**Figure 2: Schematic representation of DNA constructs used in this study.**

**Figure 3: Ectopic expression of fluorescently tagged MAP6 increased microtubule curving and lateral mobility of microtubules whereas ectopic expression of fluorescently tagged tau decreased microtubule curving and lateral mobility of microtubules, compared to ectopic expression of**

**fluorescently tagged alpha tubulin.** (A) Individual expression of fluorescently tagged alpha-tubulin, 3R or 4R tau, eMAP6, nMAP6 or mutant MAP6 in RFL6 cells. (B) Bar graph shows quantification of number of curved microtubules. P values are obtained from one-way ANOVA. \*p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. Data are expressed as mean  $\pm$  SEM. Scale bar is 10 microns. (C) Representative images from movie 1 of RFL-6 cells ectopically expressing fluorescently tagged alpha-tubulin, MAP6 or tau. (D) Quantitative analysis of number of lateral motion events. P values are obtained from one-way ANOVA. \*p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. Data are expressed as mean  $\pm$  SEM. Scale bar is 10 microns. All images were deconvolved using ImageJ to increase resolution.

**Figure 4: In RFL-6 cells ectopically expressing fluorescently tagged MAP6 and tau, these two MAPs mainly segregate on different microtubules or different domains on the same microtubules.** (A-C) Representative image of cell expressing EGFP-nMAP6 and mCherry-tau. (D) Histogram shows segregation of MAP6 and tau on same microtubule as fluorescence intensity along the microtubule length. (E-G) Representative image of cell expressing EGFP-eMAP6 and mCherry-tau. (H) Histogram shows segregation of MAP6 and tau on same microtubule as fluorescence intensity along the microtubule length. MAP6 and tau segregated onto separate microtubules decorated predominantly with either with MAP6 or Tau (arrow in panel C and H), with tau-rich and MAP6-rich domains often manifesting on individual microtubules (arrow in panel D and I). The scale bar is 10 microns. All images were deconvolved using ImageJ to increase resolution.

**Figure 5: Ectopic expression of MAP6 in RFL-6 cells caused microtubules to become more stable.** (A) Representative images of cells expressing fluorescently tagged alpha tubulin, MAP6 or tau. Cells were stained for  $\alpha$ -tubulin and detyrosinated tubulin after methanol fixation. The scale bar is 10 microns. (B) Bar graph shows relative fluorescence using the ratio of detyrosinated tubulin to total  $\alpha$ -tubulin as a readout

for microtubule stability. P values are obtained from one-way ANOVA. \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . Data are expressed as mean  $\pm$  SEM. All images were deconvolved using ImageJ to increase resolution.

**Figure 6: MAP6-rich microtubule fraction is stable whereas tau-rich microtubule fraction is labile in RFL-6 cells ectopically expressing these MAPs and Nocodazole treatment diminishes tau-rich microtubules in RFL-6 cells co-expressing fluorescently tagged MAP6 and tau.** (A) RFL-6 cells co-expressing fluorescently tagged tau and MAP6 were treated with nocodazole for 2 hours and visualized at different time points. Tau-rich microtubules began to depolymerize within minutes of adding the drug. After 2 hours, tau-rich microtubules were almost completely depolymerized (with those remaining also rich in MAP6), while MAP6-rich microtubules showed no detectable depolymerization. At 1 hour, tau-rich microtubules had MAP6-rich internal domains that curved (indicated by arrowhead). Scale bar is 10 microns. two hours after nocodazole treatment. (B) Cells after fixing and staining for alpha tubulin and detyrosinated tubulin. (C) Co-localization of MAP6 and detyrosinated tubulin. (D) Co-localization of tau and detyrosinated tubulin. The scale bar is 5 microns. All images were deconvolved using ImageJ to increase resolution.

**Figure 7: MAP6's association with microtubules turns over much more slowly than tau's association with microtubules.** FRAP analysis of RFL-6 cells expressing EGFP-MAP6 or mCherry-tau. To identify binding rate, FRAP assay was performed on cells expressing either EGFP-MAP6 or mCherry-tau and the experiment was repeated with nocodazole treatment to find diffusion rate of MAP6 and tau. (A) Representative time-lapse images of RFL6 cells expressing nMAP6 and tau. White dotted circles indicate bleached zone. Scale bar is 2  $\mu$ m. (B) Corresponding recovery graph of MAP6 and tau with and without nocodazole (NDZ) treatment. (C) Per cent fluorescence intensity of bleached region over time for MAP6 and tau.

