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**TAU PROTEINS COOPERATIVELY ASSEMBLE INTO
COHESIVE ENVELOPES THAT PROTECT MICROTUBULES
AGAINST SEVERING ENZYMES**

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DECLARATION OF THE AUTHOR

The author of this dissertation thesis hereby declares that the thesis was written independently and all resources as well as co-authors were properly indicated whenever appropriate. All work presented in the results section was done by the author of the thesis in the laboratory of Structural Proteins. Part of this work about tau phosphorylation (manuscript attached in appendix [A.3](#)) was used by Adela Karhanova to obtain a master's degree at the Charles University (<http://hdl.handle.net/20.500.11956/181446>). The major part of this work has not been used to obtain the same or any other academic degree.

Prague, March 1, 2024

ABSTRACT

Tau is a microtubule-associated protein that is preferentially found in the neuronal axons. In neurodegenerative diseases, collectively termed tauopathies, malfunction of tau and its detachment from axonal microtubules, often associated with abnormal phosphorylation of tau, are correlated with axonal degeneration and loss of microtubule mass (Kneynsberg et al., 2017). Tau can protect microtubules from microtubule-degrading enzymes such as katanin (Qiang et al., 2006) and regulate transport by molecular motors along the microtubule (Vershinin et al., 2007; Dixit et al., 2008). However, how tau carries out these regulatory functions is still unclear. Using in vitro reconstitution and TIRF microscopy, we show that tau molecules can bind to microtubules in two distinct modes: either as (i) single tau molecules independently diffusing on the microtubule surface, or (ii) cooperatively-bound tau that form a cohesive tau "envelope" enclosing the microtubule lattice (Siahaan et al., 2019; Tan et al., 2019; Siahaan et al., 2022). We found that tau envelope formation alters the spacing of tubulin dimers within the microtubule lattice, where envelope formation compacted the underlying lattice, and lattice extension induced tau envelope disassembly (Siahaan et al., 2022). Tau envelopes form a selectively permissible barrier that inhibits kinesin-1 motors while allowing dynein movement, and protects microtubules against the activity of microtubule severing enzymes such as katanin (Siahaan et al., 2019; Tan et al., 2019). Tau envelopes itself are regulated by tau phosphorylation, where phosphorylation of tau leads to destabilization of "healthy" non-phosphorylated tau envelopes and reduced protective functionality of the envelopes (Siahaan et al. (in review)). Combined, our data reveals the microtubule-dependent cooperative binding mode of tau that can constitute an adaptable protective layer on the microtubule surface. The subtle change in the microtubules lattice structure can differentially affect the affinities of other microtubule-binding proteins to the microtubule surface, thus potentially dividing microtubules into functionally distinct segments. Finally, our data suggests that a reduction in microtubule mass linked to tau hyperphosphorylation in neurodegenerative diseases, could be explained by the destabilization and impaired functionality of the tau envelopes upon tau phosphorylation.

Keywords: Cytoskeleton, microtubules, tau, in vitro reconstitution, single molecule imaging, optical trapping, phospho-regulation, microtubule-severing enzymes, neurodegeneration.

ABSTRAKT

Tau protein se přednostně se nachází v neuronálních axonech, navázaný na mikrotubuly. U neurodegenerativních onemocnění, souhrnně nazývaných tauopatie, je nesprávná funkce tau často spojována s abnormální fosforylací tau a koreluje s axonální degenerací a ztrátou celkového množství mikrotubulů (Kneynsberg et al., 2017). Tau může chránit mikrotubuly před enzymy štěpícími mikrotubuly, jako je katanin (Qiang et al., 2006) a regulovat transport molekulárními motory podél mikrotubulu (Vershinin et al., 2007; Dixit et al., 2008). Nicméně je stále nejasné, jak tau tyto regulační funkce provádí. Pomocí in vitro rekonstituce a TIRF mikroskopie jsme ukázali, že molekuly tau se mohou vázat na mikrotubuly dvěma odlišnými způsoby: buď jako (i) jednotlivé molekuly tau nezávisle difundující na povrchu mikrotubulů, nebo jako (ii) kooperativně vázané tau, které tvoří kohezivní tau "obálky" obklopující povrch mikrotubulu (Siahaan et al., 2019; Tan et al., 2019; Siahaan et al., 2022). Zjistili jsme, že tvorba tau obálky mění rozmístění tubulinových dimerů v rámci mikrotubulu, a to tak, že zhutňuje mikrotubul pod vznikající tau obálkou. Extenze mikrotubulu naopak indukuje rozložení tau obálky (Siahaan et al., 2022). Tau obálky tvoří selektivně propustnou bariéru, která inhibuje motory kinesinu-1 a zároveň umožňuje pohyb dyneinu, a chrání mikrotubuly před aktivitou enzymů štěpících mikrotubuly (Siahaan et al., 2019; Tan et al., 2019). Samotné tau obálky jsou regulovány fosforylací tau, přičemž fosforylace tau vede k destabilizaci "zdravých" nefosforylovaných tau obálek a ke snížení jejich ochranné funkce (Siahaan et al. (v recenzním řízení)). Souhrnně naše data ukazují, že kooperativní způsob vazby tau je závislý na mikrotubulech a může tvořit adaptabilní ochrannou vrstvu na povrchu mikrotubulů. Nepatrná změna v mikrotubulární struktuře může rozdílně ovlivnit afinity jiných proteinů asociovaných s mikrotubuly, a tak potenciálně rozdělit povrch mikrotubulu na funkčně odlišné segmenty. Naše data naznačují, že snížení celkového množství mikrotubulů spojené s hyperfosforylací tau u neurodegenerativních onemocnění by mohlo být vysvětleno destabilizací a zhoršenou funkčností tau obálek po fosforylací tau.

Klíčová slova: Cytoskelet, mikrotubuly, tau, rekonstituce in vitro, zobrazování jednotlivých molekul, optická pinzeta, fosfo-regulace, mikrotubul-štěpící enzymy, neurodegenerace.

PREFACE

The decision to join the lab of Zdenek and Marcus was, while poorly thought-out, one of the best and most impactful decisions I've made in my life. At the end of 2017, I joined the lab of Zdenek and Marcus for a 3-month internship that was part of the curriculum from my masters studies. Two years later, I officially started my PhD in the lab, doing research that I loved, and working with people that inspired me. During my PhD, I mostly focused on studying tau and its interaction with microtubules. Before my PhD officially started (one month, to be exact), this research lead to my first publication entitled: "Kinetically distinct phases of tau on microtubules regulate kinesin motors and severing enzymes" ([Siahaan et al., 2019](#)). Published in a journal I never even dreamt of publishing in, Nature Cell Biology, and kind of putting high expectations on my PhD career.

In the first paper, we described the unique binding mode of tau to microtubules, where tau forms high-density patches on the microtubule surface that we initially called tau "islands" and now call tau "envelopes". We described the dynamic behaviour of the tau envelopes, as well as the regulatory roles that the envelopes possess. During the work on the first paper we observed an interesting effect that the tau envelopes had on the microtubule lattice. We found that tau envelope formation leads to a compaction of the underlying microtubule lattice, which was the basis of the research that resulted in my second paper on tau envelopes, this time published in Nature Chemical Biology ([Siahaan et al., 2022](#)). The third paper, in this "series" of tau envelope papers is currently in submission and focuses on the effect that tau phosphorylation has on the envelope formation and stability. Together with students from my lab and collaborators in Prague, we found that tau phosphorylation does not only destabilize "healthy" non-phosphorylated envelopes, it even weakens their protective functionality, making them more likely to be cut by severing enzymes ([Siahaan et al. \(unpublished\)](#)).

As mentioned above, most of my work in the lab focused on tau and microtubules, however, for one project I worked together with a postdoc from the lab, Ondřej Kučera, which allowed me to work with actin filaments and an actin-binding protein called anillin. In 2021, this project lead to an exciting paper, where I am the second author, published in Nature Communications ([Kučera et al., 2021](#)).

For this thesis, I decided to focus solely on the tau envelope papers, and therefore the anillin-actin paper will not be discussed in this work. This allows me to write an in-depth biological background on tau and microtubules, and concentrate on all the information that is relevant for the reader to understand the results and discussion. The introduction will give a detailed overview of the current knowledge in the field regarding microtubules, microtubule-severing enzymes and microtubule-associated proteins, with a main focus on tau, its structure and its function. The results section will provide an overview of the data from the tau envelope papers, with a summarized description of our findings.

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INTRODUCTION

This chapter summarizes the current knowledge in the field that is relevant for the dissertation thesis. This includes an introduction on; the cytoskeleton, microtubules, the tubulin code, microtubule-associated proteins, and microtubule-severing enzymes, followed by a more in-depth review of tau; its association with disease, its structure and regulatory roles.

1.1 THE CYTOSKELETON

To carry out their versatile functions, cells need to undergo a multitude of complex processes including cell division, growth, and migration. All these processes require extreme shape changes and rearrangement of internal compartments. For cells to re-organize themselves and carry out their functions, a robust yet dynamic framework is needed. For cells this framework is called the **cytoskeleton**, from the word cyto- (meaning "cell"), and skeleton (meaning supporting framework or basic structure). The cell cytoskeleton is a network of filaments interlinked by an assembly of proteins that form a complex and dynamic structure. The main functions of the cytoskeleton are to provide structure and organization for the cell, by for instance holding the cells' organelles in the right place, and helping to maintain the cells' unique shape while at the same time assisting in rearranging this unique shape when required. On top of that, the cytoskeleton provides the basis for cellular transport within the cell. In eukaryotes, the cytoskeleton is composed of three types of filaments: the microtubules, the actin filaments, and the intermediate filaments. In this thesis the main focus will be on the first filament type, the **microtubules**.

1.2 MICROTUBULE STRUCTURE AND DYNAMICS

Microtubules (**MTs**) are long, dynamic polymers that are essential components of the cells cytoskeleton (reviewed in [Kirschner and Mitchison \(1986\)](#)). Microtubules play an important role in maintaining cell shape and facilitating intracellular transport by acting as railways for motor proteins to transport cargo throughout the cytoplasm. The microtubule structure is composed of α - and β -tubulin that form a **tubulin heterodimer** (see Fig. 1.1). These tubulin heterodimers assemble head-to-tail into a long string called a **protofilament (PF)**. Each microtubule generally consists of 13 protofilaments, but typically ranges between 12-15 protofilaments ([Wade et al., 1990](#); [Chrétien et al., 1992](#)), that are assembled side-to-side and enclose to form a hollow tube of approximately 25 nm in diameter, depending on the number of protofilaments (see Fig. 1.1).

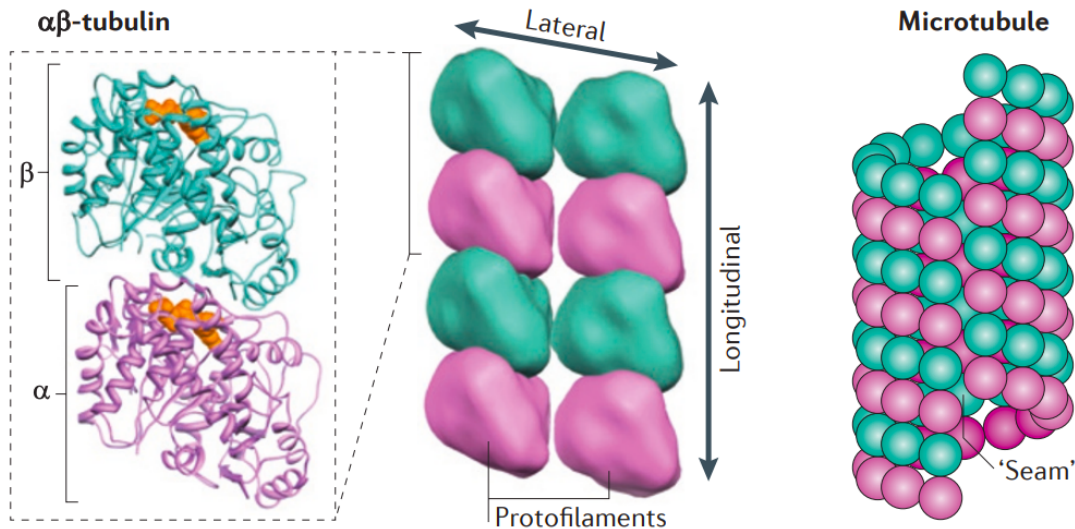


Figure 1.1: From tubulin to microtubule. The structure of the α - (magenta) and β -tubulin (cyan) when bound to GTP (orange) (left panel). The tubulin monomers combine to form the $\alpha\beta$ -tubulin heterodimer, which is the fundamental building block of microtubules. $\alpha\beta$ -tubulin heterodimers assemble through two types of contacts: (1) longitudinal contacts mediated by GTP between α - and β -tubulin to form protofilaments, and (2) lateral α - α and β - β contacts that form between protofilaments (middle panel). 12-15 protofilaments assemble laterally and enclose to form a microtubule (right panel). In microtubules, a "seam" is formed as a result of lateral α - β interactions. Figure adapted from Kollman et al. (2011).

Microtubules are polymers that can rapidly grow by the addition of tubulin (**polymerization**), or shrink by the removal of tubulin (**depolymerization**) (see Fig. 1.2). In the presence of the nucleotide guanosine triphosphate (**GTP**), microtubules polymerize by the addition of GTP-bound $\alpha\beta$ -tubulin heterodimers to their growing end. This head-to-tail polymerization gives microtubules an intrinsic polarity, whereby each microtubule has a fast growing plus-end that exposes β -tubulin, and a more stable and slow growing minus-end that exposes α -tubulin (Nogales et al., 1999). Each tubulin monomer has a nucleotide binding site in its N-terminal domain where GTP can bind (see Fig. 1.2) (Nogales et al., 1998). The GTP bound to α -tubulin is non-exchangeable and non-hydrolyzable, whereas the GTP bound to β -tubulin is exchangeable and hydrolyzable to guanosine diphosphate (**GDP**) (Berry and Shelanski, 1972; Nath et al., 1985). A tubulin heterodimer that has a GTP bound to the β -tubulin is termed as **GTP-tubulin**, where a heterodimer with GDP bound to the β -tubulin is termed as **GDP-tubulin**. Once incorporated into the lattice, GTP-tubulin, which produces a structurally stable lattice, converts into GDP-tubulin upon GTP hydrolysis of the GTP bound to the β -tubulin, and this GDP-tubulin produces a structurally unstable lattice.

During microtubule assembly, tubulin recruitment to the tip can happen at a faster rate compared to GTP hydrolysis, causing a delay in conversion of GTP- to GDP-tubulin. This delay in conversion creates a region at the growing end of the microtubule that is rich in GTP-tubulin, also called the **stabilizing cap** (David-Pfeuty et al., 1977; Carlier and Pantaloni, 1981; Erickson and O'Brien, 1992). Once the GTP hydrolysis catches up to tubulin recruitment, the underlying GDP-tubulin core gets exposed. Due to the unstable structure of the GDP-tubulin, exposure of the GDP-tubulin core triggers a sudden switch from growth to shrinkage phase, such an event is called a **catastrophe** (see Fig. 1.2). Re-establishment of the stabilizing cap is called a **rescue** event and protects the unstable GDP-tubulin core and allows the growth to resume. This phenomenon where microtubules constantly undergo phases of assembly and disassembly is known as **dynamic instability** (Mitchison and Kirschner, 1984; Brouhard and Rice, 2018), which il-

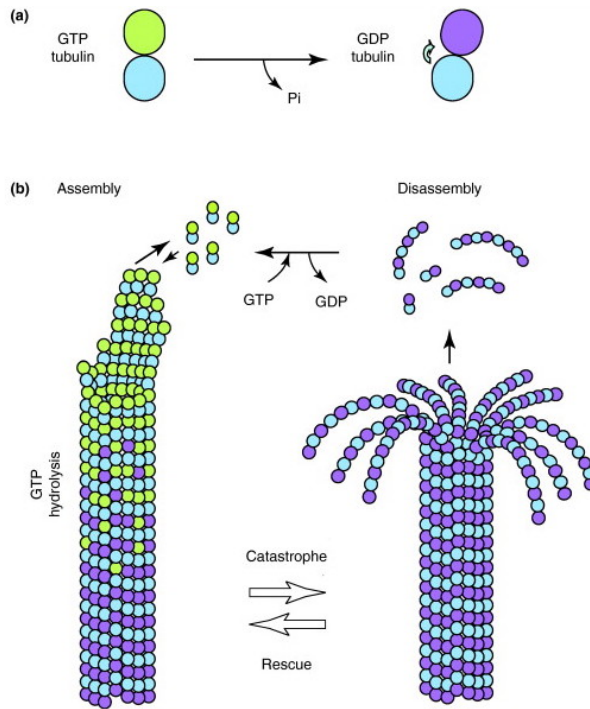


Figure 1.2: Assembly and disassembly of microtubules. (a) $\alpha\beta$ -tubulin heterodimers undergo GTP hydrolysis to convert GTP-tubulin (left) into GDP-tubulin (right). In the GTP state, α - (blue) and β -tubulin (green) produce a "straight" tubulin dimer interface. In the GDP state, the α - (blue) β -tubulin (purple) dimer interface is curved, leading to a "bent" structure. (b) During MT assembly, GTP-tubulin dimers assemble on the microtubule tips, forming a stabilizing cap of GTP-tubulin (the GTP cap). Over time, GTP hydrolysis converts GTP-tubulin in the lattice to GDP-tubulin. During MT disassembly, GDP-tubulin protofilaments curl and peel off the MT plus-ends. The transitions between growth and shrinkage states are termed catastrophe and rescue. Figure adapted from [Al-Bassam and Chang, 2011](#).

illustrates that the lengths of microtubules in cells vary constantly.

Despite the extensive amount of research done on microtubules, the structure of the stabilizing cap at the growing end of microtubules is still poorly defined. Using cryo-electron microscopy ([cryo-EM](#)), growing microtubule ends have been observed as short, blunt structures, or as ragged, tapered ends with long outwardly-curved protofilaments of varying lengths ([Mandelkow et al., 1991](#)). That some protofilaments are longer than others is mostly universally accepted (see Fig. (1.2), as well as the presence of a microtubule **seam** (see Fig. 1.1), where the lateral connections between the protofilaments are heterotypic (β -tubulin contacts α -tubulin) rather than homotypic (β contacts β , or α contacts α) and are therefore perhaps weaker ([Zhang et al., 2015](#)).

There are many factors that can influence or modulate the dynamic instability of microtubules. In the cell, microtubule dynamics is regulated by several classes of interacting and severing proteins that can either promote or obstruct microtubule stability. For instance, some end-binding proteins (**EB**) are specifically recruited to the stabilizing cap, and some microtubule polymerases, depolymerases, and kinesins cooperate or compete at the stabilizing cap to determine the microtubule's fate ([Howard and Hyman, 2007](#); [Akhmanova and Steinmetz, 2008, 2015](#)). Aside from proteins interacting with the microtubules, there are other factors that can modulate the composition of individual microtubules. These factors include different **tubulin isotypes** (or isoforms) and **post-translational modifications (PTMs)** of tubulin that modulate specific locations on a single microtubule, creating a "code" or molecular pattern that can be "read" by microtubule-interacting proteins and controls the microtubule's function. This concept is termed the "**tubulin code**" ([Verhey and Gaertig, 2007](#)) and will be described in further detail in section 1.2.1.

In vitro, microtubules can be polymerized using GTP that can hydrolyze to obtain native GDP-microtubules. However, due to the unstable nature of GDP-lattices, the need for microtubule stabilizing agents was high. There are two methods to polymerize stable microtubules in vitro

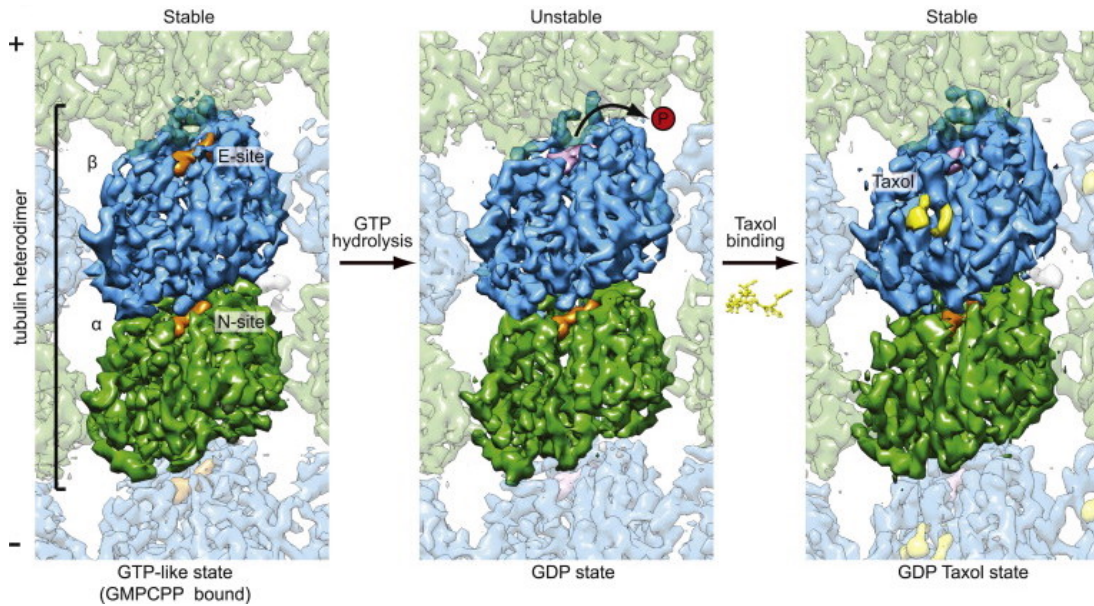


Figure 1.3: High-resolution cryo-EM structures of microtubules. Cryo-EM maps of α - (green) and β -tubulin (blue) in stable GMPCPP-bound microtubules (left panel, 4.7 Å resolution); unstable GDP-microtubules (middle panel, 4.9 Å resolution); and stable GDP-grown taxol-stabilized microtubules (right panel, 5.6 Å resolution). Tubulin are depicted as viewed from inside the microtubule lumen. GMPCPP/GTP (orange), GDP (pink), and Taxol (yellow) are visualized at their binding position. Figure adapted from Alushin et al. (2014).

that are mainly used. The first method is to polymerize microtubules in presence of guanylyl-(α,β)-methylene-diphosphonate (**GMPCPP**), a GTP analogue. **GMPCPP**, just like GTP, can bind to tubulin and incorporate into the microtubule lattice (see Fig. 1.3). However, GMPCPP hydrolyzes extremely slow after incorporation into the microtubule lattice and can therefore not easily be converted to an unstable GDP-like form. This slowly hydrolyzable property of GMPCPP ensures very stable microtubules with depolymerization rates 5000-fold slower than GDP-microtubules (Hyman et al., 1992). Early studies showed that polymerizing microtubules using GMPCPP does not change the properties of the microtubules and the GMPCPP-lattice was believed to closely mimic the GTP form of tubulin (Hyman et al., 1992, 1995), thereby making it a suitable method for in vitro polymerization of stable microtubules. The second method how to obtain stable microtubules in vitro is by stabilizing GDP-microtubules using a stabilizing drug. The first tubulin-specific drug to be discovered was **taxol** (generic name paclitaxel) (Yang and Horwitz, 2017). Taxol binds to a specific site on β -tubulin on the inside of the microtubule and is proposed to directly stabilize lateral connections between neighboring protofilaments and to allosterically stabilize the interface between neighboring heterodimers within a protofilament (see Fig. 1.3) (Nogales et al., 1999; Amos and Löwe, 1999; Alushin et al., 2014).