**Figure 8: Computational model of tau and MAP6 binding distributions along a microtubule.** (A)

Schematic of minimal model in which tau and MAP6 each bind and unbind stochastically to discrete sites along a microtubule, and the microtubule undergoes stochastic growth and depolymerization events when tau is present near the plus end. (B) Sample snapshots of the simulated evolution of protein distributions after 200 time steps (top) and 350 time steps (bottom), illustrating the initial formation of separated domains in which tau is enriched near the plus end and MAP6 is distributed along the length. See Movie 4 to watch the entire sequence for this simulation run.

**Figure 9: Computational modeling predictions of tau and MAP6 distributions.** (A) Average microtubule

length after 500 time steps as a function of tau to MAP6 binding rate ratio,  $\tau_{ON}/m_{ON}$ . (B) Fraction of binding sites bound by tau as a function of tau to MAP6 binding rate ratio,  $\tau_{ON}/m_{ON}$ . (C) Fraction of binding sites bound by MAP6 as a function of tau to MAP6 binding rate ratio,  $\tau_{ON}/m_{ON}$ . (D) Distribution asymmetry,  $DA$ , as a function of tau to MAP6 binding rate ratio,  $\tau_{ON}/m_{ON}$ . Note that the distribution asymmetry is defined as the ratio of plus-end bound fraction to fraction bound along the length, meaning that the distribution asymmetry for tau in (D) is equal to the ratio of dark blue and grey data points in (B); Likewise, the distribution asymmetry for MAP6 shown in (D) is the ratio of orange and brown data points in (C).

	Simulation parameter	Value(s) selected
Simulation scaling	Grid cell size, $dx$	$36nm$
	Simulation time step, $dt$	$0.1s$
MAP binding rates	Map6 binding rate, $m_{ON} = m_{OFF}$	$0.01s^{-1}$
	Tau binding rate, $\tau_{ON} = \tau_{OFF}$	Varied from $0.1m_{ON}$ to $10m_{ON}$
Microtubule dynamic instability parameters	Microtubule growth rate, $v_+$	$3.6\mu m/s$
	Microtubule depolymerization rate, $v_-$	$3.6\mu m/s$
	Rescue frequency, $f_{\mp}$	$50s^{-1}$
	Catastrophe frequency, $f_+$	$30s^{-1}$

**Table 1.** Input parameters for computational model.

### Movie Legends

**Movie 1:** Time-lapse movie of ectopically expressed EGFP-tubulin in RFL-6 cells. Images were captured at one second intervals over two minutes. Red arrowheads indicate laterally moving microtubules.

**Movie 2:** Time-lapse movie of ectopically expressed EGFP-MAP6 in RFL-6 cells. Images were captured at one second intervals over two minutes. Red arrowheads indicate laterally moving microtubules.

**Movie 3:** Time-lapse movie of ectopically expressed mCherry-tau in RFL-6 cells. Red arrowheads show laterally moving microtubules whereas red circles show less mobile microtubules. Images were captured at one second intervals over two minutes.

**Movie 4:** Sample simulation demonstrating the formation of distinct domains of tau and map6 binding.

## STAR★Methods

### Resource Availability

**Lead contact** Further information and requests for resources and reagents can be directed to the lead contact, Peter W. Baas (pwb22@drexel.edu).

**Materials availability** Plasmids and cell lines used in this work will be available upon request.

### Data and Code Availability

- No large dataset was generated in this study
- Any new codes for computational simulations? (Erin)

### Experimental Model and Subject Details

**Animals** 10-12 weeks old mice were obtained from Jacksons Lab (JAX stock no: 000664). These mice were used for antisense oligonucleotide injection in immunohistochemistry and western blot experiments. Only male mice were used in this study. All animal procedures employed during this study were approved by the Institutional Animal Care and Use Committee of Drexel University and are consistent with AAALAC guidelines.

**Cell Culture** RFL-6 cells (ATCC) were maintained as an adherent monolayer in F-12K media containing 20% FBS at 37 °C in a humidified environment of 5% carbon dioxide. For plasmid transfections Lipofectamine 2000 reagent was diluted in 50 µl of Opti-MEM and separately 1 µg of each plasmid was diluted in 50 µl in Opti-MEM and incubated for 5 minutes at room temperature. After incubation Lipofectamine 2000 and plasmid mixtures were combined and incubated for 20 minutes at room temperature. The mixture was added dropwise to the cells and incubated for 24 hours. For live cell imaging media was replaced with FluoroBrite™ DMEM (Thermo Scientific) containing 2% FBS and 25 mM HEPES (pH.7.2) 24 hours after transfection. Cells were fixed with cold methanol 24 hours after transfection for immunostaining. Cells were treated with 2 µg/mL of nocodazole.