Recently, it was shown that following GTP hydrolysis and phosphate release, the dimers in the microtubule lattice **compact** and shorten the length of a heterodimer by 3 Å through a movement of a subdomain of α -tubulin (see Fig. 1.4) (Alushin et al., 2014). This revealed that native GDP-microtubules, as found in vivo, have a different lattice spacing between heterodimers at the GDP-rich lattice compared to the GTP-rich cap, and importantly, the GDP-lattice will also differ in lattice spacing when compared to GMPCPP-microtubules which mimic the GTP-like extended state of microtubules. Additionally, stabilizing microtubules using taxol restores the compacted GDP-lattice to a GTP- or GMPCPP-like state and therefore likewise expands the axial spacing between tubulin heterodimers within the microtubule lattice (see Fig. 1.4) (Alushin et al., 2014;

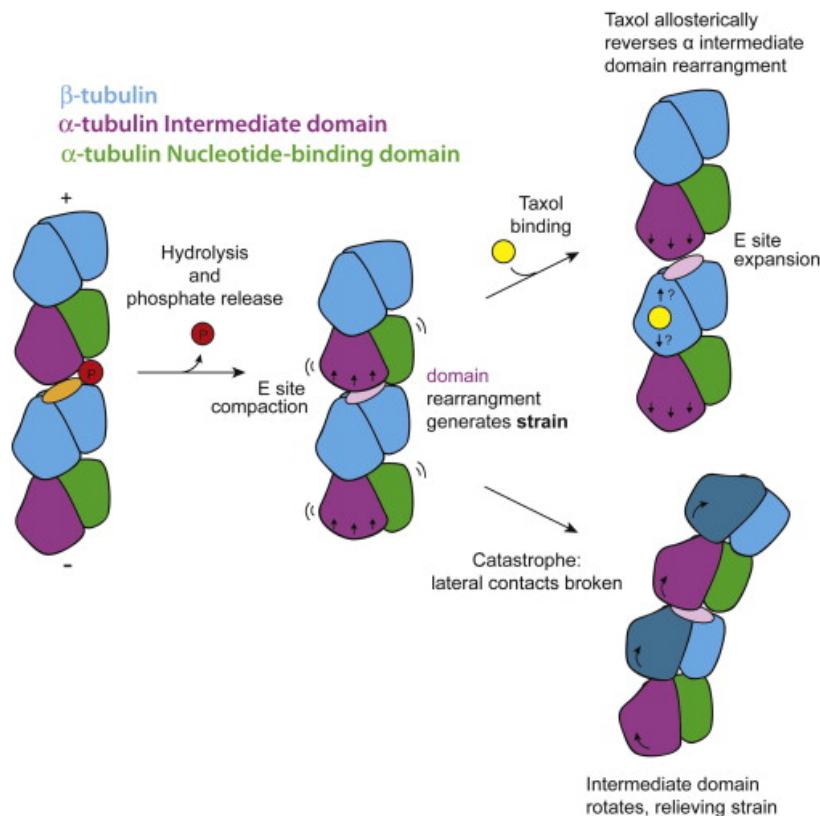


Figure 1.4: Cartoon of the proposed model of structural transition in the microtubule lattice. Nucleotide hydrolysis and phosphate release leads to compaction of the E-site and rearrangement of the α -tubulin (left to middle), generating destabilizing strain in the lattice. Taxol binding (right, top) leads to a reversal of the compaction and α -tubulin rearrangement. Subtle structural changes can propagate across the interdimer-interface (up arrow), within the dimer (down arrow) or both. In the absence of binding by a stabilizing agent, strain would disappear by tubulin bending during catastrophe (right, bottom), and the α -tubulin (and β -tubulin intermediate domain, dark blue) can undergo rotation due to the relief of constraints. Figure from [Alushin et al. \(2014\)](#).

[Kamimura et al., 2016](#)). However, there are reports that state that the extent of the extension due to taxol varies greatly and is greater when adding taxol to tubulin during microtubule assembly compared to adding taxol to preformed microtubules ([Kellogg et al., 2017](#)). Therefore, GMPCPP-microtubules, as well as taxol-stabilized microtubules to varying degrees, contain an **extended** microtubule lattice, whereas native GDP-microtubules contain a **compacted** microtubule lattice.

The differences in microtubule structure are important since proteins and enzymes that bind to the microtubule lattice can be affected by changes in the lattice structure. For instance, as mentioned above, microtubules possess a stabilizing GTP-cap at the growing plus-end of the microtubule where GTP has not hydrolyzed yet. The finding that GTP hydrolysis induces a lattice compaction indicates that GDP-microtubules contain a segment at the growing end of the microtubule where the lattice is still in the GTP-like extended state. Some microtubule-binding proteins have been shown to be sensitive to the conformational change in the MT lattice as GTP hydrolyzes. This includes end-binding (EB) proteins ([Zanic et al., 2009](#)) as well as TPX2 ([Roostalu et al., 2015](#)) that both prefer the extended lattice and the MT cap; doublecortin (DCX) that is excluded from the cap and was found to prefer the compacted GDP-like lattice ([Ettinger et al., 2016](#)); and CAMSAP3 that was found to prefer the extended microtubule lattice ([Liu and Shima, 2023](#)).

Reports on proteins not only preferring a specific lattice conformation but also altering it into their preferred state came in 2018 when Peet et al. worked with GDP-microtubules and kinesin and found that binding of kinesin-1 to GDP-microtubules increases the axial lattice spacing by 1.6% ([Peet et al., 2018](#)). This 1.6% lattice extension is less than the difference between compacted GDP-tubulin and extended GMPCPP-tubulin which was measured to be 2.4% ([Alushin](#)

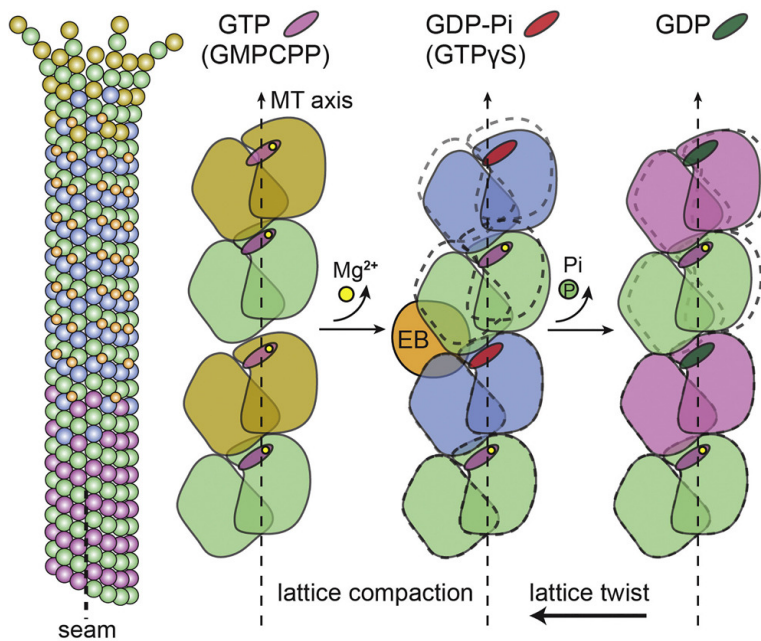


Figure 1.5: Cartoon of the proposed model describing the effect of EB Proteins on MTs. EB protein (orange) binds to the interface between α - (green) and β -tubulin (different colors). From GMPCPP (mimicking GTP) to GTP γ S (mimicking GDP) state, changes around the E-site nucleotide upon GTP hydrolysis and Mg^{2+} release accompany a lattice compaction at the interdimer-interface (viewed from the side of the MT), where EB promotes and preferentially binds to the compacted intermediate GDP-Pi state. A lattice twist upon phosphate release results in reduced EB affinity (viewed from the lumen of the MT). Figure adapted from [Zhang et al. \(2015\)](#).

[et al., 2014](#); [Zhang et al., 2018](#)). Therefore, it appears that kinesin-1 binding promotes a semi-extended state rather than one that mimics the fully extended state of a GTP-like lattice. Additionally, in the same year, Zhang et al. showed that a member of the EB proteins, EB3, was able to partially compact GMPCPP-microtubules and induce a **MT lattice twist** (see Fig. 1.5), and the compaction of the GMPCPP-lattice was dependent on the density of EB3 bound to the microtubule while the lattice twist was induced regardless of the EB3 density ([Zhang et al., 2018](#)).

1.2.1 THE TUBULIN CODE

In addition to the GDP/GTP dual nature of tubulin, **post-translational modifications (PTMs)** of tubulin and the existence of different **tubulin isotypes** can additionally modulate microtubule stability and function (reviewed in [Janke and Magiera \(2020\)](#)). The specific pattern that these PTMs and tubulin isotypes create along a microtubule lattice can influence the binding of microtubule effectors and have the potential to locally adapt microtubules for specific functions. This concept of tubulin diversity altering microtubule properties is referred to as the "tubulin code" ([Verhey and Gaertig, 2007](#)). How cells write and read the tubulin code is largely unknown but a vast amount of in vitro and in vivo work has been done to decipher this complex concept (reviewed in [Roll-Mecak \(2020\)](#)).

Many different post-translational modifications have been reported to occur on tubulin (see Fig. 1.6). Some PTMs occur specifically on α -tubulin, such as **acetylation** of Lys40 and tubulin **tyrosination/detyrosination**, while some PTMs occur mostly on β -tubulin, such as **phosphorylation** of Ser172 and **polyamination** at Glu15. Other PTMs occur on either α - or β -tubulin, including **polyglutamylation** which is the addition of glutamine-side chains to glutamine residues on the tubulin C-terminal tail (**CTT**). Tubulin PTMs have the potential to directly affect MT dy-

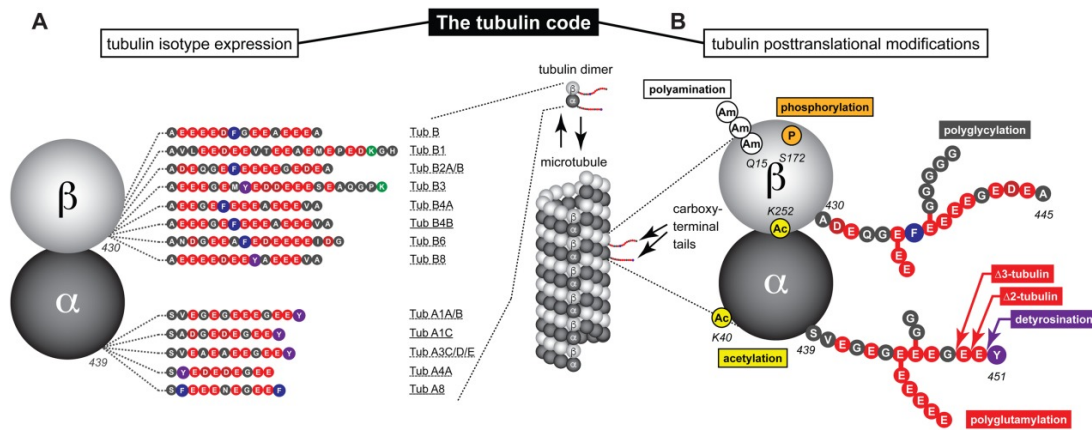


Figure 1.6: Schematic representation depicting the elements of the tubulin code. After MT polymerization, C-terminal tails of α - and β -tubulin are projecting away from the microtubule lattice. Processes that can influence the tubulin diversity can be divided into two categories: (A) by expression of different tubulin isotypes, or by (B) the presence of different tubulin PTMs. **A** The amino acid sequence of the C-terminal tails of α - and β -tubulin of the different tubulin isotypes is schematically drawn. Acidic amino acids (aspartate: D, glutamate: E) are shown in red. Note that there is little variation within the C-terminal regions of α -tubulin isotypes, whereas more variety is found within the β -tubulins. **B**: Tubulin PTMs can occur at different location on the tubulin. Acetylation of lysine (K) 40 is localized in the lumen of polymerized MTs, whereas polyglutamylation, polyglycylation, detyrosination, and follow-up modifications take place on the C-terminal tails of tubulin, thereby altering the surface of assembled MTs. Figure from [Chakraborti et al. \(2016\)](#).

namics and organization by inducing changes in the microtubule lattice structure, however, most of the variations in MT function due to tubulin PTMs are attributed to alterations in the interaction of microtubule effectors with the microtubule surface. Modulations in interaction of microtubule-associated proteins (MAPs) with microtubules can change their stability by recruiting MT severing proteins or polymerization promoting factors to the microtubule, or their function by repelling MAPs that, for example, bundle microtubules (reviewed in [Janke and Magiera \(2020\)](#)).

Mammals have at least nine α - and nine β -tubulin isotypes and all of them have a highly conserved N-terminal globular domain and an unstructured **C-terminal tail** that extends outwards along the polymerized microtubule surface (see Fig. 1.6). This exposed C-terminal tail is acidic, giving microtubules an overall negative charge on their surface ([Nogales et al., 1999](#)). Where some isotypes are ubiquitous (expressed everywhere), some isotypes have cell- or tissue-specific expression, suggesting that different isotypes might have specialized functions ([Ludueña, 2013](#)). There have been findings that support the hypothesis for distinct function of tubulin isotypes ([Fukushige et al., 1999](#)), however, inability to directly visualize distinct tubulin isotypes in vivo and low success-rate in expressing individual isotypes for in vitro studies complicates the research done on this matter. Recently, the laboratory from Robert Cross found that taxol differentially binds to tubulin isotypes ([Chew and Cross, 2023](#)). They showed that microtubules prepared from $\alpha 1 \beta 4$ isotypes extend upon taxol stabilization, while microtubules prepared from $\alpha 1 \beta 3$ isotypes stay compacted upon taxol stabilization due to lower affinity of taxol to the latter isotype. However, when using GMPCPP, both isotypes will be driven to extension, indicating that extension of the lattice is possible but the potentially lower taxol occupancy on $\alpha 1 \beta 3$ -microtubules is insufficient to induce extension of the lattice. These findings indicate that specific tubulin isotypes can show unique interactions with binding partners that can differentially affect their functionality.

1.3 MICROTUBULE-ASSOCIATED PROTEINS

The precise organization of MTs, including their composition, stability, and spacing, is essential for the correct development and function of cells. As discussed in the previous section, this organization is controlled by many different factors including post-translational modifications of the tubulin, different tubulin isoforms, but also by outside factors binding to the microtubule lattice. Proteins that interact with microtubules are collectively referred to as **microtubule-associated proteins**, or **MAPs**.

There are different ways how to classify or divide MAPs into distinct groups, but most generally, MAPs are divided based on their mode of action (reviewed in [Bodakuntla et al. \(2019\)](#)), leading to the following division: The first group of MAPs contains the "**motile MAPs**", which are MT-based **molecular motors proteins**, such as kinesin and dynein motors (reviewed in [Hirokawa et al. \(2010\)](#)). The second group consists of factors that break or **depolymerize microtubules**, such as **microtubule-severing enzymes** katanin and spastin (reviewed in [McNally and Roll-Mecak \(2018\)](#)). The third group contains proteins that act as **microtubule nucleators**, which include **microtubule polymerases** (reviewed in [Roostalu et al. \(2015\)](#)). The fourth group consists of **end-binding proteins** that associate with plus- or minus-ends of microtubules (reviewed in [Akhmanova and Steinmetz \(2015\)](#)). And finally, the fifth group is the so-called "**structural MAPs**" group which was originally defined as proteins that bind and stabilize microtubules, but the group was eventually named due to their ability to control the structure of microtubule assemblies. This last group of MAPs is the most broad group that cannot be defined by a single function, since new functions of the structural MAPs keep emerging and a systemic view of their functions is still missing. Figure 1.7 gives an overview of some of the known functions of structural MAPs, which include **crosslinking microtubules** with other cytoskeletal filaments, promoting **microtubule bundling**, stabilizing and polymerizing MTs, and regulating other microtubule-associated factors (reviewed in [Bodakuntla et al. \(2019\)](#)). The structural MAPs group includes the MAP2/tau family of MAPs, which includes all isoforms of MAP2, MAP4, and tau, and their homologs in other animals (reviewed in [Dehmelt and Halpain \(2005\)](#)).

Structural MAPs, also referred to as classical MAPs, were originally isolated from mammalian brains by co-purification with MTs (reviewed in [Schoenfeld and Obar \(1994\)](#)). In vitro studies showed that all structural MAPs have a **microtubule-binding region** and most MAPs bind along the MT lattice where they regulate microtubule-related processes such as MT polymerization and stabilization. However, the binding characteristics and effects on MTs and MT-associated factors differ among MAPs (see Fig. 1.8). For instance, MAP2, MAP4, and tau bind along the outer ridges of the protofilament (PF) ([Al-Bassam et al., 2002](#); [Kellogg et al., 2018](#); [Shigematsu et al., 2018](#)) and MAP7 was originally thought to bind at the same region (see Fig. 1.8), but has recently been shown to bind halfway between the outer ridge and the site of lateral contacts of the protofilaments ([Ferro et al., 2022](#)). Doublecortin (**DCX**) was found to attach to the grooves between PFs which is thought to provide lateral and longitudinal stability to the microtubules ([Moores et al., 2004](#)) and MAP9 has been speculated to have the same binding site as DCX ([Monroy et al., 2020](#)). MAP6 has recently been shown to bind to the microtubule lumen when

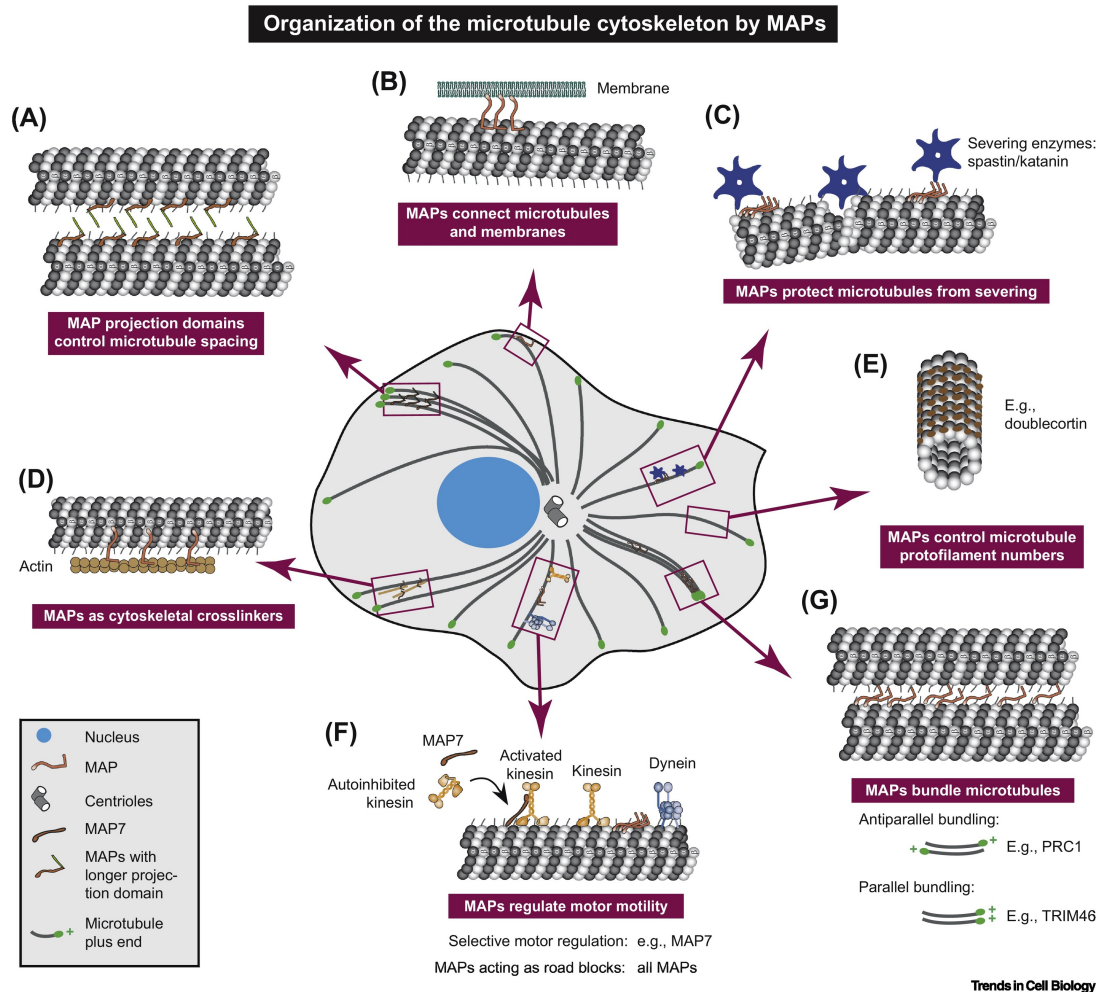


Figure 1.7: Functions and organization of the microtubule cytoskeleton by structural MAPs. Structural MAPs have many different functionalities and can be divided into: (A) MAPs that bind to microtubules and regulate spacing between microtubules. Typically these MAPs have structures containing long projection domains that regulate the spacing; (B) MAPs that crosslink and connect microtubules with membranes; (C) MAPs that protect microtubules from microtubule-severing enzymes, and thus from disassembly; (D) MAPs that crosslink microtubules with different cytoskeletal elements, such as actin; (E) MAPs that control the protofilament number of microtubules; (F) MAPs that affect the binding and motility of motor proteins either by forming a complex with the motor or by acting as a roadblock on the microtubule surface; (G) MAPs that promote microtubule bundling by neutralizing the acidic C-terminal tails of tubulin. Figure adapted from Bodakuntla et al. (2019).

co-polymerizing microtubules in presence of MAP6 (Cuveillier et al., 2020). Additionally, most MAPs, like tau and MAP2, only need a single protofilament for MT binding, while MAP4 has been shown to interact with adjacent protofilaments (Al-Bassam et al., 2002; Kawachi et al., 2003).

Some structural MAPs, like tau and MAP2, induce MT bundling, whereas others like MAP4 do not have bundling activity (Lewis et al., 1989; Nguyen et al., 1997). Many MAPs can, aside from binding MTs, also bind to other cytoskeletal filaments including actin (Griffith and Pollard, 1982), which is another important factor in cytoskeletal regulation and neural development. Localization of structural MAPs differs greatly for each MAP family or even individual members of a family. MAPs such as MAP1a, MAP2, DCX, and MAP9 are overall **dendrite-specific**, while MAPs such as tau are **axon-specific**, with MAP4 and MAP7 localized at the branchpoints along axons. Other MAPs localize to both the dendrites and axons throughout development, includ-

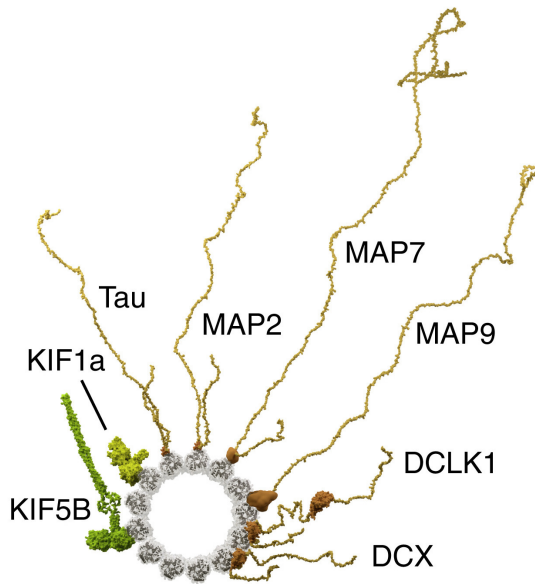


Figure 1.8: Known or predicted binding sites of MAPs to microtubules. View of the microtubule from the minus-end on, showing the 13 protofilaments (grey). The modeled MAPs and kinesin motors are visualized at their known or predicted binding sites to the microtubule. KIF5B and KIF1a motor proteins have been shown to attach to the protofilament edge. Tau and MAP2c have been shown to bind along the outer PF ridge. Plausible MT-binding location for MAP7 and MAP9 were modeled (predicted binding sites shown). DCX and its paralog doublecortin-like kinase-1 ([DCLK1](#)) attach to the grooves between the PFs. MAPs are shown as full-length pseudo-models, with projection domains and domain linkers modeled as intrinsically disordered. Figure adapted from [Monroy et al. \(2020\)](#).

ing MAP1b and MAP6 (reviewed in [Ramkumar et al. \(2018\)](#)). The differential localization of specific MAPs can explain the large variety in the known functions and regulatory roles of the MAPs.

1.3.1 THE MAP CODE

The "**MAP code**" is a model that was proposed in recent years as an addition to the existing tubulin code that was introduced in 2007 ([Verhey and Gaertig, 2007](#); [Monroy et al., 2020](#)). The tubulin code describes the concept that different post-translation modifications ([PTMs](#)) and tubulin isotypes control the properties and functions of the microtubule cytoskeleton (reviewed in [Verhey and Gaertig \(2007\)](#); [Janke and Chloë Bulinski \(2011\)](#)). The MAP code adds another layer of complexity to this regulations and aims to describe the concept that the binding of different MAPs likewise control the properties and function of the microtubule cytoskeleton and thereby regulate the interactions of other factors with the microtubules. Additionally, PTMs such as phosphorylation of MAPs can further regulate the MAPs interaction with the microtubules and thereby alter the microtubule structure and function.

Tau and MAP2 are known to inhibit kinesin-driven motility along the microtubule lattice ([Ebnet et al., 1998](#); [Trinczek et al., 1999](#); [Seitz et al., 2002](#)). In contrast, MAP7 family members are established to be **positive regulators** of kinesin-1 ([Sung et al., 2008](#)), that have been shown to activate kinesin motility ([Hooikaas et al., 2019](#); [Monroy et al., 2020](#)). Additionally, despite the different binding sites of tau and MAP7, it has recently been shown that tau and MAP7 compete for binding on taxol-stabilized microtubules. This created a patch-like binding pattern of separate tau and MAP7 domains decorating the microtubule lattice, and these domains differentially promote or inhibit kinesin-1 stepping ([Monroy et al., 2020](#)). Kinesin-1 motor heads bind at the intratubulin-dimer interface between the α - and β - tubulin ([Shigematsu et al., 2018](#)), and this binding site is a weak binding site for tau ([Kellogg et al., 2018](#)). The weak overlap in binding could be a factor contributing to the inhibition of kinesin-1 motility in tau-rich areas, however, kinesin-1 has been shown to be recruited to microtubules decorated with MAP7 despite kinesin-1 and MAP7 competing for the same binding site ([Ferro et al., 2022](#)). Tracking kinesin-1

1.4. MICROTUBULE-SEVERING ENZYMES

stepping with nanometer precision revealed an increase in the probability of sideways and backwards stepping of kinesin in the presence of MAP7 on the microtubules (Ferro et al., 2022). Therefore, the proposed model for kinesin stepping on MAP7 decorated MTs was described as a "**tethered diffusion**" model, where the projection domain of MAP7 tethers kinesin to the MT surface, allowing it to rebind the MT at available sites. This model was further substantiated by the finding that when the MT surface was nearly saturated with MAP7, the frequency and length of kinesin-1 runs was reduced, likely due to the scarcity of empty binding sites.

Post-translational modifications (PTMs) of structural MAPs, such as **phosphorylation**, have been shown to affect the binding of MAPs to microtubules (reviewed in Ramkumar et al. (2018)) and it was shown that there are three general classes of effects that phosphorylation has on the MAPs:

- (1) **MAP dissociation** from the MT: was found for DCX, MAP2, MAP4, MAP6, tau.
- (2) **MAP relocation** within the cell: was found for DCX, MAP2, MAP6, MAP7, MAP9.
- (3) **Altered MAP function**: was found for MAP1a, MAP1b, MAP9, tau.

The differential effects of phosphorylation alone indicate the complexity of the system. Combining it with other PTMs of the MAPs and all the effects described in the tubulin code creates a demanding task to elucidate the exact mechanisms of organization of microtubule-related processes in neurons and other cells.

1.4 MICROTUBULE-SEVERING ENZYMES

Microtubule-severing enzymes, also called **severases**, are a class of microtubule regulators that include katanin, spastin and fidgetin (reviewed in McNally and Roll-Mecak (2018)). These enzymes are known to sever (or cut) microtubules into shorter fragments that can repolymerize after the severing event, indicating that the severing activity of the enzyme does not modify or denature the tubulin (McNally and Vale, 1993).

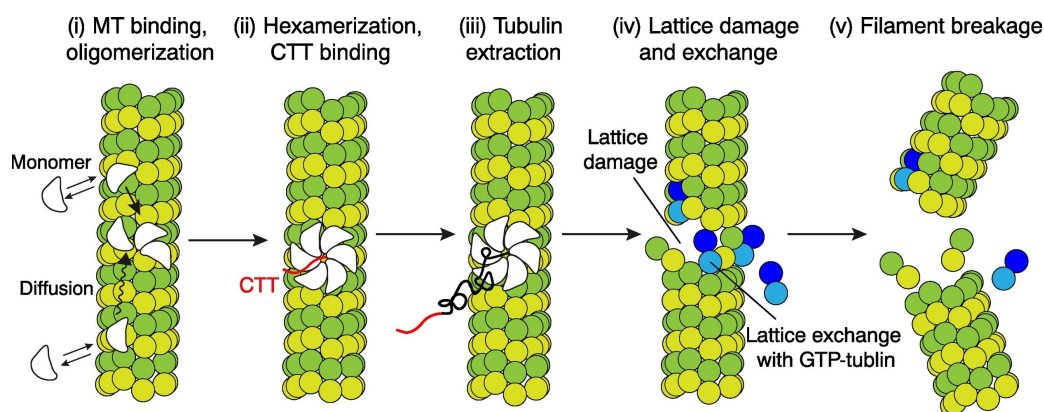


Figure 1.9: The model of microtubule severing. Microtubule severing requires several steps: (i) enzyme monomers diffuse on the microtubule lattice and assembly into hexamers, (ii) hexamers enclose the tubulin C-terminal tail (CTT), (iii) the CTT gets pulled through the pore of the hexamer, (iv) removal of tubulin subunits creates lattice defects, (v) lattice defects can lead to microtubule breakage. GTP-tubulin (blue) can be added to the defects, leading to lattice exchange and potential repair. Figure adapted from Kuo and Howard (2021).

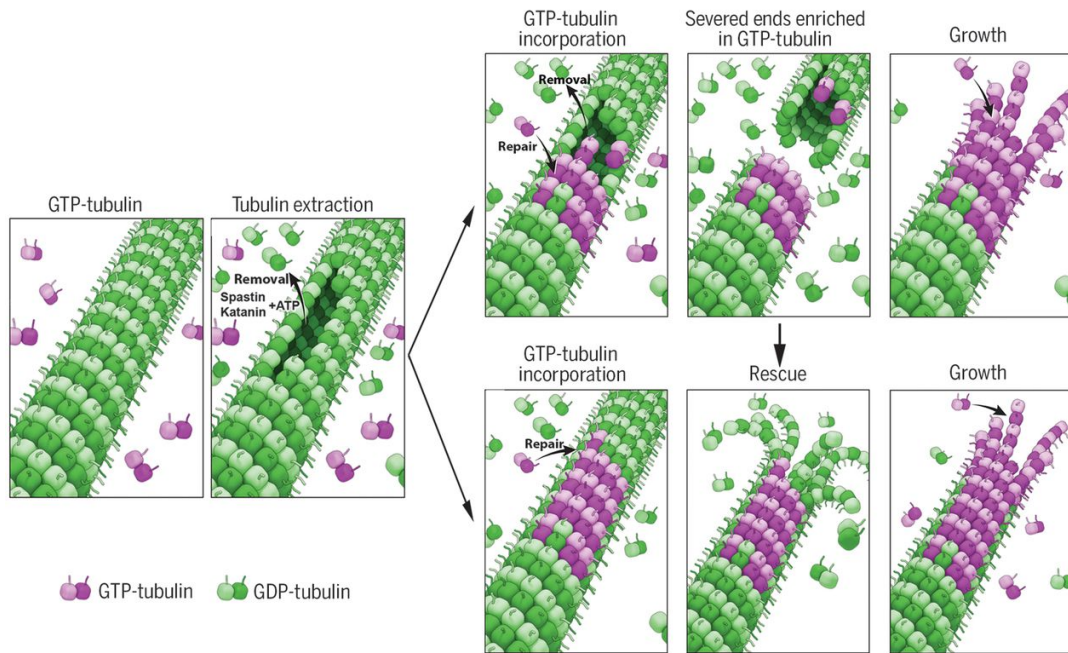


Figure 1.10: Katanin and spastin increase microtubule mass through two processes. Severing enzymes katanin and spastin create nanoscale damage sites along the microtubule lattice that lead to incorporation of GTP-tubulin (purple). This leads to two processes: (top) upon microtubule breakage, severed ends are enriched in GTP-tubulin and are therefore more stable and likely to grow; (bottom) GTP-tubulin islands create stable sites on the microtubule lattice with increased rescue frequency. Figure adapted from [Vemu et al. \(2018\)](#)

Microtubule severing induces internal breaks in microtubules through an oligomerization process (reviewed in [Kuo and Howard \(2021\)](#)). Severing enzymes assemble into a **hexamer** on the microtubule lattice with a ring-like structure that encircles the tubulin C-terminal tail (**CTT**) (see Fig. 1.9). Once the hexamer encloses the CTT, it pulls the tail through the pore of the hexamer, thereby deforming the tubulin monomer and loosening the inter-dimer interactions to free the subunit from the microtubule. The microtubule is fully severed when enough tubulin subunits have been removed. While there is no direct evidence that microtubule-severing enzymes generate force on the CTT, several studies show that removal of CTTs inhibits severing by katanin ([Johjima et al., 2015](#); [McNally and Vale, 1993](#)) as well as spastin ([White et al., 2007](#); [Roll-Mecak and Vale, 2005](#)).

It is intuitive to think of microtubule severing enzymes as disassembly factors and disruptors of microtubule networks, and this is the case when the newly generated microtubules are unstable. Nevertheless, recent studies have shown that severing enzymes are powerful **promoters** of microtubule growth. This is due to the fact that katanin and other severases increase the number of microtubule fragments that can act as seeds to generate new microtubules ([Srayko et al., 2006](#); [Vemu et al., 2018](#); [Kuo et al., 2019](#)). For spastin, another explanation for the increase of microtubule mass comes from spastin accumulating at the shrinking end of microtubules, thereby facilitating the conversion from shrinking microtubules to growing ones ([Vemu et al., 2018](#); [Kuo et al., 2019](#)). Additionally, it was shown that katanin and spastin induce damage-sites along the microtubule lattice that can be repaired by the incorporation of GTP-tubulin ([Vemu et al., 2018](#)). The incorporation of GTP-tubulin along the lattice of the microtubule give rise to a GTP-rich region **GTP islands**. When severing activity leads to complete microtubule breakage, the GTP islands create stabilized GTP-rich microtubule ends on the newly formed microtubule fragments

1.4. MICROTUBULE-SEVERING ENZYMES

(see Fig. 1.10). When complete breakage is not achieved, the GTP islands create stabilized sites with increased rescue frequency (see Fig. 1.10) (Dimitrov et al., 2008; Tropini et al., 2012; Aumeier et al., 2016; Vemu et al., 2018). These findings indicate that severing enzymes katanin and spastin can produce stabilized microtubule ends and elevated rescue rates, both leading to increased microtubule mass and number.

1.4.1 KATANIN

Katanin was initially purified from sea urchin eggs and was the first microtubule-severing enzyme to be discovered (Vale, 1991). Due to its cutting activity, katanin is named after the Japanese word "katana", referring to the Japanese samurai sword. Katanin belongs to a large family of **ATPases**, called the AAA (ATPases associated with various cellular activities) ATPases, where "AAA" refers to the presence of a highly conserved ~ 230 -amino-acid "AAA domain" that is responsible for ATP binding and hydrolysis. ATPases are enzymes that use the energy produced from ATP hydrolysis to drive their activity. In the case of katanin and other severases, this activity is the severing of microtubules (McNally and Vale, 1993). Katanin consists of two subunits, a p60 and p80 subunit both named for their size in kilodaltons (60 and 80kDa, respectively). The p60 subunit is the enzyme that contains the AAA domain and is therefore responsible for the removal of tubulin subunits and the eventual severing of the microtubule. The p80 subunit, in contrast, has no microtubule-severing properties of its own, it enhances binding of katanin to microtubules and regulates the p60 subunit (McNally and Vale, 1993; Yu et al., 2005). Combined, the p60 and p80 subunits form a stable complex in vivo (Wang et al., 2017).

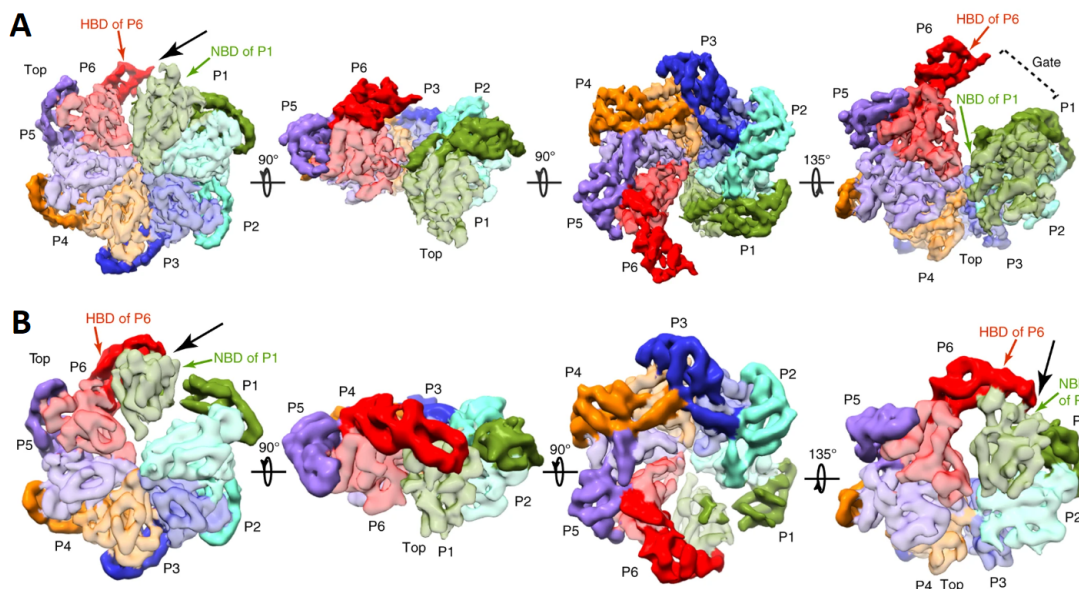


Figure 1.11: Cryo-EM structure of the two configurations of the katanin hexamer. A. 3D model of the katanin open spiral conformation with the rotation angles between the different views indicated with arrows. Katanin monomers are numbered P1-P6 and are shown in distinct colors. HBD, helix bundle domain; NBD, nucleotide-binding domain. Black arrow indicates the open gate between P1 and P6, most clearly visible in the fourth view. B. 3D model of the katanin closed ring conformation, where the gate between P1 and P6 is closed. Figure adapted from Zehr et al. (2017).

In 2017, the structure of the katanin hexamer was resolved using cryoEM and it was shown

that the hexamer switches between an **open spiral** and a **closed ring** conformation depending on the ATP-binding state (see Fig. 1.11) (Zehr et al., 2017). It is hypothesized that the tubulin extraction process fits a hand-over-hand mechanism, where each p60/p80 dimer within the hexamer can undergo ATP hydrolysis which moves the structure from the open to the closed conformation. This conformational change causes the dimer to move towards or away from the microtubule surface (reviewed in Kuo and Howard (2021)). With the movement of one dimer perpendicular to the microtubule surface, the hydrolysis of ATP is triggered in the neighboring dimer, creating a propagating up-and-down motion of the dimers within the hexamer. Due to the binding of the CTT to the pore of the hexamer, it is hypothesized that each open-to-closed conformational change leads to the extraction of an additional two amino acids of the CTT through the pore of the katanin hexamer (Zehr et al., 2017).

Due to the direct interaction of katanin with the CTT, it is not surprising that tubulin PTMs on the CTT affect katanin activity. It was found that katanin responds differently to glutamylation on the α - and β -tubulin tails, where glutamylation on α -tubulin strongly enhances katanin binding while glutamylation on β -tubulin lead to either enhancement or inhibition of katanin binding (Szczesna et al., 2022). No effect was found on katanin binding upon Lys40 acetylation, however, they found that katanin is inhibited by tubulin detyrosination and this inhibition can be overcome by glutamylation, demonstrating that the regulation of katanin by tubulin PTMs is differential and combinatorial.

1.5 TAU PROTEIN

Tau is a prominent structural MAP that is regulated by extensive phosphorylation. Tau is an intrinsically disordered protein that was first discovered in the laboratory of prof. Kirschner in 1975 (Weingarten et al., 1975). The tau protein has gained a lot of interest in research due to its implications in neurodegenerative diseases, which in turn is linked to tau phosphorylation. To understand the importance of studying tau, this section will first describe the connection that tau has with many neurodegenerative diseases, collectively called tauopathies (reviewed in Lee et al. (2001)). After that, a more in-depth review will be given about the tau protein structure and its roles and function under physiological (healthy) conditions.