## Method details

Antisense oligonucleotide treatment of adult mouse brain. Mouse tau antisense oligonucleotides (ASO) or control ASOs were administered intracerebroventricular (ICV) as a single dose (800 µg/mouse) to 10-12 weeks old C57BL/6J mice. ASOs optimized and validated for tau depletion on mice brain (as well as the control ASOs) were provided as a gift from Ionis Pharmaceuticals. Mice were sacrificed 4 weeks after ICV injection.

Adult mouse tissue preparation for western blotting and immunohistochemistry (IHC). Mice (male C57BL/6J, 10-12 weeks old) were anesthetized with Euthazol-III solution by intraperitoneal injection. After animals became unresponsive to toe pinch, the brain and spinal cord were dissected, and tissues were kept frozen at -80°C overnight for western blotting. For IHC, anesthetized animals were perfused with 0.9% w/v of NaCl solution and then with 4% PFA in PBS. Brains were dissected and were kept at 4°C overnight in 4% PFA and the next day the PFA solution was replaced with 15% sucrose solution in PBS for overnight incubation, and then changed to 30% sucrose solution in PBS. The next day brains were placed in a Tissue-Tek cryomold, embedded in M-1 Shandon embedding matrix on dry ice, and kept at -80°C until sectioning. The brains were sliced coronally at 30 µm thick using a HM500 OM Series cryostat at -20°C onto microscope slides. Slides were dried overnight at room temperature and then stored at 4°C.

Western Blot analysis of adult mouse tissues. Tissue samples of different brain regions and spinal cord were obtained from mice 4 weeks after ICV injection of control or tau ASOs. Tissues were disrupted and homogenized by sonication in cold RIPA lysis buffer with protease and phosphatase inhibitors using a Sonic Dismembrator (Fisher Scientific) at 20W power with 2 times 10 second pulses following 30 seconds of cooling on ice. Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Fisher). 30 µg of protein from each sample were denatured in 6X protein sample loading buffer (Bio-Rad) and heated

at 95°C for 10 minutes. Proteins were separated on 4-15% gels (Bio-Rad). Following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto PVDF membrane using a wet transfer system overnight at 20V. Membranes were blocked in Intercept TBS blocking buffer (LI-COR) for 1 hour at room temperature and incubated with primary antibodies (Tau R1 1:5000, GAPDH 1:10000) at 4°C overnight. Membranes were washed 3 times with TBS-T and probed with IRDye secondary antibodies (LI-COR) for 1 h at room temperature. Membranes were then washed with TBS-T and visualized using the Odyssey® CLx Imaging System (LI-COR).

IHC on adult mouse brain tissue. IHC was used to assess microtubule levels as well as markers of microtubule stability in axon-rich regions of the adult mouse brain tissue. For tissue staining, mounted mouse brain sections were permeabilized with 0.3% Triton X-100 for 1 h and then sections were quenched using a mixture of hydrogen peroxide in 100% ice-cold methanol for 1 hour. Sections were then blocked for 1 hour with normal goat serum and incubated with primary antibodies ( $\beta$ III tubulin 1:2000, acetylated tubulin 1:5000 and tyrosinated tubulin 1:5000) overnight at 4°C followed by secondary antibodies (goat anti-rabbit AlexaFluor-488, goat anti-mouse AlexaFluor-555 and goat anti-rat AlexaFluor-647 1:1000) for 2 hours at room temperature. DAPI (Thermo Fisher Scientific, D1306) was used at 1:20,000 concentration to stain the nuclei. Images of the tissues were acquired as Z-stack acquisitions using the Leica True Confocal System SP8 with 63X oil objective. All images were analyzed using ImageJ and prepared for publication using Adobe Photoshop.