1.5.1 TAU PATHOLOGY IN NEURODEGENERATIVE DISEASES

Under physiological conditions, tau is bound to microtubules where it carries out a multitude of microtubule-related functions. Tau malfunction has long been correlated with **neurodegenerative diseases**, which most famously includes Alzheimer's disease (**AD**) (reviewed in Iqbal et al. (2005)). One of the hallmarks of AD is the accumulation of tau into aggregates or bundles that are referred to as **neurofibrillary tangles (NFTs)**. NFTs are bundles of tau filaments that accumulate in dendrites and axons and that are a burden for neurons (see Fig. 1.12). The filaments that constitute NFTs are build up of tau monomers that are stacked together to form a long fiber. Two of these fibers can twist around each other in a helical fashion to form a paired helical filament (**PHF**) or a straight filament (**SF**) (Kidd, 1963; Crowther, 1991), differing in the arrangement of the fibers (see Fig. 1.12). The tau proteins that constitute the PHFs and SFs have often been found to be highly phosphorylated, linking tau phosphorylation to NFT formation and neurodegeneration (reviewed in Wesseling et al. (2020)). Aside from NFT formation,

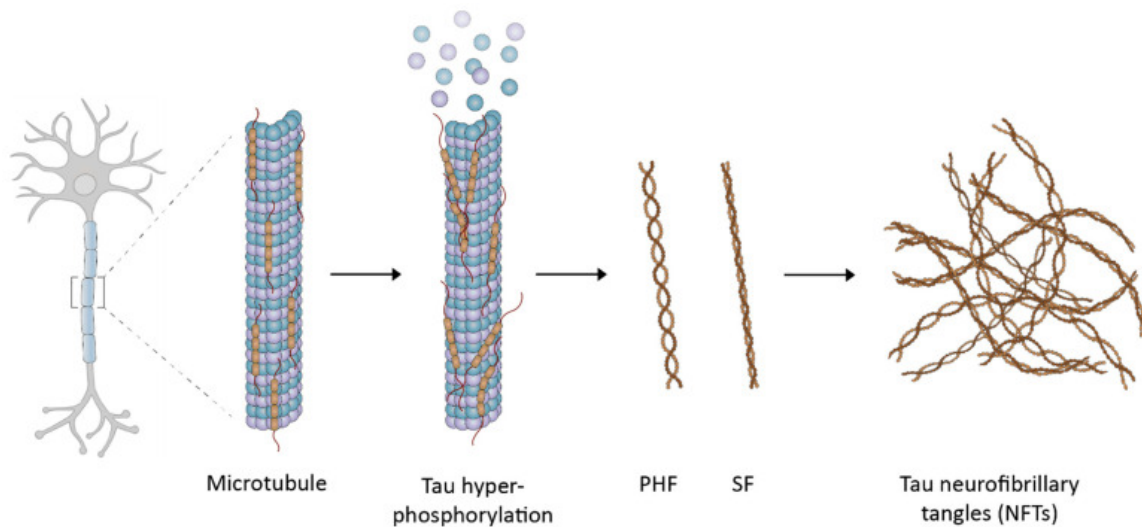


Figure 1.12: The development of tau neurofibrillary tangles. Under physiological conditions tau binds to microtubules in neuronal axons. Upon tau phosphorylation, tau detaches from microtubules, causing microtubule destabilization, and formation of PHFs or SFs from hyperphosphorylated tau monomers. The PHFs and SFs bundle together forming neurofibrillary tangles **NFTs**. Figure adapted from [Jie et al. \(2021\)](#).

another hallmark of AD is the reduction of microtubule density which is often attributed to tau detachment from microtubules ([Kneynsberg et al., 2017](#)), however, this microtubule reduction does not correlate with tangle pathology ([Cash et al., 2003](#)). Additionally, cognitive decline starts before the detection of mature NFTs, and cognitive improvement can occur even while tangles are still present ([Santacruz et al., 2005](#); [Nelson et al., 2009](#)). Therefore, it appears that the accumulation of tau into NFTs does not directly lead to neurodegeneration, even though its presence does not improve matters either. Most likely, tau-mediated neurodegeneration results from the combination of tau's losses of function and toxic gains-of-function, which are caused by tau mislocalization, malfunction, aggregation, and formation of NFTs.

Generally, a proven way to establish causation in diseases is by using genetics. However, up until 1998 no genetic linkage between tau and neurodegenerative conditions had been found. In 1998, several groups identified a direct genetic link between a mutation in the tau gene and frontotemporal dementia with parkinsonism linked to chromosome 17 (**FTDP-17**) ([Spillantini et al., 1998](#); [Hutton et al., 1998](#); [Clark et al., 1998](#)). FTDP-17 is a collection of neurodegenerative dementia's with symptoms similar to AD and other tauopathies, which include neuronal cell death and the presence of insoluble tau aggregates that are similar to NFTs. As will be discussed in more detail in section 1.5.3, there are **six isoforms of tau**, and through alternative splicing (specifically of exon 10) three of the isoforms contain 3 microtubule-binding repeats, referred to as **3R-tau**, and three isoforms contain 4 microtubule-binding repeats, referred to as **4R-tau**. Many of the mutations of tau related to FTDP-17 occur in the microtubule-binding region and in exon 10, which has been shown to decrease tau binding to microtubules ([Dayanandan et al., 1999](#)), enhance tau self-aggregation ([Nacharaju et al., 1999](#)), and affect exon 10 splicing and therefore the production and ratio of 3R- or 4R-tau ([Grover et al., 1999](#); [Varani et al., 1999](#)). In healthy adult brains, the ratio of 3R-tau and 4R-tau is roughly 1:1 ([Goedert et al., 1989](#); [Kosik et al., 1989](#); [Capano et al., 2022](#)), and alteration of this ratio is linked to neurodegeneration (reviewed in [Ghetti et al. \(2015\)](#)). An imbalance in 3R- and 4R-tau can cause deleterious effects since 3R- and 4R-tau are differentially involved in tangle formation, where 4R-tau is solely found in so-called "pretangles" (the preliminary structures that eventually develop into NFTs) ([Uchi-](#)

[hara, 2014](#)). Additionally, an increase in 4R- over 3R-tau was found to cause the formation of insoluble tau aggregates ([Capano et al., 2022](#)) and in the majority of FTDP-17 patients, the filaments assembled from tau only contain the 4R-tau isoform ([Heutink, 2000](#)), suggesting that 4R-tau may be more prone to form aggregates.

One major clue that could allow us to understand the cause of many tauopathies is that in all of the diseases, tau appears to become **abnormally phosphorylated**. In particular, the redistribution or mislocalization of tau into somatodendritic compartments is considered a hallmark of early tauopathy development and is attributed to tau phosphorylation (discussed in more detail in section 1.5.6) ([Andorfer et al., 2003](#); [Braak et al., 2011](#)). The increased phosphorylation state of tau could be a cause of the compromised activity of tau-targeting protein phosphatase 2A (PP2A) that was found in the brain tissue of AD patients ([Gong et al., 1993](#)), along with an increased activity of several tau-targeting kinases including cyclin-dependent kinase 5 (Cdk5) ([Patrick et al., 1999](#)).

Tau phosphorylation causes **detachment** of tau from microtubules that is often linked to loss of microtubule mass and therefore to neurodegeneration, but to date there is very little information on how tau phosphorylation leads to microtubule destabilization. One explanation is that tau normally protects microtubules against severing enzymes, so by losing that protection, microtubules start to disintegrate. Additionally, filamentous tau is abnormally hyperphosphorylated ([Wesseling et al., 2020](#)), however, it remains to be proven whether phosphorylation is the trigger of tau to form aggregates or if tau aggregation precedes (hyper-)phosphorylation. Nonetheless, hyperphosphorylation of tau has been shown to promote tau aggregation and lead to tau filament formation in vitro ([Alonso et al., 2001](#)), and dephosphorylation of tau from AD brain inhibited the self-assembly of tau ([Alonso et al., 1994](#)), indicating that phosphorylation was necessary for self-assembly.

The relationship between tau phosphorylation and tau aggregation is complex, since phosphorylation at distinct sites that are known to result in tau detachment from microtubules, have been shown to additionally prevent tau from aggregation ([Schneider et al., 1999](#)). To complicate matters even more, aside from increased phosphorylation, aggregated tau from AD brains was also found to be increasingly acetylated and ubiquitinated in the microtubule-binding repeats ([Wesseling et al., 2020](#)), indicating that phosphorylation is not the only PTM to be involved in neurodegeneration. Combined, it has been shown that tau phosphorylation plays a key role in neurodegeneration and a lot of substantial research has been done to understand the role of tau in neurodegenerative diseases. Nonetheless, the mechanism by which tau, and specifically tau phosphorylation, affects pathological effects such as tau malfunction, aggregation, and mislocalization, is still not well understood. Therefore, in order to reduce the number of patients affected, a lot more work needs to be done to unravel the process of neurodegeneration and the role that tau (phosphorylation) has in the manner.

1.5.2 MISSORTING OF TAU

Missorting of tau is considered an early sign of neurodegeneration as observed in Alzheimer's disease. In healthy neurons, tau is predominantly found in the **axons**, while in disease, tau appears in the **somatodendritic compartment** (see Fig. 1.13) (reviewed in [Zempel and Mandelkow \(2014\)](#)). This **mislocalization of tau** was shown to occur before tau aggregation appears and correlates with destruction of microtubules in dendrites and loss of spines and synapses ([Bal-](#)

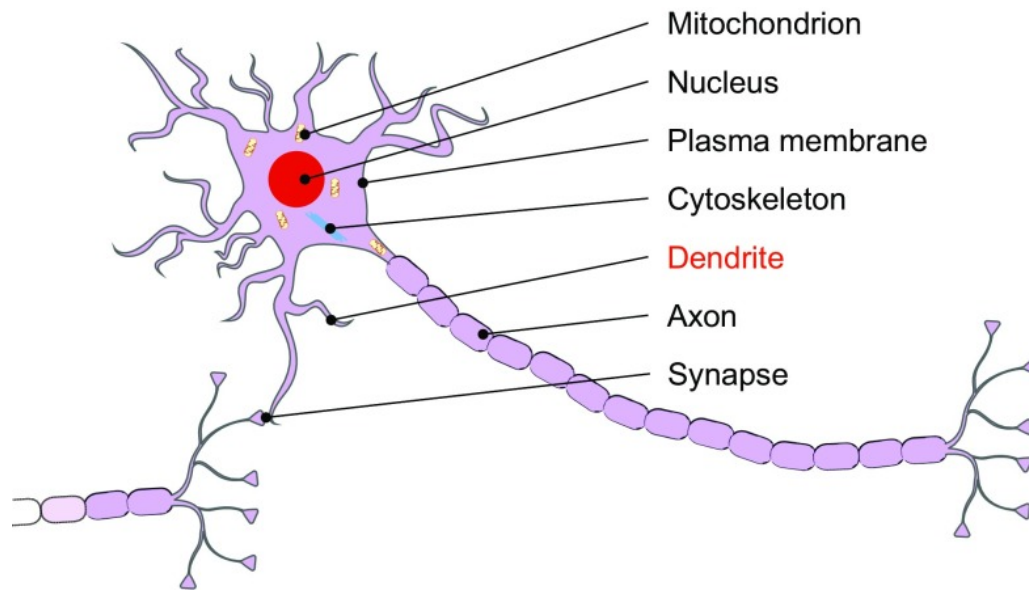


Figure 1.13: Tau localisation in neurons. Schematic of a neuron and the differing locations where tau can be found. Tau is preferentially found associated with the microtubule cytoskeleton in axons, but can also be found in the somatodendritic compartment, including in mitochondria, the nucleus, plasma membrane, and in the synapses. Tau is found at elevated levels in dendrites (red) in tauopathies. Figure adapted from [Guo et al. \(2017\)](#)

[latore et al., 2007](#); [Haass and Selkoe, 2007](#); [Zempel et al., 2010](#)). Therefore, a clear correlation between tau missorting and pathological effects has been determined. So what are the causes and what are the consequences of tau missorting? Many studies have aimed to answer these questions, and while the consequences might be easier to describe, the causes are not as readily found.

There are many studies that aim to describe the mechanism that leads to the missorting of tau. One of the mechanisms that was found to induce tau missorting in mature primary neurons was the presence of extracellular adenosine triphosphate (ATP), which functions as a stress signal and induces Ca^{2+} influx ([Zempel et al., 2010](#)). Another cause leading to tau missorting was described in 2013, when [Zempel et al.](#) found that after the exposure of neurons to amyloid- β ($\text{A}\beta$) oligomers, tau becomes missorted in the somatodendritic compartment ([Zempel et al., 2013](#)). Additionally, in a tau knock-in mouse model with 27 different phosphomimetics that model hyperphosphorylation, axonal tau mislocalized into the somatodendritic compartment ([Gilley et al., 2016](#)), indicating that tau mislocalization is attributed to hyperphosphorylation of tau. Combined, these findings indicate that there are different factors that appear to be implicated in tau missorting, which suggests that the process of missorting of tau might be caused by a series of events.

To explain the consequences of tau missorting, it is important to realize that missorting of tau does not only cause impaired physiological functions in the axons, it additionally enables tau to gain toxic pathological function by, for example, interacting with dendritic proteins that are usually out of reach. It was, for instance, shown that missorted and phosphorylated tau co-aggregated with cofilin and actin rods in dendrites ([Whiteman et al., 2011](#)). Additionally, missorting of tau could divert the activity of kinases or phosphatases away from other proteins, and thereby change the balance of phosphorylation and additionally the activity of these proteins. Early experiments with overexpressed tau showed that tau mislocalization to the dendrites

resulted in transport inhibition of vesicles and organelles, loss of ATP supply, and decay of synaptic spines (Thies and Mandelkow, 2007). Moreover, missorted tau is free to form toxic oligomers and aggregates. Another toxic gain-of-function linked to tau mislocalization due to A β exposure is the promotion of translocation of tubulin tyrosine ligase-like enzyme 6 (TTL6) into dendrites, where it induces polyglutamylation of microtubules, a trigger for subsequent recruitment of spastin which causes excessive microtubule severing (Lacroix et al., 2010; Zempel et al., 2013). Tau knockout (KO) neurons were shown to be resistant to A β -induced microtubule loss, indicating that the breakdown of microtubules is tau-dependent (Zempel et al., 2013). Furthermore, tau depletion in neurons was found to increase **neuronal branching** due to increased severing activity (Yu et al., 2008), raising the hypothesis that the preferential expression of tau in axons may maintain a non-branched structure of the axon. This is in contrast to the highly branched dendrites where tau shows low expression levels (Yu et al., 2008) and can also explain why tau mislocalization in pathology is catastrophic for axons since it may lead to inappropriate branching. Finally, tau is phosphorylated in a compartment-specific manner in differentiated neurons, and therefore tau mislocalization can lead to phosphorylation at ill-favored sites that can be irreversible, which in turn can lead to pathological effects caused by tau mislocalization.

1.5.3 TAU PROTEIN STRUCTURE

Tau is a highly soluble and natively unfolded protein that is classified as an **intrinsically disordered protein (IDP)**. IDPs are proteins that are unable to fold spontaneously into stable, well-defined, globular three-dimensional structures (reviewed in Dyson and Wright (2005)). Instead, IDPs, such as tau, have a highly flexible structure and tau has been shown to have an unusually low content of secondary structure (Mukrasch et al., 2009). When considering the structure of the molecule, tau can be subdivided into four domains (see Fig. 1.14):

- (1) the **N-terminal domain**,
- (2) the **proline-rich domain**,
- (3) the **microtubule-binding region**,
- (4) and the **C-terminal domain**.

The microtubule-binding region (MTBR) (also called the microtubule-binding domain (MTBD)) consists of three or four partially repeated sequences, the repeat domains, that directly interact with the microtubule surface. Additionally, tau contains a "**pseudo-repeat domain**" (R'), which is weakly homologous to the other repeat domains and has been shown to bind to microtubules but only weakly influences the functionality of tau (Gustke et al., 1994). The N-terminal region with the proline-rich domain and the C-terminal domain are considered the **projection domains** that project away from the microtubule surface.

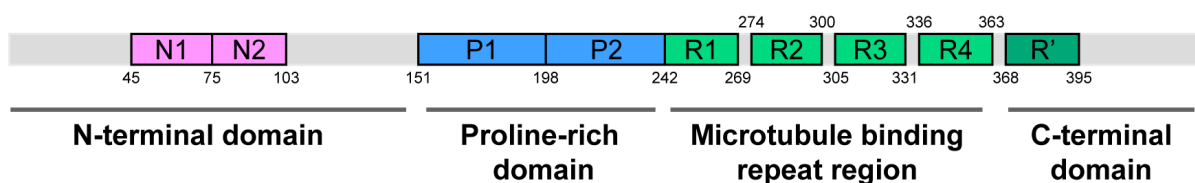


Figure 1.14: Structure of the longest isoform of tau. The longest of the six isoforms of tau (2N4R-tau) consists of 2 N-terminal inserts (pink) in the N-terminal domain; 2 inserts in the proline-rich domain (blue); 4 MT-binding repeats (green) in the MT-binding region; and 1 pseudo-repeat (R', dark green) in the C-terminal domain.

1.5. TAU PROTEIN

Approximately 26% of the residues in the longest tau isoform are charged amino acids with a slight dominance of positively charged residues, giving tau an **overall basic** structure. However, when considering the specific regions separately, we can observe a slight difference in charge. The MT-binding region is mostly positive, therefore binding preferentially to the negatively charged microtubule surface. The C-terminal domain has a mix of positive and negative charges, while the N-terminal domain is mostly negatively charged and the proline-rich domain is mostly positively charged. Since the positively charged proline-rich domain is directly connected to the microtubule-binding region, this explains why the projection domain branches away from the negatively charged microtubule surface.

Using fluorescence resonance energy transfer (FRET) and electron paramagnetic resonance (EPR) to examine the proximity of the tau domains and the global folding, a **paperclip-like conformation** was proposed for tau in solution (Jeganathan et al., 2006; Mukrasch et al., 2009). In this conformation, the C-terminal domain of tau folds over the microtubule-binding repeats and the N-terminal domain folds back over the C-terminal domain, bringing the two termini in close proximity to one another (see Fig. 1.15). This close proximity is however reduced upon tau binding to the microtubule (Rapoport et al., 2002). It has been proposed that the formation of the paperclip structure protects tau from aggregation. Post-translation modifications such as truncation and phosphorylation of tau can result in the disruption of the paperclip structure, thereby promoting aggregation (Alonso et al., 2001). Moreover, proline-directed tau phosphorylation variably results in loosening or tightening of the paperclip structure, and this may depend of the specific sites where tau gets phosphorylated (Leroy et al., 2002).

Other than the microtubule binding region interacting with microtubules, it remains largely unclear what the specific functions of the domains of tau are. The N-terminal region of tau binds to the C-terminus of the p150 subunit of the dynein complex, which mediates the association of the dynein motor with membranous cargoes (Magnani et al., 2007). Additionally, the N-terminal flanking region is thought to modulate the formation of microtubule bundles (Chen et al., 1992; Rosenberg et al., 2008), and the number of N-terminal inserts can influence the microtubule spacing within a bundle or the spacing between microtubules and other cell components (Chen et al., 1992; Frappier et al., 1994). Tau is also able to bind to actin filaments through a minimum of two microtubule binding repeats. This would allow tau to bind both actin and microtubules simultaneously, providing a **molecular tether** between the two types of cytoskeletal filaments

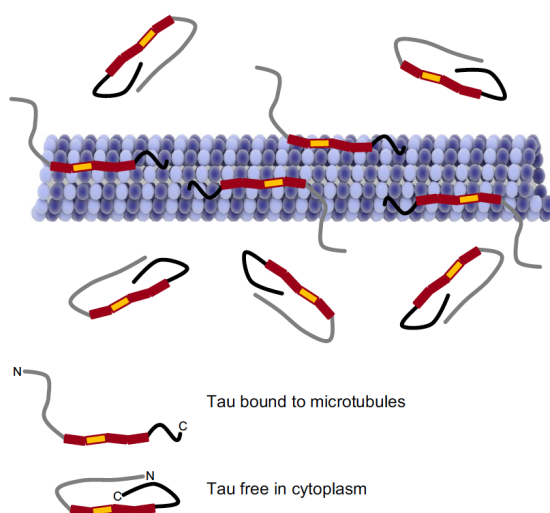


Figure 1.15: Tau adopts a paperclip-like conformation in solution. Tau binds to microtubules primarily through the microtubule binding domain (red), comprising either three or four repeats (the second repeat R2 depicted in yellow). The N- and C-termini of tau are in close proximity when tau is free in the cytoplasm giving rise to a proposed "paperclip" conformation of tau. Upon binding to microtubules, the N-terminal domain projects away from the microtubule surface. Figure adapted from Guo et al. (2017).

(Elie et al., 2015). Regarding the C-terminal domain of tau, neither its function nor its potential binding partners have been well established. However, some studies suggest that changes within the C-terminal region can influence other domains of tau, including their availability for phosphorylation and their interaction with binding partners (Connell et al., 2001; Reynolds et al., 2008).

1.5.4 TAU GENE AND ISOFORMS

Human tau is encoded by a single gene called the microtubule-associated protein tau (MAPT) gene, which comprises 16 exons and is located on chromosome 17q21. The adult human brain contains **six main tau isoforms**, ranging from 352 to 441 amino acids in length and 37 to 46 kDa in size, generated by alternative splicing of exons 2, 3, and 10 (see Fig. 1.16). The six isoforms differ in the number of 29 amino acid (aa) N-terminal inserts (N1, N2), which are encoded by exon 2 and 3 (exon 3 is not transcribed in the absence of exon 2), resulting in isoforms containing either zero (0N), one (1N), or two N-terminal inserts (2N). Additionally, alternative splicing of exon 10 results in the presence or absence of the second microtubule-binding repeat (R2, 31 aa) generating isoforms with either three (3R), or four (4R) microtubule-binding repeat sequences (Lee et al., 1988). Combined, this results in the six main isoforms found in the adult human brain: 0N3R (352 aa), 1N3R (381 aa), 2N3R (410 aa), 0N4R (383 aa), 1N4R (412 aa), and 2N4R (441 aa) (Goedert et al., 1989).

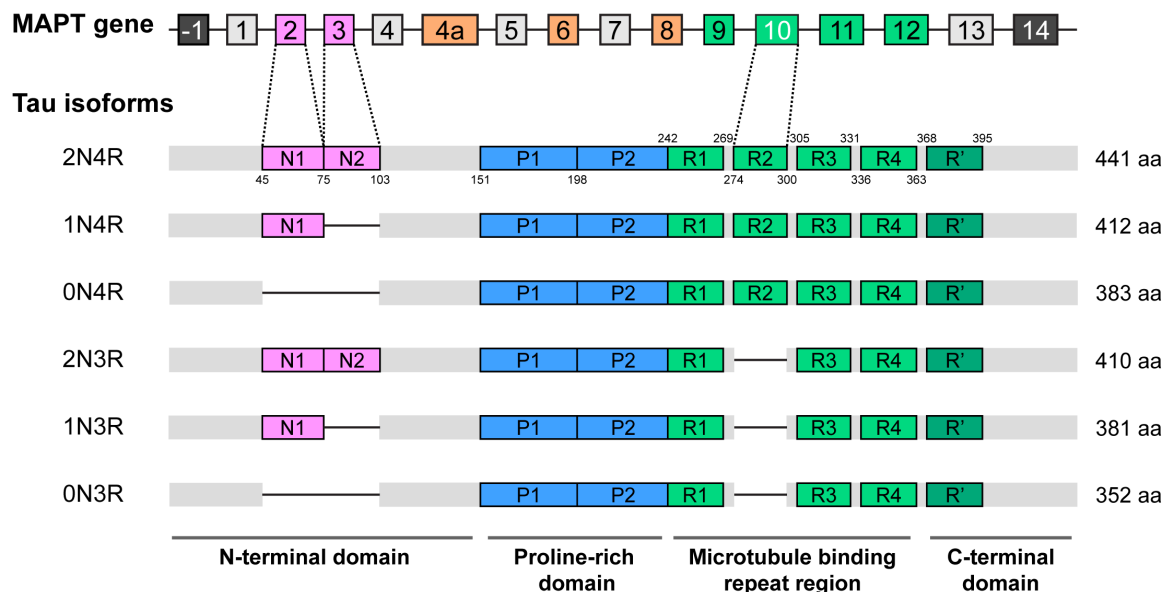


Figure 1.16: Tau protein isoforms in the human brain. Human tau is encoded by the MAPT gene that gives rise to 6 tau isoforms in the adult human brain through different combinations of the splicing of exons 2, 3, and/or 10. Tau isoforms all consist of an N-terminal domain, a proline-rich domain, 3 or 4 microtubule-binding repeat domains, and a C-terminal domain that includes a pseudo-repeat domain (R'). N-terminal insert N1 and N2 (pink) are produced from exons 2 and 3, respectively. Exon 10 encodes the second microtubule-binding repeat domain (R2, green), R2. Depending on the presence of the R2 domain, tau proteins are either produced as 3R- or 4R-tau. The exons depicted in orange give rise to the "Big tau" isoforms which will be described in the discussion.

In the central nervous system (CNS), tau expression is developmentally regulated. In the adult human brain, all six isoforms of tau are expressed, whereas in the fetal brain, only the shortest tau isoform (0N3R) is expressed (Bullmann et al., 2009). The fetal 0N3R-tau is initially evenly

distributed in the cell body and neurites, however, when axons start to emerge and the neurons are polarized, tau becomes enriched in the **axons**. In the adult human brain, the amount of 3R and 4R isoforms is approximately equal (Goedert et al., 1989; Kosik et al., 1989; Capano et al., 2022), however, 2N tau isoforms are underrepresented in comparison to 0N and 1N tau, where 0N, 1N, and 2N tau isoforms comprise 37, 54, and 9% of the total tau in the CNS (Goedert and Jakes, 1990). The differential expression of the tau isoforms during development and in the adult brain suggests that the six isoforms are likely to have distinct physiological roles.

1.5.5 TAU ISOFORM LOCALIZATION

In adult neurons, tau is preferentially found in the axons and in much lower densities in the somatodendritic compartments, including the tiny protrusions that can be found on the dendrites termed the **dendritic spines** (see Fig. 1.13) (reviewed in Morris et al. (2011)). One of the mechanisms that can explain the polarized distribution of tau is preferential degradation of tau in non-axonal compartments. This theory came from the finding that after micro-injection of tagged tau into neurons, initially tau was distributed over all cell compartments, however, after four days, labeled tau was only found in the axons (Hirokawa et al., 1996). **Compartment-specific degradation** of tau could be a result of differential interactions with degradation pathways, or could depend on distinct post-translational modifications. Aside from compartment-specific degradation, there are several other mechanisms, both at the protein level as well as the mRNA level, that could explain the polarized distribution of tau in neurons. At the mRNA level, the 3'-untranslated region (**UTR**) of the **MAPT** gene contains a sequence motif that directs the mRNA into the axon for **local translation** (Aronov et al., 2001). Additionally, the 5'UTR of the tau gene contains a motif which enhances translation of tau in axons (Morita and Sobue, 2009). Nevertheless, exogenous tau that did not contain these motifs was also sorted in a polarized fashion (Zempel et al., 2017), suggesting that the mRNA-based mechanisms cannot solely account for the sorting of tau. At the protein level there are multiple mechanisms that contribute to a polarized distribution of tau in neurons: cytosolic tau can translocate to axons either through free diffusion between the cytosol of different departments (Konzack et al., 2007), by diffusion along the microtubule lattice (Hinrichs et al., 2012), or by active transportation facilitated by motor proteins such as kinesin family members (Utton et al., 2005). Retention of tau in the axon is guaranteed by two mechanisms. Firstly, tau shows a higher binding affinity to the microtubules in axons compared to the microtubules in dendrites (Kanai and Hirokawa, 1995), which could be due to differential post-translation modifications in the different compartments. Tau phosphorylation, for instance, is much lower in the axons versus dendrites (reviewed in Hoogenraad and Bradke (2009)), which could account for the higher binding affinity of tau to axonal microtubules compared to, for instance, dendritic microtubules. Additionally, the presence of a **microtubule-dependent retrograde barrier** in the axon initial segment (**AIS**) allows tau to enter the axon while preventing it from travelling back towards the soma and dendrites (Li et al., 2011). Both mechanisms, however, break down due to hyperphosphorylation of tau which causes tau to detach from the microtubules and enables tau to pass through the retrograde barrier (Li et al., 2011). This phenomenon links (hyper)phosphorylation of tau to the pathological hallmarks of tau missorting in neurodegenerative diseases. Another factor that might account for the sorting of tau in the axons is preferential degradation of tau in non-axonal compartments (reviewed in Lee et al. (2013)), that could depend on post-translational modifications of tau.

Interestingly, sorting of tau appears to be isoform-specific. In mice, tau 0N, 1N, and 2N isoforms each show distinct subcellular distribution, where the 1N4R-tau isoform preferentially

localizes to the nucleus and less to the soma and dendrites but not the axons (Liu and Götz, 2013). Aside from the developmentally regulated expression, the isoform-specific localization further suggests **distinct functional properties** of the different isoforms. It has already been shown that the shortest (0N3R) and longest (2N4R) isoforms of tau regulate the initiation of microtubule polymerization and microtubule stability to different extents (Bunker et al., 2004), where 4R-tau increased microtubule stability by suppressing dynamicity of both growing and shortening events with threefold higher capability compared to 3R-tau. Most of these differences can be attributed to the additional microtubule-binding repeat of the 4R-tau that increases the affinity to the microtubule of the longest isoform compared to the 3R-tau (Gustke et al., 1994; Goode et al., 2000). Another example came from overexpression of 0N3R- and 2N4R-tau in primary mouse cortical neurons which reduced the amount of mitochondria localized to the axon, where the 2N4R-tau had a larger effect than 0N3R-tau (Stoothoff et al., 2009). Additional *in vitro* studies have shown that tau inhibits kinesin-mediated in an isoform-specific manner (Vershinin et al., 2007; Dixit et al., 2008), however, in these studies the longest isoform appeared to be less potent to inhibit kinesin motility. Moreover, both studies revealed no significant change in kinesin velocity indicating that once a motor binds it will walk with the same velocity. Combined, these results reveal that tau regulates kinesin-mediated transport along the microtubule lattice, and this regulation appears to be isoform-specific, however, the exact mechanism by which tau controls kinesin transport remains yet to be uncovered.

1.5.6 TAU PHOSPHORYLATION

To ensure proper dynamics of the cytoskeleton system, tau and other MAPs are tightly regulated by a number of factors, including post-translational modifications. Tau is subject to many PTMs, including phosphorylation, acetylation, methylation, truncation and isomerization (reviewed in Martin et al. (2011)). Of these PTMs, **phosphorylation** is the most commonly described for tau. Phosphorylation events generally occur on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues. Full-length (2N4R) tau possesses 45 serine, 35 threonine, and 5 tyrosine residues, adding up to a total of **85 potential phosphorylation sites** for tau (see Fig. 1.17). Given this large number of theoretical phosphorylation sites on the tau sequence, it is not surprising that phosphorylation has a profound impact on its physiological function and localization and abnormal phosphorylation levels of tau have been linked to several pathological effects.

Phosphorylation is a chemical process in which a single phosphate group (Pi) is added to a protein; **dephosphorylation** is the process of removal of the phosphate group. Phosphorylation is performed by **protein kinases** while dephosphorylation is performed by **protein phosphatases**, and the balance between the activity of protein kinases and phosphatases is tightly controlled (reviewed in Hanger et al. (2009)). The addition of the phosphate group adds a local negative charge to the amino acid residue. Various research groups use phospho-mimicking techniques as a powerful tool to examine site-specific phosphorylation. Phospho-mimicking, or pseudo-phosphorylation, is the technique of substituting phosphorylatable residues with negatively charged glutamate or aspartate.

Since the microtubule surface is mostly negatively charged, addition of negative charge to tau by phosphorylation affects the binding of tau for microtubules. Indeed, it has been shown that tau phosphorylation reduces the affinity for microtubules (Drewes et al., 1997; Schneider et al., 1999; Cho and Johnson, 2003), which can result in decreased functioning of tau leading to e.g. microtubule destabilization (discussed in more detail in section 1.5.9). Phosphorylation of tau

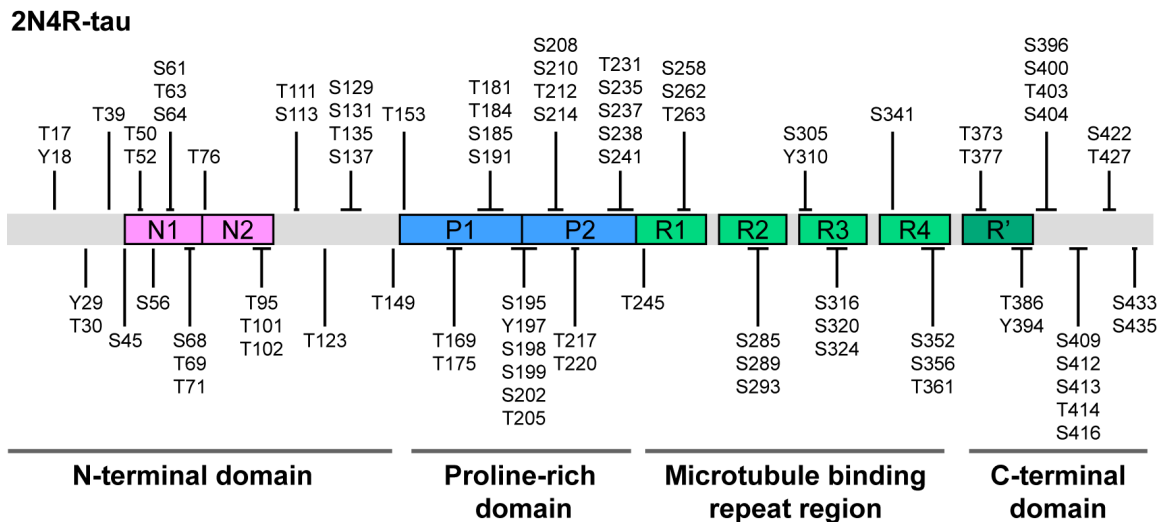


Figure 1.17: Tau phosphorylation sites. Schematic showing the longest isoform of tau (2N4R, 441 aa) with all potential phosphorylation sites (all Ser, Thr, Tyr residues), many of which are found in Alzheimer’s disease (Wesseling et al., 2020). Note that a high number of potential phosphorylation sites are located in the proline-rich domain (blue).

within the microtubule-binding repeats, specifically at Ser262, has been shown to decrease tau binding to microtubules (Drewes et al., 1995; Sengupta et al., 1998; Schneider et al., 1999). However, in vitro studies have further shown that phosphorylation outside of the microtubule-binding repeats, specifically at Thr212, Ser214, Thr231, and Ser235 (within the proline-rich domain), also contributes to dissociation of tau from microtubules (Sengupta et al., 1998; Schneider et al., 1999; Ksiezak-Reding et al., 2003), indicating that the regions outside the microtubule-binding domain of tau also influence the association of tau to microtubules. Phospho-mimicking replacements at Ser262 and Ser356 (within the MTBD) effectively reduced MT binding (Biernat et al., 1993), indicating that these sites can specifically regulate to binding of tau to microtubules.

Aside from influencing microtubule binding, tau phosphorylation also promotes aggregation of tau into tangles (Alonso et al., 2001), and this increased rate of aggregation was even found for pseudo-phosphorylated mutants (Haase et al., 2004). Furthermore, tau phosphorylation can cause missorting of tau from the axons, which can lead to abnormal phosphorylation of tau. As discussed in section 1.5.2, tau is phosphorylated in a compartment-specific manner, where in axons phosphorylation of tau at S393/S404 is strong, while in dendrites, tau is strongly phosphorylated at S262 (Kishi et al., 2005; Zempel et al., 2010). Factors that could account for the compartment-specific phosphorylation of tau include the preferential localization of tau phosphatase PP2A in axons (Zhu et al., 2010) that can dephosphorylate tau at S262 but not S396/S404 (Qian et al., 2010); the localization of Microtubule Affinity Regulating Kinase (MARK) and Synapses of Amphids Defective (SAD) kinases in the dendrites and spines that phosphorylates tau at S262 (Kishi et al., 2005); the ubiquitous presence of glycogen synthase kinase-3 β (GSK3 β) which phosphorylates tau at S396/S404 (Muyllaert et al., 2008); and the dendritic presence of tau phosphatase PP2B that dephosphorylates tau at S396/S404 (Braithwaite et al., 2012). These findings suggest that missorting of tau can lead to inappropriate, and possible even irreversible, phosphorylation of tau which in turn can directly or indirectly lead to pathological effects.

Another effect of tau phosphorylation is that it can disrupt tau’s **intracellular route of degra-**

dition. For example, once tau is phosphorylated at Ser262 or Ser356 (within the MTBD) it is no longer recognized by a specific proteasome and is therefore protected against degradation (Dickey et al., 2007). Additionally, tau can undergo conformational changes including **trans-to-cis isomerization** which reduces the affinity of tau to microtubules, and phosphorylation of tau at Thr231 has been shown to induce this conformational change (Lu et al., 1999). Moreover, cis-tau has been shown to display toxic gains-of-function and losses of normal tau function related to tau pathology (Nakamura et al., 2013). This conformational change in the tau structure can be converted back to the trans-conformation by peptidyl-prolyl cis-trans isomerase (PPIase) Pin1, and interestingly, Pin1 has been found downregulated in AD neurons (Sultana et al., 2006), proposing a link between conformational changes of tau and pathological effects resulting in neurodegeneration. Combined, these findings indicate that tau phosphorylation can have many deleterious effects, ranging from disruptions in the route of degradation, to tau mislocalization, and conformational changes of tau that induce toxic gains-of-function.

1.5.7 TAU PROTEIN KINASES AND PHOSPHATASES

As mentioned in the previous section, tau phosphorylation is tightly controlled by the balance between protein kinases and phosphatases. A disruption of the balance has been suggested to be the origin of abnormal tau phosphorylation as observed in neurodegenerative diseases (Hanger et al., 2009). The proteins involved in phosphorylation and dephosphorylation of tau are protein kinases and phosphatases, respectively (see Fig. 1.18).

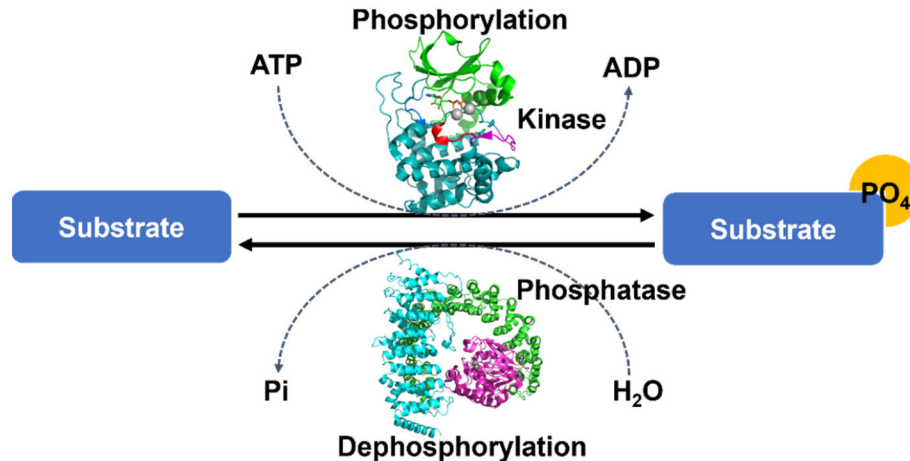


Figure 1.18: The process of protein (de)phosphorylation. The overall mechanism of protein phosphorylation regulated by protein kinases and dephosphorylation regulated by protein phosphatases. Protein kinases covalently attach a phosphate group from ATP to a protein substrate and protein phosphatases remove the phosphate group from a phosphorylated protein substrate. These processes are reversible. Figure adapted from Seok (2021).

Kinases add a phosphate group to their protein substrate by transferring the terminal phosphate group of ATP (or GTP) to its substrate. Typically, protein kinases are composed of a non-conserved regulatory domain and a conserved catalytic core (Taylor et al., 2012). The catalytic core of the kinase is where the phosphorylation reaction occurs, and this functionally active domain is highly conserved for all protein kinases. The catalytic core consists of an N- and C-terminal lobe (see Fig. 1.19), and the active site, containing the ATP/GTP binding pocket and a site where the substrate to be phosphorylated is anchored, lies between the two lobes. When the

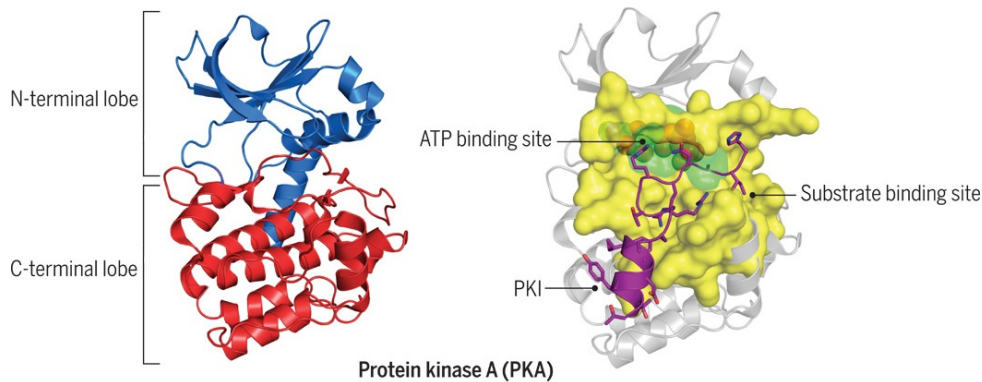


Figure 1.19: Structure of the catalytic domain of PKA. The structure of the catalytic domain of protein kinase A (PKA) is shown on the left (N-terminal lobe in blue, C-terminal lobe in red). The same structure highlighting the functional aspects of the catalytic domain is depicted on the right. The ATP binding site (green), the substrate binding site (yellow), and the binding site for protein kinase inhibitor (PKI) (purple) are depicted in the structure. Figure adapted from [de Oliveira et al. \(2016\)](#).

ATP or GTP as well as the substrate are anchored to the kinase, the phosphate transfer occurs by hydrolysis of ATP/GTP into ADP/GDP + Pi. Kinase activity itself can be regulated by (i) binding of ligands, (ii) post-translational modifications on the kinase including phosphorylation, or (iii) prior phosphorylation of the substrate catalyzed by other kinases which is a process called **substrate priming**.

Tau kinases can be classed into three broad groups:

- (1) Proline-directed serine/threonine protein kinases (**PDPK**),
- (2) Non-proline-directed serine/threonine protein kinases (**non-PDPK**),
- (3) Tyrosine protein kinases (**TPK**).