Nocodazole-based assay for microtubule stability on adult mouse brain slices. Adult (male C57BL/6J, 10-12 weeks old) mouse brains were prepared similarly to the preparation used previously for whole-cell patch clamp electrophysiology (McEachern et al, 2020). Mice were anesthetized, until unresponsive to toe pinch, with Euthasol-III solution by Intraperitoneal injection. The mice were then decapitated, and the brain was



dissected and kept in ice-cold sucrose solution (in mM: 87 NaCl, 75 sucrose, 2.5 KCl, 2 CaCl<sub>2</sub>, 7 MgCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 25 dextrose) bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The brain was then sliced coronally at 300 microns thick using a Leica VT-1200 S Vibratome (Leica Microsystems, Wetzlar, Germany). Brain slides were collected and incubated at 37°C in the sucrose solution and bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> for 30 minutes. Brain slides were incubated with in a solution containing 2 µg/mL of either DMSO (as control) or nocodazole (Sigma# M1404-2MG) for 15, 30 or 90 minutes. Immediately following nocodazole treatment, the brain slides were fixed and sectioned at 30 microns thick using a Cryostat. Then brain slices were then prepared for IHC for  $\beta$ III-tubulin (1:2000). Images were captured with the Zeiss microscope indicated earlier. Quantification of  $\beta$ III-tubulin fluorescence was performed using Zeiss blue software.

Cell culture and transfection. RFL-6 rat fibroblasts were used for these studies because they are relatively flat, enabling high-resolution imaging of microtubules, because they transfect with relatively high efficiency (roughly 40%), and because they were used for previous studies from our laboratory in which neuronal MAPs and other microtubule-related proteins were ectopically expressed<sup>26,40-42</sup>. For the present work, we cultured and transfected these cells with Lipofectamine 2000 in a similar fashion to our earlier studies. Each plasmid was transfected individually or in various MAP6/tau combinations. Expression levels were optimized to roughly correlate with endogenous levels of each MAP in the axons of cultured neurons.

DNA constructs. Used for these studies were plasmids containing human alpha tubulin, 3R tau, 4R tau, eMAP6, or nMAP6 DNA, as well as that of a mutant form of MAP6 in which the N-terminal 3 lysine was substituted with alanine to prevent membrane interaction. Alpha tubulin plasmid with EGFP tag on its N-terminus was purchased from ClonTech. 3R tau and 4R tau plasmids with N-Terminal mCherry tag were kindly provided by Dr. I. Gozes. eMAP6 and nMAP6 plasmids with C-terminal EGFP were kindly provided by Dr. A. Andrieux. Because the nMAP6 and EGFP coding sequences (CDs) are separated by a stop codon,

we removed the stop codon between nMAP6 and EGFP CDs. Because the spacer length between MAP6 and EGFP was different in size for between the eMAP6 and nMAP6 constructs, we made the spacer length equal by deleting a 110 bp nucleotide sequence, via site-directed mutagenesis using the primers: MAP6\_N\_delStop\_F: 5'-ATTGAGAGCTCCCCTCACTCACCCCTTGAC-3' and MAP6\_N\_delStop\_R: 5'-GTCAAGGGGTGAGTGAGGGGAGCTCTCAAT-3'. In a small number of experiments, fluorescent tags were swapped to ensure that the results were not affected by the properties of the different tags. Fluorescent tags are on the N-terminus of tau to avoid the potential for affecting its binding to microtubules, whereas with MAP6, there was no such issue because the microtubule binding domain of MAP6 is not situated at either the C-terminus or the N-terminus.

Imaging studies on fibroblasts. For live-cell imaging, 18 hours after transfection, RFL-6 cells were imaged at 37°C on a Zeiss Axio observer Z1 microscope equipped with a stage-top incubation unit using a 63x/1.4 NA objective lens. Images were acquired at 1 second intervals over a period of 1 minute using dsRed and GFP FITC filter cubes. Laser power and exposure times were minimized to avoid photobleaching and photodamage. To determine stability properties of microtubules, cells were treated with nocodazole and visualized at different time points up to two hours. Microtubule loss was assessed in the same cell over time, using the fluorescence signal of the MAPs, and also by comparing microtubule levels in fixed cells immunostained with a total (alpha) tubulin antibody (1:2000), with additional information provided by double-staining with an antibody against detyrosinated tubulin (1:10000).

To analyze and quantify observed microtubule movements, each image was deblurred, background was removed and each frame in the movie was subtracted from the next using ImageJ to follow the trail. The number of lateral displacements of microtubules was then calculated. Microtubule sliding events were observed and recorded, but too rare to quantify.