The first group of kinases, **PDPK**, are kinases targeting serine and threonine residues that precede a proline residue (SP or TP motif). The second group of kinases, **non-PDPK**, target serine and threonine residues that do not precede a proline residue (SX or TX residues). The third group of kinases, **TPK**, target tyrosine residues. There are many protein kinases that are known to phosphorylate tau and many of them are linked to neurodegenerative diseases including AD (reviewed in [Martin et al. \(2013\)](#)). Two of the most well-known kinases from the PDPK group are **GSK3**, and **Cdk5**, which will be described in further detail below. The non-PDPK group contain tau-tubulin kinase 1/2 (**TTBK1/2**) which phosphorylates tau at 10 sites and all of these sites were found phosphorylated in AD brains (reviewed in [Sato et al. \(2006\)](#)), and dual-specificity tyrosine phosphorylation-regulated kinase 1A (**DYRK1A**) which is a kinase that has increased mRNA levels in AD brains and is therefore a promising target in AD treatment ([Kimura et al., 2007](#)). The TPK group is distinct group of tau-targeting kinases since tau can only be phosphorylated at 5 tyrosine residues. The most well-known protein kinases from this group are the Src family kinases (**SFK**) which include Fyn and lymphocyte-specific protein tyrosine kinase (**Lck**). Fyn and Lck have both been shown to phosphorylate tau at Tyr18 and this phosphorylation site has been implicated with AD ([Lee et al., 2004](#)).

Protein phosphatases reverse kinase activity by removing phosphate groups from their substrates through hydrolysis of phosphoric acid monoesters into a phosphate ion, which leaves behind a free hydroxyl (-OH) group. Phosphatases are generally classified into three groups:

- (1) Phosphoprotein phosphatase (**PPP**),
- (2) Metal-dependent protein phosphatase,
- (3) Protein tyrosine phosphatase (**PTP**).

Tau phosphatases belong to the **PPP** group, with members PP1, PP2A/B and PP5, as well as the **PTP** group, that only contains PTEN. In healthy human brain, phosphatase activity is predominantly accounted for by PP2A (around 71%), compared to other phosphatases such as PP2B (around 7%), and PP5 (around 11%) (Millward et al., 1999; Liu et al., 2005). In AD brains, the total phosphatase activity has been shown to reduce by half, suggesting that tau hyperphosphorylation can be attributed to phosphatase inactivity (Gong et al., 1993).

1.5.7.1 GSK3

Glycogen synthase kinase-3 (GSK3) consists of two isoforms, GSK3 α and GSK3 β , and alternative splicing of the GSK3 β isoform generates two isoforms; β 1 and β 2, that account for 85% and 15% of GSK3 β , respectively (Mukai et al., 2002). Tau phosphorylation by GSK3 occurs at a staggering 42 sites and 29 of these are found phosphorylated in AD brains (reviewed in Hanger et al. (2009)). Additionally, an increase in GSK3 β activity has been found in AD brains and GSK3 β has been involved in the acceleration in AD pathology. Therefore, GSK3 β has long been considered a promising therapeutic target against tauopathies like AD and many GSK3 inhibitors are being tested for clinical use, so far, however, without promising outcomes (reviewed in Arciniegas Ruiz and Eldar-Finkelman (2022)).

1.5.7.2 Cdk5

Cyclin-dependent protein kinase-5 (Cdk5) is a kinase that needs to form a complex with an activator to be enzymatically active. The co-activators of Cdk5 are p39/p35 (p35 in short), and its cleaved version p29/p25 (p25 in short). The most active form of Cdk5 is when it forms the Cdk5/p25 complex, and therefore the cleavage of p35 to p25 has been shown to result in pathological tau hyperphosphorylation (Lee et al., 2000). Additionally, Cdk5 is involved in substrate priming for GSK3 β , signifying that phosphorylation of tau by Cdk5 makes tau a better substrate for further phosphorylation by GSK3 β . This cross-talk between Cdk5 and GSK3 β is reported to be dependent on ageing (reviewed in Engmann and Giese (2009)), and gives Cdk5 activity a central role in AD pathology.

1.5.7.3 PP2A

Since protein phosphatase 2A (PP2A) accounts for more than 70% of phosphatase activity in the brain and PP2A can override the kinase activity of GSK3 β and Cdk5 with respect to tau (Planel et al., 2001), PP2A is considered the main tau phosphatase. In AD brain, PP2A activity is reduced by \sim 20% in the grey matter and \sim 40% in the white matter (Gong et al., 1993). This decrease in activity is thought to be caused by post-translational modifications in the catalytic domain of the phosphatase, decreased expression of PP2A, as well as increased levels of PP2A inhibitors I1PP2A and I2PP2A (Sontag et al., 2004). Additionally, it has been shown that PP2A does not dephosphorylate tau peptides with phosphorylated Thr231 residue when it is in the cis-conformation as opposed to the trans-conformation, which further indicates that cis- but not trans-tau can contribute to hyperphosphorylation (Zhou et al., 2000).

1.5.8 TAU AS A MICROTUBULE-ASSOCIATED PROTEIN

Microtubules are the main binding partners of tau molecules, but how and where does tau bind to microtubules and what conformation does tau have once it binds? Because of the biological relevance of tau binding to microtubules there have been numerous investigations including many different techniques that tried to shed a light on the tau-MT complex. However, due to the dynamic nature of the tau-MT interaction, this complex has proven to be difficult to visualize and the different studies have led to distinct models describing the interaction. What does seem to be universally accepted is that tau binds microtubules through the microtubule-binding repeats, and the binding affinity of tau to microtubules increases with the number of repeats ([Butner and Kirschner, 1991](#)).

As discussed in section [1.5.3](#), tau was found to adopt a paperclip-like conformation in solution which was lost when bound tau bound to microtubules ([Jeganathan et al., 2006](#); [Mukrasch et al., 2009](#)). Additional FRET experiments in cells, using a tau protein labeled at its N- and C-termini with fluorophores, showed that tau also exhibits a paperclip-like conformation when bound to the microtubule in a cellular environment ([Di Primio et al., 2017](#)). These experiments were performed on cells treated with silicon rhodamine-labeled tubulin ([SiR-tubulin](#)) to visualize the microtubules and control experiments were done to show that the FRET signal was due to **intramolecular interactions** (within a single molecule), and not **intermolecular interactions** (between neighboring molecules). Nuclear magnetic resonance (NMR)-based analysis of the tau structure on taxol-stabilized microtubules further indicated that although tau is largely extended when bound to MTs and does not fold into a single globular structure, distinct regions of tau fold into a defined conformation upon binding to MTs ([Kadavath et al., 2015b](#)). In this study a hairpin-like conformation, which resembles a molecular hook, was suggested to be adopted by tau when bound to the microtubules.

Even though most studies show that tau binds on the outside of the microtubule lattice, a [cryo-EM](#) study revealed that tau has two distinct binding sites on microtubules ([Kar et al., 2003](#)). One tau binding sites overlaps with the binding site of taxol to microtubules which is on a specific site on β -tubulin on the inside of the microtubule, while the other binding site is on the outside of the microtubule lattice. Later on, it was suggested that these distinct binding modes may be the result of differences in the polymerization of the microtubules, where binding of tau to the lumen of the microtubule only arises when microtubules are co-polymerized with tau in solution, while addition of tau to pre-assembled microtubules reveals tau binding on the outside of the microtubule lattice ([Makrides et al., 2004](#)). The exact binding mode of tau to microtubules remains controversial to this day. Most studies suggest that tau proteins solely align along the protofilaments ([Al-Bassam et al., 2002](#); [Schaap et al., 2007](#); [Kadavath et al., 2015a](#); [Kellogg et al., 2018](#)), while tau binding across protofilaments (lateral binding) as well as along protofilaments has been observed on a single taxol-stabilized microtubule using cryo-EM ([Santarella et al., 2004](#)). The observation that tau can bind laterally across protofilaments is consistent with findings that tau can promote the formation of tubulin rings ([Duan et al., 2017](#)).

Ultimately, a breakthrough study on the tau-MT interaction came in 2018 from using a combination of cryo-EM at near-atomic resolution and Rosetta modeling ([Kellogg et al., 2018](#)). In this study, tau was added to preformed microtubules, or to polymerizing tubulin, in both cases in the absence of stabilizing agents. Tau was never observed in the microtubule lumen, moreover, they found that tau attaches longitudinally with the full MTBR along the protofilaments of the

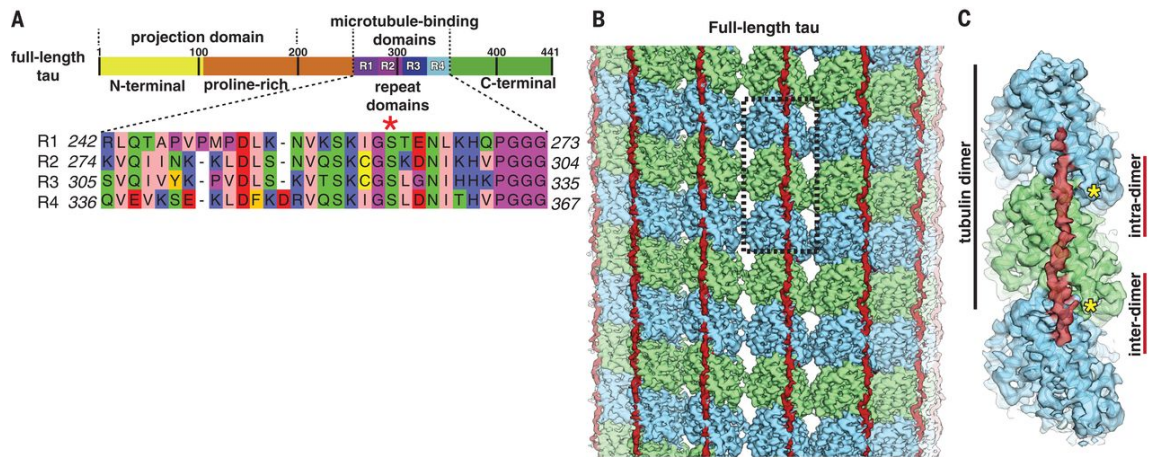


Figure 1.20: Tau binding to microtubules. (A) Schematic of the full-length tau structure and the sequence alignment of the four microtubule binding repeats, R1-R4. Ser262 is marked by a red asterisk (B) Cryo-EM density map (4.1 Å overall resolution) of a microtubule decorated with full-length tau. Tau (red) binds as a nearly continuous stretch along the MT protofilaments (α -tubulin in green, β -tubulin in blue). (C) Zoom-in of a single tau repeat spanning three tubulin monomers (one full α -tubulin and two half β -tubulin on either end), covering both intra- and inter-dimer tubulin interfaces. C-termini positions of tubulin are indicated with yellow asterisks. Figure adapted from Kellogg et al. (2018).

microtubules following the H11 and H12 helices that form a ridge at the microtubule surface (see Fig. 1.20). This result is in line with earlier cryo-EM findings at lower resolution that indicated that both MAP2c and tau bind along and not across the protofilaments of taxol-stabilized microtubules (Al-Bassam et al., 2002). The new findings show that the MTBR of a single tau molecule covers one α -tubulin subunit while contacts with β -tubulin are detected on both sides. This suggests that tau can promote association between tubulin subunits by acting as a "stapler" connecting three tubulin monomers, thereby promoting microtubule stability.

Generally, in tau-related studies using single-molecule resolution total internal reflection microscopy (TIRF), tau has been found to diffuse along the microtubules bi-directionally with rapid on- and off-rate, described as a kiss-and-hop interaction (Hinrichs et al., 2012). However, multiple reports have shown that tau has two different **binding modes** on the microtubule lattice, which are described as a diffusive and a static state (Makrides et al., 2003; Hinrichs et al., 2012; McVicker et al., 2014). The diffusing population was shown to be sensitive to pH, ionic strength, subtilisin treatment (treatment that removes the C-terminal tails of MTs), and the nucleotide state of the microtubules (Hinrichs et al., 2012; McVicker et al., 2014). On taxol-stabilized microtubules, 0N3R-tau was shown to prefer the static conformation with complexes of 2-3 molecules, while 2N4R-tau predominantly existed as single molecules equally distributed as static or diffusing populations (McVicker et al., 2014). However, on GMPCPP-microtubules both isoforms favored the diffusing binding state and did not form static complexes composed of more than one tau molecule (McVicker et al., 2014). Additionally, on taxol-stabilized microtubules the population of static tau molecules increased for both isoforms upon increasing tau concentration, while on GMPCPP-microtubules the population of static molecules remained constant for both isoforms upon increasing tau concentration (McVicker et al., 2014). This ability of tau to recognize the different nucleotide states of microtubules has been shown in a multitude of studies (Bechstedt et al., 2014; Ettinger et al., 2016; Duan et al., 2017). Recently, Castle et al. showed that tau avoids the GTP cap of dynamic microtubules, indicating a preference of tau for the GDP-lattice (Castle et al., 2020).

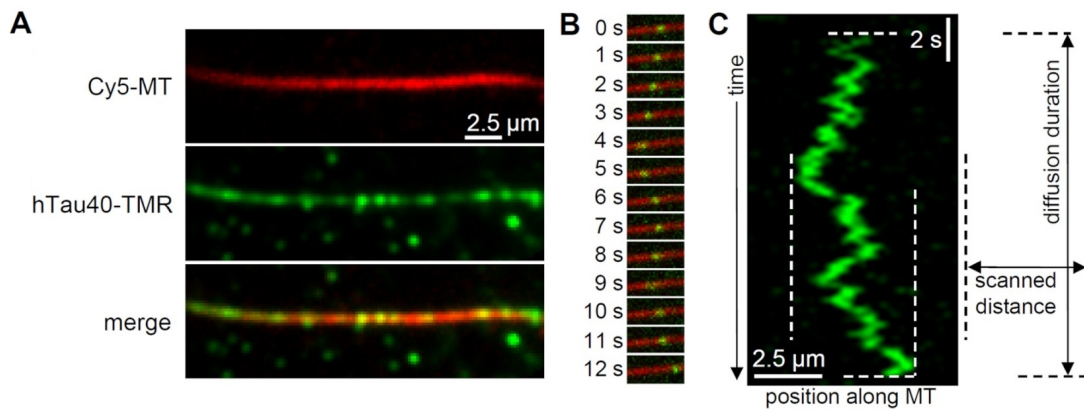


Figure 1.21: Tau interaction with microtubules. (A) TIRF microscopy images of TMR-labeled tau (full-length human tau, green) bound to a Cy5-labeled MT (red). (B) sequential frames of a tau molecule (green) moving along an immobilized Cy5-labeled MT (red). Time intervals as indicated. (C) Kymograph of the movement of the tau shown in B. Horizontal dashed lines indicate start and end of diffusive interaction, respectively. Vertical dashed lines indicate the extreme positions along the MT that the tau molecules reaches during this diffusive encounter. Figure adapted from [Hinrichs et al. \(2012\)](#).

Research done two decades ago showed that tau can oligomerize on the microtubule, and that two distinct binding modes of tau can co-exist on microtubules ([Makrides et al., 2003](#)). In this work, tau bound to taxol-stabilized microtubules was visualized using atomic force microscopy (AFM) and the two binding modes were described as (1) clusters of low organization consisting of single tau monomers, and (2) a single layer of tau oligomers that form a ring-like structure encircling the microtubule lattice. The height of the tau layer bound to the microtubules as observed using AFM methodology was too high to represent tau molecules that were fully extended while attached to the microtubule, indicating that part of the tau structure is projected away from the microtubule surface.

In cells, tau has generally been shown to be evenly distributed along the lengths of individual microtubules. However, there was a significant increase in tau density at regions of higher microtubule curvature ([Samsonov et al., 2004](#); [Ettinger et al., 2016](#)). Samsonov et al. hypothesized that this increase in tau density may increase the local flexural rigidity of the MT to resist compressive forces acting on the MT lattice ([Samsonov et al., 2004](#)). In the work of Ettinger et al. both tau and DCX were shown to bind preferentially at microtubule regions with higher curvature, which was attributed to the local compaction of the microtubule lattice due to bending of the microtubule (see Fig. 1.22) ([Ettinger et al., 2016](#)). Additionally, Samsonov et al. showed that addition of taxol to fibroblasts or neurons induced rapid dissociation of tau from MTs ([Samsonov et al., 2004](#)), a finding that remained ill-understood but was confirmed by Ettinger et al., where it was attributed to the sensitivity of tau to the MT geometry and its preference for curved MTs ([Ettinger et al., 2016](#)). However, the exact mechanism can only be speculated.

1.5.9 FUNCTIONALITY OF TAU PROTEIN

Tau is most famously known as a "microtubule stabilizing protein" and this knowledge mainly comes from early research done on the tau protein. In 1976, Witman et al. showed that tubulin requires tau for both the initiation and growth of microtubules ([Witman et al., 1976](#)). In 1977, Murphy et al. showed that tau promotes the nucleation of microtubule in vitro, however they

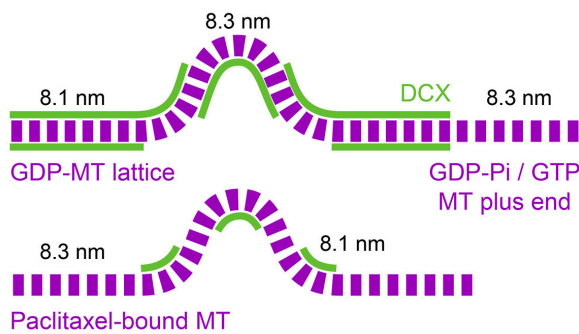


Figure 1.22: Cartoon explaining DCX (green) binding to straight and curved MT segments based on tubulin dimer (magenta) spacing in the GDP-MT lattice (8.1 nm) and in paclitaxel-bound (taxol-bound) MTs (8.3 nm). A curvature of $1 \mu\text{m}^{-1}$ was assumed which would result in a 2.5% larger lattice spacing in the outside compared with the inside of the lattice. Tau was found to bind similarly to DCX to the lattices, however, on GDP-MTs, tau was found preferentially on the high curvature regions, while DCX was found preferentially on the straight regions. Figure adapted from [Ettinger et al. \(2016\)](#).

found that the addition of tau was not an absolute requirement for microtubule elongation ([Murphy et al., 1977](#)). In 1986, Drubin and Kirschner found that after microinjection of tau protein into fibroblast cells, the microtubule mass increased significantly and more long microtubules were observed compared to in its sister cell which served as the control ([Drubin and Kirschner, 1986](#)). Furthermore, they found that microtubules coated with tau were more resistant against nocodazole-treatment. From these findings, it was concluded that tau protein stabilizes microtubules and promotes microtubule assembly. In 1992, in vitro reconstitution assays showed that the addition of tau to dynamic microtubules increased the growth rate of microtubules and decrease the shrinkage rate, overall showing that tau generates stable but still dynamic microtubules ([Drechsel et al., 1992](#)). Other research has shown that tau enables tubulin to form oligomers including single and double rings, which improve microtubule assembly and suggests that tau is a microtubule inducer in addition to a microtubule stabilizer ([Weingarten et al., 1975](#); [Devred et al., 2004](#)).

Due to the extensive research done on the topic, it is nearly universally accepted by the scientific community that tau is a microtubule stabilizer protein. However, over the years there appears to be some research that opposes this apparent fact. For instance, research done on cultured neurons showed that there is no difference in microtubule stability after microinjection of tau ([Tint et al., 1998](#)). Additionally, in rat brain neurons, there was no obvious change in axon length or morphology after tau depletion ([Qiang et al., 2006](#); [Yu et al., 2008](#); [Sudo and Baas, 2011](#)) and research done on tau KO mice showed no microtubule-related defects, except for very mild changes in neurite outgrowth and axon caliber ([Harada et al., 1994](#); [Dawson et al., 2001](#)). Recent work from the laboratory of Peter Baas suggests that tau's actual role in the neurons is to allow microtubules to grow and remain dynamic, rather than stabilizing them ([Qiang et al., 2018](#)). In fact, tau seems to be doing the opposite of stabilizing microtubules, it seems to prevent the dynamic regions of microtubules from becoming stable and allows them to lengthen. This is an important distinction since a lot of drugs currently in clinical trials for treating neurodegenerative diseases are based on the presumption that patients with neurodegenerative diseases are losing microtubules because they are becoming less stable. However, by treating these diseases with microtubule-stabilizing drugs, the potential exists that it makes matters worse rather than better. Combined, this shows that a lot of research still needs to be done to explain the exact role of tau in microtubule stabilization and polymerization.

Another well-described role of tau is its ability to protect microtubules against severing enzymes. The regulation of katanin was shown to be related to phosphorylation ([McNally and Roll-Mecak, 2018](#)), however, katanin itself appeared not to be phosphorylated, suggesting that katanin activ-

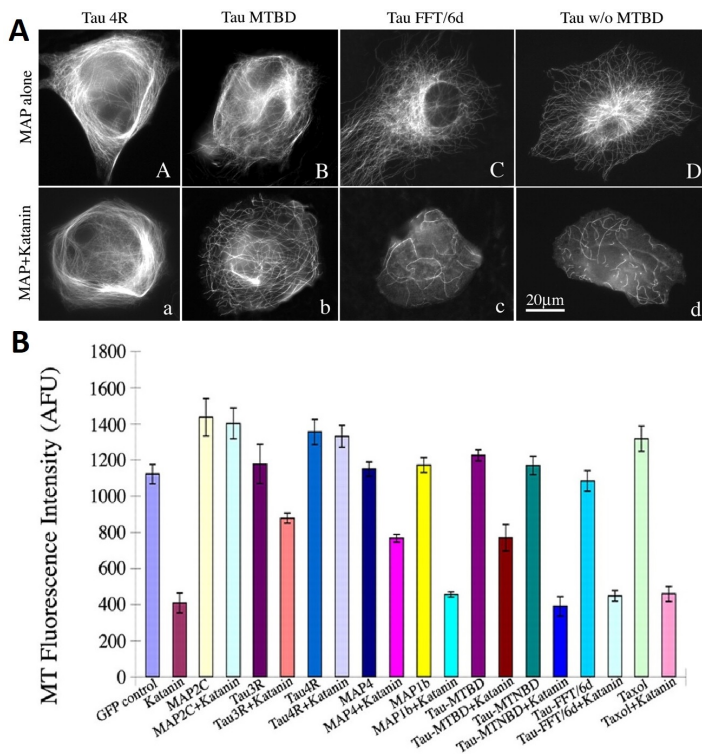


Figure 1.23: Tau protects microtubules against katanin-mediated severing. A. Fibroblast cells were transfected with: tau alone (top row); tau + p60-katanin (bottom row). 4R-tau generates microtubule bundling (A) and no microtubule severing (a). The MT-binding domain alone (Tau MTBD) generates minor bundling (B) and provides some protection against severing (b). Two versions of tau that lack the MT-binding domain: one naturally occurring (C, c) and one generated by experimental truncation (D, d), show no protection against severing. w/o, Without. Scale bar: 30 μm . B. Average fluorescence intensity of microtubule total mass. From left to right each bar shows a pair of: MAP alone (left), and MAP + p60-katanin expression (right). "GFP control" represents a control where the GFP-tag alone is expressed. Figure adapted from Qiang et al. (2006).

ity is regulated by the phosphorylation of other proteins that in their turn regulate the activity of katanin (McNally and Roll-Mecak, 2018). Due to its presence in neurons and tight regulation by phosphorylation, tau was a primary candidate to study for the role of protecting microtubules against katanin-mediated severing. Depletion of tau in neurons, as well as overexpression of tau in fibroblast cells, showed a similar ability of tau to protect microtubules against severing activity by katanin (Qiang et al., 2006). It was also shown that 4R-tau (all three isoforms) was more effective in protection compared to 3R-tau (all three isoforms), and a truncated construct of tau that lacked the N-terminal projection domain was still able to protect the microtubules against katanin severing, but at much lower ability compared to 3R- and 4R-tau (see Fig. 1.23A,B). Additionally, they showed that MAP2 protects microtubules to a similar degree compared to 4R-tau, while MAP4 showed similar but reduced protection compared to 3R-tau, and MAP1b did not protect microtubules at all. In the case of spastin, similar experimental techniques showed that tau also protects microtubules against severing by spastin, however, the protection is weaker compared to the protection against katanin (Yu et al., 2008). The presence of tau on axonal microtubules would provide a high level of protection against severing activity and the loss of microtubule mass in tauopathies may be a result of increased microtubule severing due to dissociation of tau from microtubules. The exact mechanism by which tau carries out its protective function is, however, still unclear.

In neurons depleted of tau, increased branching was observed while decreased branching was observed in neurons depleted of spastin (Yu et al., 2008). Interestingly, in neurons depleted of both tau and spastin the number of branches was indistinguishable compared to control neurons. These results indicate that while the presence of tau protects the neurons against excessive branching, and spastin increases the number of branching, the tau-based mechanism for axonal branching can not be attributed to protection against spastin. A likely explanation is that tau protection focuses mainly on katanin. Additionally, in neurons overexpressing katanin, tau depletion did not lead to complete loss of microtubule mass in the cell body and dendrites (Yu

et al., 2005), suggesting that there are other factors involved in microtubule protection against katanin-mediated severing.

The binding of tau to microtubules has been shown to regulate motor proteins such as kinesin and dynein (Hagiwara et al., 1994; Vershinin et al., 2007; Dixit et al., 2008), as discussed in subsection 1.3.1. In vitro, tau has been shown to inhibit kinesin-1 motility by acting as an obstacle on the microtubule surface (Vershinin et al., 2007; Dixit et al., 2008). The ability of tau to inhibit kinesin was, however, lost when using GMPCPP-stabilized microtubules (McVicker et al., 2014), suggesting that either tau or kinesin-1 is sensitive to the nucleotide-state of the microtubule. Dynein motility on the other hand is only inhibited at high levels of tau (Ebner et al., 1998; Vershinin et al., 2007; Dixit et al., 2008). Additionally, it was found that higher levels of the longest isoform of tau (2N4R) was needed to achieve a comparable reduction in motor activity compared to the shortest isoform of tau (0N3R) (Seitz et al., 2002; Vershinin et al., 2007), suggesting functional differences between the different tau isoforms. Furthermore, in vivo, tau overexpression in epithelial and neuronal cells lead to change in cell shape, loss of polarization, and change in mitochondrial distribution (Ebner et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Soundararajan and Bullock, 2014). In these experiments, mitochondria became clustered near the nucleus of the microtubule organizing center (MTOC), which was attributed to the preferential inhibition of tau to plus-end directed transport, causing minus-end directed transport (by dynein that walks towards the MTOC) to predominate. The perturbation of the balance in transport could be suppressed by addition of taxotere, a taxol derivative, and this perturbation was ascribed to the randomization of the microtubule network caused by nucleation of new microtubules in random configuration after taxol addition, however this remained to be proven.

Overall, tau is involved in many microtubule-related processes that range from regulating molecular motor transport to protection against severing enzymes. However, the mechanism by which tau carries out these regulatory roles remain elusive. In order to understand the downstream effects of tau malfunction, mislocalization and phosphorylation that are often associated with neurodegeneration, we first need to discover how tau functions in a physiological way.

This thesis aims to describe the work that was performed by the author of the thesis in the "Laboratory of Structural Proteins" for the PhD project entitled: "Tau proteins cooperatively assemble into cohesive envelopes that protect microtubules against severing enzymes". During the PhD project, it was found that tau can bind cooperatively to microtubules, forming a cohesive layer surrounding the microtubule lattice that was termed a **tau envelope**. The overall goal of this thesis is to describe the mechanisms underlying the cooperative assembly of tau proteins into cohesive envelopes, and to investigate the functional implications of these envelopes in protecting microtubules against severing enzymes. More specifically, the main goals can be outlined as follows:

- **Characterize the distinct binding modes of tau on microtubules**

Investigate the distinct binding modes of tau on microtubules using *in vitro* reconstitution and TIRF microscopy, and specifically the dynamic properties of the binding modes. To describe the dynamic properties of the envelopes we want to characterize; how the envelopes form, if they grow and disassemble, and how single tau molecules behave within the envelope structure.

- **Explore the mechanism of tau envelope formation**

Preliminary data suggests that tau envelope formation affects the underlying microtubule lattice. Our goal is to examine the effects of tau envelope formation on the spatial arrangement of tubulin dimers within the microtubule lattice. Using single molecule resolution TIRF microscopy we can study potential compaction of the microtubule lattice upon tau envelope formation, and using optical trapping we can reverse the compaction of the microtubule lattice to establish whether the induced effects are a requirement for tau envelope formation.

- **Investigate the regulatory roles of tau envelopes**

Study the effects of tau envelopes on molecular motors, such as kinesin-1, and microtubule severing enzymes, such as katanin using *in vitro* reconstitution and TIRF microscopy. This will reveal the potential impact of tau envelopes on kinesin-driven transport as well as microtubule stability.

- **Study the effect of tau phosphorylation on tau envelope integrity and function**

Using mass spectrometry analysis we can study the phosphorylation state of our tau samples, and combining this with our *in vitro* TIRF assays, we can study how the phosphorylation state affects tau envelope formation and stability. Additionally, we can study the

impact of tau phosphorylation on the regulatory functions of the envelopes, specifically on kinesin-driven transport and katanin-mediated severing.

By addressing these aims and objectives, this thesis intends to provide a comprehensive understanding of the binding mode of tau to microtubules and the mechanism behind the cooperative assembly of tau into cohesive envelopes. This will offer insights into the role of tau, and specifically tau envelopes, in regulating microtubule-related processes and how tau phosphorylation affects these properties.

LIST OF PUBLICATIONS

3

3.1 PUBLICATIONS RELATED TO THIS THESIS

All publication related to, and discussed in, this thesis can be found in appendix [A](#), along with the author contributions of the author of this thesis.

- **Siahaan, V.**, Krattenmacher, J., Hyman, A.A., Diez, S., Hernández-Vega, A., Lansky, Z., Braun, M. Kinetically distinct phases of tau on microtubules regulate kinesin motors and severing enzymes. *Nat Cell Biol* 21, 1086–1092 (2019).

Impact factor in 2019: 20.0

Publication and author contributions can be found in appendix [A.1](#).

- **Siahaan, V.**, Tan, R., Humhalova, T. Libusova, L., Lacey, S. E., Tan, T., Dacy, M., Ori-McKenney, K. M., McKenney, R. J., Braun, M., Lansky, Z. Microtubule lattice spacing governs cohesive envelope formation of tau family proteins. *Nat Chem Biol* 18, 1224–1235 (2022).

Impact factor in 2022: 14.8

Publication and author contributions can be found in appendix [A.2](#).

- **Siahaan, V.**, Weisssova, R., Lanska, E., Karhanova, A., Dostal, V., Henriot, V., Janke, C., Libusova, L., Braun, M., Balastik, M., Lansky, Z. Tau phosphorylation impedes functionality of protective tau envelopes. (unpublished)

Note: Manuscript is currently in review at Nature Chemical Biology (2024), and can be found in appendix [A.3](#) along with the author contributions.

3.2 PUBLICATIONS UNRELATED TO THIS THESIS

All publication unrelated to, and not discussed in, this thesis can be found in appendix [B](#), along with the author contributions of the author of this thesis.

- Kučera, O., **Siahaan, V.**, Janda, D. Dijkstra, S. H., Pilátová, E., Zatecka, E., Diez, S., Braun, M., Lansky, Z. Anillin propels myosin-independent constriction of actin rings. *Nat Commun* 12, 4595 (2021).

Impact factor in 2021: 17.7

Publication and author contributions can be found in appendix [B.1](#).

In 2019, our laboratory in collaboration with the laboratory of Richard McKenney¹, discovered that tau can bind cooperatively to microtubules, forming a protective layer on the microtubule surface that we called a **tau envelope**. In this chapter, I will describe the work that I, in collaboration with my fellow colleagues, have done during the course of my PhD to obtain the knowledge on tau envelopes that we have to this day. Whenever work was done in collaboration with a fellow student or collaborator, I acknowledge their input and cite their work. The chapter is divided in three section, each representing the work from a single publication related to this thesis (two published and one unpublished, attached in appendix [A](#)).

4.1 KINETICALLY DISTINCT PHASES OF TAU ON MICROTUBULES REGULATE KINESIN MOTORS AND SEVERING ENZYMES

During the start of my work in the laboratory of Zdenek Lansky², I got introduced to a project that involved tau liquid-liquid phase separation. Previous work done by Amayra Hernandez-Vega³ and Marcus Braun⁴ showed the remarkable ability of tau to form liquid droplets under conditions with crowding agents ([Hernández-Vega et al., 2017](#)). They found that tubulin partitions inside these droplets and, after addition of GTP, can nucleate microtubules. During their work on tau liquid droplets, Amayra and Marcus observed the formation of "high-density regions" or "domains" of tau on taxol-stabilized microtubules using TIRF microscopy, these domains turned out to be what we later termed tau envelopes. The initial aim of my PhD project was to reestablish the formation of the tau domains and to describe their formation with single molecule resolution using TIRF microscopy.

Reestablishing the formation of tau envelopes proved relatively straightforward. Having one of the lead scientists of the work, Marcus Braun⁴, as my supervisor made troubleshooting very effortless, and within a couple of weeks I had purified fluorescently tagged tau from insect cells (tau-meGFP and tau-mCherry) and was able to visualize tau envelopes, allowing me to start studying their dynamic behavior. I performed most of the preliminary work on the dynamics of tau envelopes during my internship in the laboratory of Zdenek Lansky². All experiments were

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²Z. Lansky: Institute of Biotechnology, Czech Academy of Sciences, BIOCEV, Prague West, Czech Republic

³A. Hernández-Vega: Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Germany

⁴M. Braun: Institute of Biotechnology, Czech Academy of Sciences, BIOCEV, Prague West, Czech Republic

then repeated over a timespan of several months to gather enough data to publish. In collaboration with a fellow PhD student from the lab, Jochen Krattenmacher⁵, who was very skilled with Matlab script writing, the data was analyzed and prepared for publication. The article was published in September 2019 in Nature Cell Biology (Siahaan et al., 2019). The published article and the material and methods discussed in the following section can be found in appendix A.1.

4.1.1 TAU COOPERATIVELY FORMS COHESIVE ENVELOPES ON THE MICROTUBULE LATTICE

The first objective that we set out to investigate was to find out how the tau envelopes form and if they nucleate from single spots, dynamically growing along the microtubule lattice without preference for a specific region of the lattice, or if they form as a full compartment at once and remain static throughout time. To study the formation of tau envelopes, we immobilized fluorescently labelled microtubules on a coverslip surface and added insect cell expressed tau-meGFP or tau-mCherry to the microtubules while observing the interaction of the tau and microtubules using TIRF microscopy (see Fig. 4.1A). After addition of 20 nM tau to surface-immobilized microtubules, we observed the immediate binding of tau to microtubules at low densities resembling diffusive tau. Interestingly, within 10 seconds, we could observe the formation of high-density regions nucleating at different locations along the microtubules lattice and at different timepoints after addition of tau (see Fig. 4.1B,C). These high-density regions, now called tau envelopes, grew from their boundaries in both directions at random growth rates with occasional pausing intervals (see Fig. 4.1C). Furthermore, we found that when two growing envelopes meet, they **merge** into a larger envelope while the density of tau within the envelopes remained constant during the merging event (see Fig. 4.1D), suggesting that the envelopes grow by the addition of tau molecules to their boundaries and the structure as a whole has a 2d arrangement that does not grow in the third dimension.

Upon removal of tau from solution, the tau bound to microtubules in the regions outside the envelopes disappeared from the microtubule lattice instantly, while the envelopes remained for several minutes while slowly disassembling from their boundaries with a dissociation rate of about 1,300 seconds (see Fig. 4.1E). Occasionally, we observed fission events within the boundaries of the envelope during disassembly. Again, when studying the density of tau during disassembly we found that the density remained constant throughout the procedure (see Fig. 4.1D). Combined, these findings strongly suggest that tau molecules within the envelope region bind cooperatively and can grow and shrink by the addition or removal of tau molecules from their boundaries. Incidental fission events happen on the rare occasion when tau unbinds from the center of an envelope.

To confirm that tau envelope formation is indeed a cooperative process, we measured the **Hill coefficient** of the process, which is a common way to test if a system is subject to cooperative binding. To measure the Hill coefficient, we used the Hill-Langmuir equation:

$$Y = \frac{K \cdot [X]^n}{1 + K} \cdot [X]^n \quad (4.1)$$

⁵J. Krattenmacher: Institute of Biotechnology, Czech Academy of Sciences, BIOCEV, Prague West, Czech Republic

4.1. TAU FORMS COHESIVE ENVELOPES ON MICROTUBULES

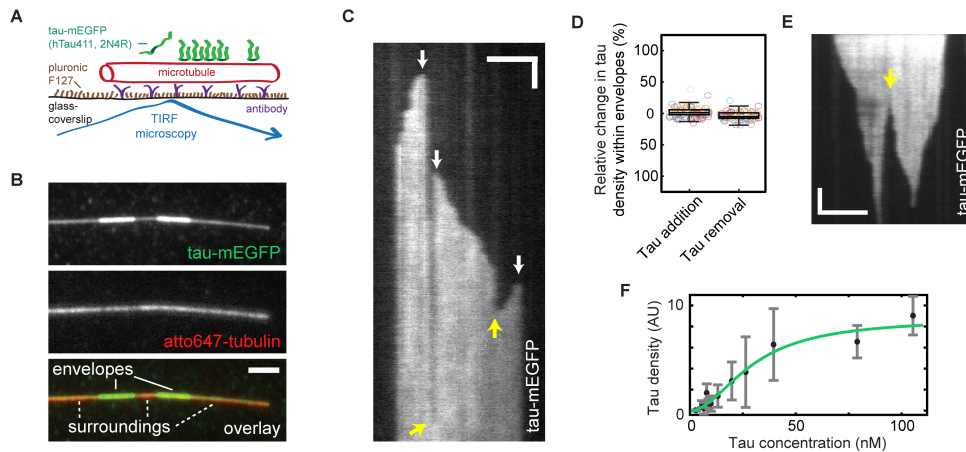


Figure 4.1: Tau forms cohesive envelopes on microtubules. **A.** Schematics of the in vitro TIRF assay. **B.** Multi-channel fluorescence micrograph showing envelopes of high-density tau–GFP (bright green) surrounded by regions of low-density tau–GFP (less intense green) on an Atto647-labelled microtubule (red). Images taken 5 min after the addition of 20 nM tau–GFP. **C.** Kymograph showing the fluorescence signal of tau–GFP on a microtubule after the addition 20 nM tau–GFP. Initially, the microtubule is covered by low tau density. Over time, high-density tau envelopes start to nucleate (indicated by white arrows) and assemble. Orange arrows indicate the merging of neighboring envelopes growing towards each other. **D.** Relative difference in tau density within envelopes just after tau envelope nucleation (left), and after the removal of tau from solution (right) ($n=91$ microtubules in 16 experiments). Data analyzed by Jochen Krattenmacher⁵. **E.** Kymographs showing the fluorescence signal of tau–GFP on the microtubule after the removal of tau from solution, visualizing envelope disassembly. The yellow arrow indicates a fission event. Vertical scale bar: 50s. **F.** From Siahhaan et al. (2022). Quantification of cooperative binding of tau to taxol-stabilized microtubules (mean \pm s.d., $n=652$ microtubules, 60 experiments, 95% confidence bounds, $r^2 = 0.9633$, gray), Hill–Langmuir equation fit (green). AU, arbitrary units. Unless stated otherwise, all scale bars: vertical 5s, horizontal 2 μ m.

Here, Y is the fraction of the receptor protein concentration bound by the ligand protein, X is the ligand concentration, K is the ligand concentration producing half occupation, and n is the Hill coefficient. Generally, this equation is used to describe how well a ligand binds to a macromolecule or receptor protein. In our case we can use this equation to test how well tau (our ligand) binds to microtubules (our receptor protein). The Hill coefficient will give a measure of cooperativity of the system. If the Hill coefficient $n < 1$, the system is **non-cooperative**. If the Hill coefficient $n > 1$, the process is considered **cooperative**, meaning that the binding of one ligand molecule makes it easier for the next ligand molecule to bind. To obtain the Hill coefficient of our tau-microtubules system, we immobilizes taxol-stabilized microtubules on the coverslips surface and varied the tau concentration in solution while measuring the tau density along the length of the microtubules (including tau envelope and non-envelope regions). The density on the microtubule was then plotted against the concentration in solution, and fitted against the Hill-Langmuir equation. This gave us a Hill-coefficient of around 2 (see Fig. 4.1F), indicating that tau envelope formation is a cooperative process, which states that the binding of one tau molecule facilitates the binding of the next tau molecule (Siahhaan et al., 2022).

We still, however, did not know how single tau molecules within an envelope behave. Can single tau molecules in the envelope exchange with tau molecules in solution? And are these envelope-bound tau molecules dynamic? Do they diffuse around within the envelope region, or do they remain statically bound to a specific binding site on the microtubule lattice? To study whether tau molecules in the envelope can exchange with tau molecules in solution, we made use of our two differently colored tau stocks (tau-meGFP and tau-mCherry). We formed tau envelopes using 20nM tau-mCherry on surface-immobilized taxol microtubules, and incubated the tau for

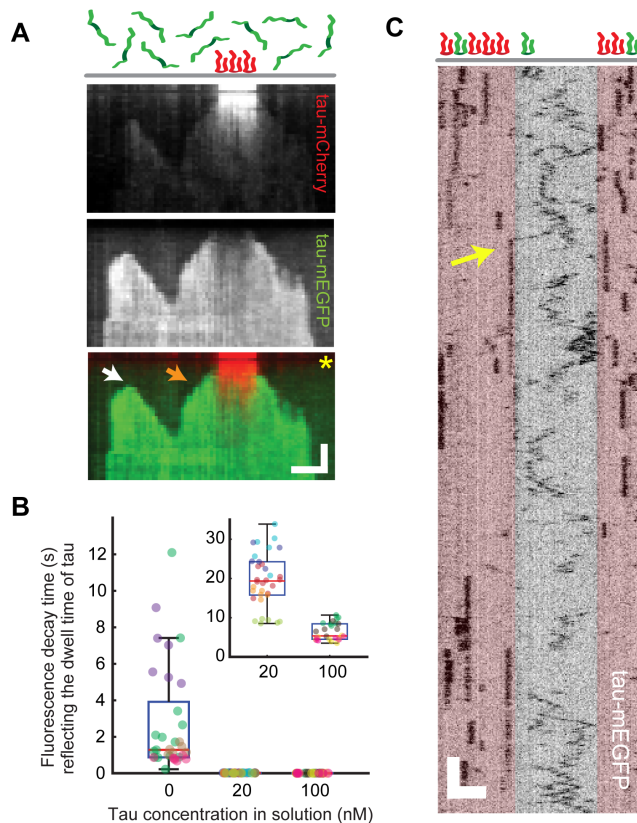


Figure 4.2: Tau molecules in envelopes are stationary and exchange for tau in solution. **A.** Multichannel fluorescence kymograph of 20 nM tau-mCherry (red) exchanged for 20 nM tau-meGFP (green) at timepoint indicated by the yellow star. Initially, tau-mCherry exchanges for tau-meGFP within the envelope region while elongating the existing envelope from the boundaries (orange arrow). Additionally, new envelopes nucleate at different locations on the microtubule lattice (white arrow). Scale bars: vertical 20 s, horizontal 2 μ m. **B.** The dwell time of tau within the envelopes decreases with increasing concentration of tau in solution. Analyzed by Jochen Krattenmacher⁵. Datapoints are color-coded by experiments. **C.** Kymograph showing single tau-meGFP molecules (black) interacting with a microtubule containing two tau-mCherry islands (beige transparent boxes). Tau diffuses outside envelope regions, whereas within the envelopes, tau is stationary. Occasionally, diffusing tau molecules become stationary when associated with an envelope boundary (yellow arrow). Scale bars: vertical 1 s, horizontal 2 μ m.