FRAP (Fluorescence Recovery After Photobleaching) was used to determine on/off rates of tau and MAP6 association with microtubules in RFL-6 cells ectopically expressing each MAP. In order to obtain diffusion rate, cells were treated with nocodazole for 1 hour prior to imaging and kept in media containing nocodazole during imaging to create microtubule free environment. FRAP experiments were performed on a Leica SP8 confocal microscope with 63X oil immersion objective using FRAP-wizard option, with cells kept at 37°C on a stage incubator. Bleaching was performed using 488nm and 514 nm lines of argon laser at 80% laser power and 1 iteration was used for bleach pulse. Fluorescence recovery was monitored at 1% laser power at 79 millisecond intervals using the bidirectional scan mode. 2 X 200 iterations were used for post-bleach recording. FRAP analysis was done using ImageJ and related plugins (create\_spectrum.jru, combine\_all\_trajectories.jru, normalize\_trajectories.jru, batch\_FRAP\_fit.jru and average\_trajectories.jru). After subjecting 10 individual traces to photobleaching, their average was calculated and subsequently plotted as a function of time. Briefly, for each cell analyzed the bleached region was selected with an ROI and “create spectrum” plugin was run with average spectrum statistics. After obtaining all spectral plot for each data set, plots were combined into one window using “combine all trajectories” plugin. Then for the ease of fitting combined trajectories were normalized using “normalize trajectories” plugin with min-max normalization option. To generate a best fitting curve “batch FRAP fit” plugin was run, and the average recovery half-time ( $t_{1/2}$ ) values were calculated from the obtained data. Finally, the average curves were generated using “average trajectories” plugin.

All images were analyzed using ImageJ and processed using Parallel Spectral Deconvolution plugin for deblurring and prepared for publication using Adobe Photoshop.

Experimental design and statistical analysis. For animal studies, experiments were repeated 3 times and for expression studies in RFL-6c cells, experiments were repeated at least 3 times, and 20 cells were analyzed for each experiment. Analyses were focused on regions near the cell periphery where individual microtubules could be better discerned. GraphPad Prism 10 was used to perform statistical analysis and graph construction. Multiple group comparison was performed by one way ANOVA. Student's t-test was performed to compare means of two groups of parametric data sets. Data are shown as mean  $\pm$  SEM. For statistical analyses, the mean difference was significant if  $p < 0.05$ .

Computational modeling. We introduce a discretized one-dimensional minimal model to simulate the evolution of tau and MAP6 distributions along the length of a microtubule (Figure 8A). The microtubule is treated as a 1D array of discretized binding sites of size  $dx$ , where  $dx$  corresponds to the estimated length of microtubule occupied by a single MAP (either tau or MAP6). We assume that each site can only be occupied by one protein at a time, although the model is not designed to distinguish whether competition between tau and MAP6 is mediated through direct competition for binding sites (a mechanism we consider unlikely due to dissimilarity of the binding domain structures) or through some other mechanism for cooperativity such as the posited "lattice gating". During each time step of the simulation, we use a Monte Carlo algorithm to simulate stochastic binding and unbinding events. Each protein population is characterized by tunable values of their on/off rates. We define a region of the microtubule made up of the five binding sites closest to the plus end as the "plus end region" of the microtubule. To incorporate the phenomenological observation that plus-end binding of tau promotes microtubule dynamic growth, we introduce the rule that when more than half of the plus-end region is occupied by tau, then the microtubule undergoes dynamic instability. As an output of the model, we quantify the fraction of sites occupied by each type of map in the "plus-end region" vs "along the length" (all other binding sites outside of the plus-end region), to characterize the degree to which tau and MAP6 separate into distinct spatial

regions. We incorporate an established stochastic model for dynamic instability (Dogterom, 1993) in which the microtubule stochastically switches from growth to shrinking (catastrophe) at a frequency,  $f_{\pm}$ , and from shrinking to growth (rescue) at a frequency,  $f_{\mp}$ . We initialize each simulation with a very short microtubule ( $0.36\mu m$  or  $10dx$  in length), with no tau or MAP6 initially bound, and we run the simulation long enough for the protein distributions to reach a steady state ( $50.0s$  or  $500dt$ , where  $dt$  is the program time step). Parameter values examined in this study are listed in Table 1. In the absence of precise measurements of each of these parameters in live axons, we select reasonable approximations of the order of magnitude, and hold most system parameters fixed to focus our investigation on how the difference in binding rates for tau and MAP6 impacts their spatial distribution along the microtubule. While different values of the dynamic instability parameters would change the resulting microtubule lengths for a given run-time, we expect that the key qualitative conclusions of the modeling in terms of tau and MAP6 spatial organization would not be highly sensitive to these parameters.

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