5 minutes on the microtubules to establish medium-sized tau envelopes. After 5 minutes of incubation, we removed the tau-mCherry from solution by flushing in 20nM tau-meGFP. The tau-mCherry outside the envelopes was rapidly replaced by tau-meGFP, with a time constant of about 3 seconds (see Fig. 4.2A). The tau-mCherry within the envelopes, however, exchanged with a much slower rate of about 20 seconds, which is still faster than the dissociation rate upon complete removal of tau (which was 1,300 seconds) (see Fig. 4.2B). This exchange rate was dependent on the concentration of tau in solution, since the dissociation rate further decreased when increasing the concentration of tau-meGFP tau to 100nM (see Fig. 4.2B). These findings indicate that tau molecules within the envelopes can turn-over and exchange with tau in solution and the rate of dissociation depends on the concentration of tau in solution.

To study the movement of single tau molecules within the tau envelope, we performed dual-color imaging using TIRF microscopy with single molecule resolution. We formed tau envelopes with a mixture of 20 nM tau-mCherry and a very low (about 1 nM) concentration of tau-meGFP. This technique allows us to follow the movement of single tau-meGFP molecules inside and outside envelopes predominantly formed by tau-mCherry molecules. Outside the envelopes, we observed single tau-meGFP molecules rapidly diffusing on the microtubule lattice as indicated by the wiggly lines that can be observed in the kymograph (see Fig. 4.2C). By contrast, within the envelope regions, single tau molecules remained mostly stationary, indicated by the straight lines of the molecules in the kymograph (see Fig. 4.2C). Occasionally, we observed a tau-meGFP molecule that initially diffused outside of the tau envelope, become stationary when it associated with the envelope boundary. Combined, we found that single tau molecules within the envelope regions are stationary and remain roughly in one location within the envelope, but they can exchange for tau in solution.

4.1.2 FUNCTIONALITY OF TAU ENVELOPES

The next thing we were interested in was to study whether tau envelopes had any regulatory roles that could explain the regulatory functions of tau found in many other studies (discussed in section 1.5.9). We therefore tested the effect of the presence of tau envelopes on molecular motor kinesin-1 and microtubule-severing enzyme katanin. To test the regulatory roles of tau envelopes on kinesin-1, we prepared envelopes using tau-mCherry and added GFP-labelled kinesin-1 to the envelope-covered microtubules. We found that kinesin-1 was able to walk in regions outside of the tau envelope, but when a kinesin-1 motor encountered the boundary of an envelope, the motor dissociated from the microtubule lattice (see Fig. 4.3A). We never observed any processive movement of the molecular motor within a tau envelope, indicating that tau envelopes inhibit transport by kinesin-1.

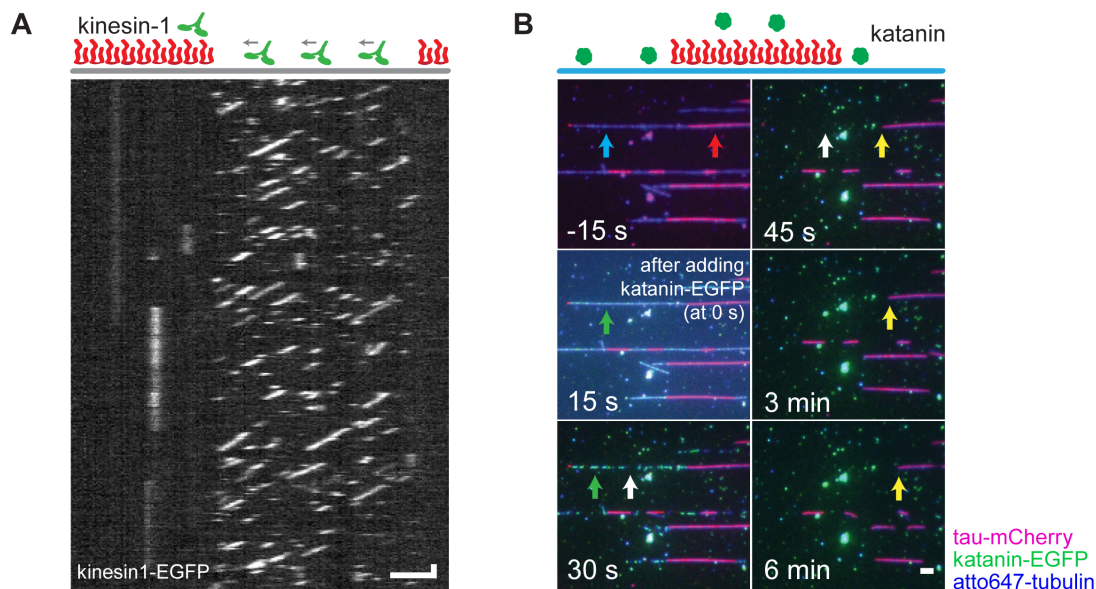


Figure 4.3: Tau envelopes regulate access to the microtubule surface. **A.** Fluorescence kymograph showing kinesin-1-GFP molecules (white) moving processively outside the envelope regions and dissociating from the microtubule lattice when encountering the envelope boundary. Locations of the envelopes are indicated by the schematics above the kymograph. Scale bars: vertical 1s, horizontal 2 μm. **B.** Multichannel fluorescence micrographs showing katanin-GFP (green) severing an Atto647-labelled microtubules (blue) covered by tau-mCherry (magenta) envelopes (red arrow) and non-envelopes (blue arrow). Katanin binding (green arrow) and microtubule severing (white arrow) initially occurs outside the envelope boundaries.

To study the effect of tau envelopes on the microtubule-severing enzyme katanin, we formed envelopes using 20 nM tau-mCherry and after 5 minutes of incubation added 200 nM katanin-GFP (p60 and p80 subunits) in presence of 20 nM tau-mCherry to the envelope-covered microtubules. We observed katanin-GFP binding predominantly at regions of microtubules not covered by a tau envelope, leading to the severing of these regions (see Fig. 4.3B). Within one minute of incubation, the katanin had bound to and severed all non-enveloped regions on the microtubules while the envelope-covered regions prevailed on much longer timescales and no katanin binding within the enveloped regions was observed. Over a much longer timescale the tau envelopes were eventually severed mostly from their boundaries with occasional severing events within the boundaries of the envelope. These findings indicate that tau envelopes are able to locally

prevent katanin from binding to the microtubule surface and thereby protect the microtubule lattice from being severed by the enzyme. Additionally, we found that an equally dense layer of diffusing tau did not protect the microtubule against severing by katanin, indicating that it is specifically the cooperative binding mode of tau that prevents katanin from binding and severing the microtubule lattice (Siahaan et al., 2019).

In summation, the work done for the Nature Cell Biology paper of 2019 (attached in appendix A.1) was a great effort that was led by Jochen Krattenmacher⁵ and myself, under supervision and with great support from Zdenek Lansky² and Marcus Braun⁴. The story was published back-to-back with the laboratory of Richard Mckenney¹, where the work was led by Ruensern Tan⁶ (Tan et al., 2019), establishing a great collaboration between our labs. The highlight of both stories shows that tau forms a cohesive and protective layer on microtubules, that we now name **tau envelopes**. The dynamics and functionality of these envelopes were described by both labs, complimenting and validating each other's work (Siahaan et al., 2019; Tan et al., 2019).

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4.2 MICROTUBULE LATTICE SPACING GOVERNS TAU ENVELOPE FORMATION

Our knowledge on the dynamics and functionality of the tau envelopes had grown significantly in a short period of time. The lingering question that remained was what the mechanism is behind tau envelope formation. Studies regarding the mapping of the domains of tau that are necessary for envelope formation concluded that the projection domains of tau are hugely important in the ability of tau to bind cooperatively (Siahaan et al., 2019; Tan et al., 2019). Therefore, tau-tau interaction through the projection domains seemed to be the leading mechanism for tau envelope formation. However, we wondered if there is an additional mechanism that facilitates tau envelope formation which involves the structure of the microtubule lattice. For this story, I performed all in vitro experiments using TIRF microscopy and optical trapping that are described in this thesis. Additionally, work done on the different members of the tau/MAP2 family were performed in collaboration with the laboratory of Richard McKenney¹. And finally, all in vivo experiments were performed by Tereza Humhalova⁷ from the laboratory of Lenka Libusova⁸. This article was published in November 2022 in Nature Chemical Biology (Siahaan et al., 2022). The published article and the materials and methods discussed in the following section can be found in appendix A.2.

4.2.1 TAU ENVELOPE FORMATION INDUCES A LOCAL MICROTUBULE LATTICE COMPACTION

Work done in the laboratory of Richard McKenney¹ showed that tau envelopes were not able to form on GMPCPP-microtubules (Tan et al., 2019), indicating that the nucleotide state of the microtubules influenced the ability of tau to bind cooperatively. Additionally, studies on microtubule lattices had shown that the nucleotide state of the microtubules affects the structure of the lattice (Alushin et al., 2014; LaFrance et al., 2022). More precisely, native GDP-microtubules have a compacted conformation of the lattice while microtubules grown using the slowly hydrolyzable GTP-analog GMPCPP, or microtubules stabilized using taxol, have an extended conformation of the lattice. Knowing that, the finding that tau envelopes could not form on GMPCPP-microtubules suggests that tau envelope formation is sensitive to the structure of the microtubule lattice, and that tau envelopes may prefer a compacted microtubule lattice over an extended one. The fact that tau envelopes grow on taxol-stabilized microtubules, that are also in the extended state, could be explained by the fact that taxol can unbind from the microtubule lattice whereas GMPCPP cannot, making the extension of the microtubule lattice due to taxol **reversible**, while the extension of the microtubule lattice by GMPCPP is (nearly) **irreversible** since GMPCPP cannot unbind from the tubulin after polymerization. Hydrolysis of GMPCPP could, however, induce a compaction of the lattice, but due to the slowly hydrolyzable property of GMPCPP, this is unlikely to occur within our experimental timeframes.

The hypothesis that tau envelopes may prefer a compacted microtubule lattice became increasingly plausible when we found that tau envelopes form much more rapidly on natively compacted

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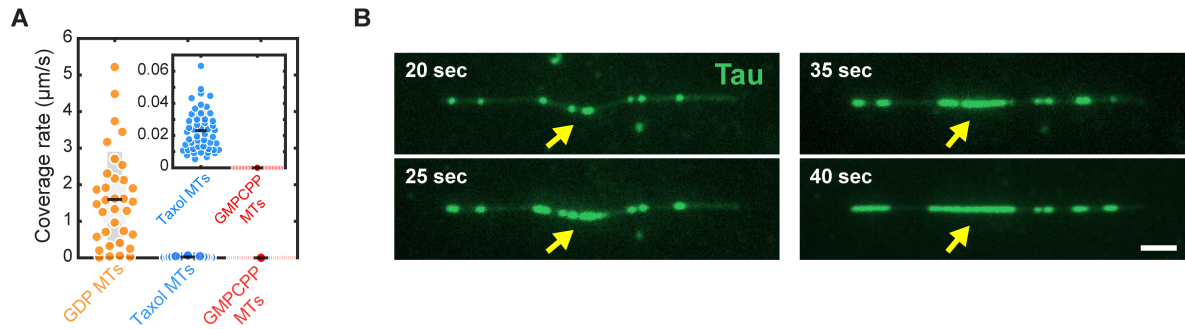


Figure 4.4: Tau envelope formation induces straightening of the microtubule lattice. **A.** Coverage rate of tau envelopes on different microtubule lattices. Coverage rate on GDP-lattice microtubules (orange) was $1.6 \pm 1.3 \mu\text{m/s}$; on taxol-stabilized microtubules (blue) $23.0 \pm 12.8 \text{ nm/s}$; and no envelope formation was observed on GMPCPP-lattice microtubules (red). **B.** Fluorescence micrographs of 20 nM tau-mCherry (green) added at $t=0$ min to an immobilized taxol-stabilized microtubules that initially has a bent structure (yellow arrows), but upon tau binding straightens out. Scale bar: $2 \mu\text{m}$.

GDP-microtubules, suggesting that tau envelopes indeed prefer a compacted microtubule lattice (see Fig. 4.4A). This also explains why tau is often observed at higher density in bends of microtubules (Samsonov et al., 2004; Ettinger et al., 2016), where the lattice is assumed to be partly compacted on the inside of the bend. Additionally, we observed that tau envelope formation on taxol-stabilized microtubules induced straightening of the microtubule lattice at locations that initially had a bent structure (see Fig. 4.4B). As one can imagine, a bend would be a highly unfavorable position when compacting the microtubule structure and if outside factors would induce a compaction, it is not unlikely to assume that a bend would be straightened out. Therefore, we hypothesized that tau did not only have a preference for compacted microtubules lattices, the formation of tau envelopes could even induce a compaction of the microtubule lattice.

In order to visualize length changes of the microtubule lattice we polymerized so-called "**speckled microtubules**" (Mitra et al., 2020). These microtubules are polymerized from mostly unlabeled tubulin with a very low content of fluorescently labeled tubulin. The resulting microtubules appear speckled when using TIRF microscopy (see Fig. 4.5A). This polymerization technique allows us to track single tubulin molecules inside the microtubule lattice and since tubulin molecules are fixed within the microtubule lattice, we can now track how a single speckle, and therefore a single location within the microtubule lattice, is moving with respect to neighboring

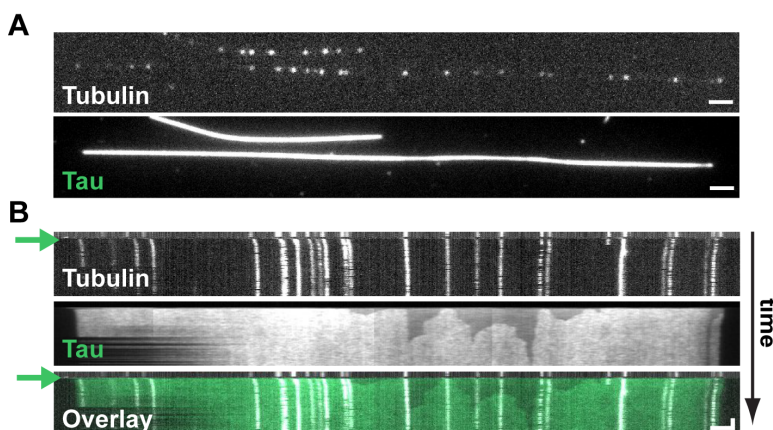


Figure 4.5: Speckled microtubules enable visualization of MT lattice compaction. **A.** Fluorescence micrographs of a speckled Atto-647-labeled microtubule (top) after addition of 400 nM tau-mCherry (bottom). **B.** Multichannel kymograph corresponding to **A** showing individual speckles (white) moving closer to each other upon tau envelope formation (green), indicating microtubule lattice compaction. Timepoint of tau addition is marked by green arrows. All scale bars: $2 \mu\text{m}$, 1 min.

4.2. MICROTUBULE LATTICE SPACING GOVERNS TAU ENVELOPE FORMATION

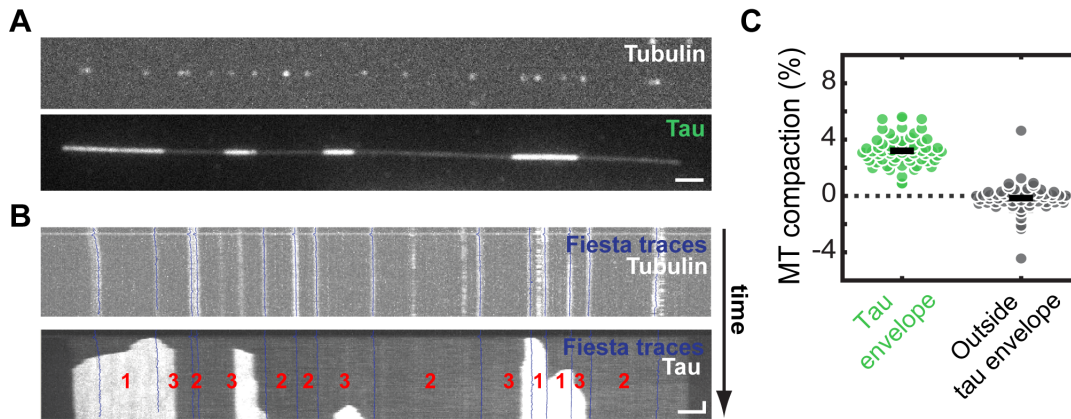


Figure 4.6: Microtubule lattice compaction measured using FIESTA tracking of speckled MTs. **A.** Fluorescence micrograph of an Atto647-labeled speckled microtubule (top) after addition of 20 nM tau-mCherry (bottom). **B.** Visual representation of compaction analysis using FIESTA tracking software. The kymographs correspond to the experiment from **A** and show the individual speckles on the microtubule lattice (top, tubulin in white) and the corresponding traces found by FIESTA (blue). Using the kymograph of the tau signal (bottom, tau in white), areas between the traces are assigned the corresponding type of event between the speckles. Event types indicated by red numbers. 1: Tau envelope region, 2: Non-envelope region, 3: Not measured (both events occurred). **C.** Compaction of the microtubule lattice by tau within the envelope regions: $3.2 \pm 1.1\%$; outside envelope regions: $-0.1 \pm 1.1\%$. All scale bars: horizontal 2 μm , vertical 1 min.

speckles along the lattice. If a microtubule lattice would be compacting, this would result in neighboring speckles moving closer to each other.

We performed the tau envelope formation assay by immobilizing speckled taxol-stabilized microtubules on the coverslip surface and adding a high concentration (400 nM) of tau to ensure full coverage and therefore maximum effect of the tau envelopes. We observed that upon tau envelope formation, individual speckles indeed moved closer to each other as evident from the kymograph (see Fig. 4.5B). The question that remained was whether this compaction of the lattice could be attributed to the cooperative binding mode of tau, or whether compaction happened in non-enveloped regions as well. Therefore, we repeated the experiment, this time with a lower concentration (20 nM) of tau to ensure partial coverage of tau envelopes on the microtubules (see Fig. 4.6A,B). Using FIESTA tracking software (Ruhnnow et al., 2011), we were able to track single tubulin molecules over time, allowing us to quantify the change in distance between neighboring speckles after tau addition. We then separated the results based on whether the speckles were found in regions where a tau envelope formed, or regions where no tau envelope formed (see Fig. 4.6B). We found that the microtubule lattice compaction appeared locally within tau envelope regions, and was to the extent of $3.2 \pm 1.0\%$, while locations outside tau envelopes did not compact (see Fig. 4.6C). These findings indicate that the cooperative binding mode of tau induces a **local** lattice compaction that is localized specifically to the area of the microtubule where the tau envelope formed.

As this compaction of the microtubule lattice reverses the taxol-induced extension of the lattice, we wondered if the cooperative binding of tau replaces the taxol bound to the microtubule. To visualize the taxol on the microtubule we used a fluorogenic taxane called SiR-tubulin that binds and stabilizes polymerized microtubules. We then polymerized microtubules using 10 μM taxol with additional 2 μM SiR-tubulin and immobilized these SiR-tubulin microtubules on the coverslip surface. Strikingly, after addition of 20 nM tau-mCherry, we observed that the initially uniform distribution of SiR-tubulin showed a decrease in density in the regions specif-

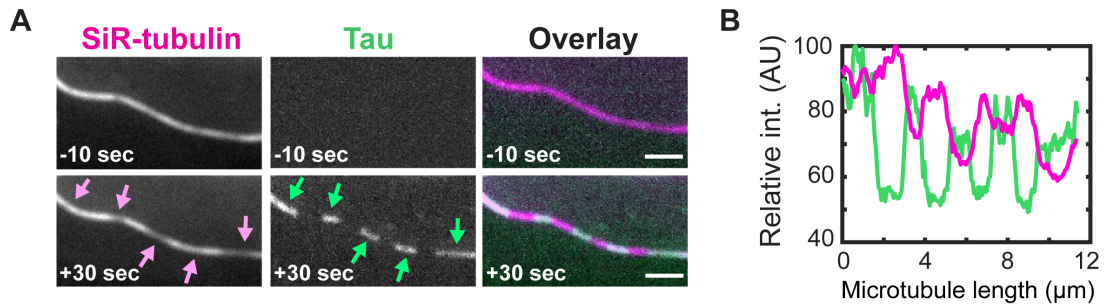


Figure 4.7: Tau envelope formation displaces taxol on the microtubule. **A.** Fluorescence micrographs of 2 μM SiR-tubulin (magenta, left) bound to a MT, 10 seconds before (top) and 30 seconds after (bottom) the addition of 20 nM tau-mCherry (green, middle). Green arrows indicate tau envelopes, pink arrows indicate the corresponding local decrease in SiR-tubulin density. Local decrease in SiR-tubulin density within tau envelope regions compared to non-envelope regions was $21.7 \pm 12.9\%$. Scale bar: 2 μm . **B.** Fluorescence intensity profile of SiR-tubulin (magenta) and tau-mCherry (green) on the microtubule shown in **A** 30 seconds after tau addition.

ically where a tau envelope formed (see Fig. 4.7A,B). This local decrease happened rapidly after tau envelope formation and indicates that cooperative binding of tau induces a removal of taxol on the microtubule lattice. Combined, these findings show that tau envelope formation can overcome the taxol-induced lattice extension by displacing taxol from the microtubule lattice.

4.2.2 MICROTUBULE LATTICE EXTENSION INDUCES DISASSEMBLY OF TAU ENVELOPES

To understand whether the compaction of the microtubule lattice is required for the cooperative binding mode of tau, we wanted to see if reversal of the compaction would induce disassembly of the tau envelopes. Therefore, we extended the microtubule lattice in two ways: either locally, or globally. **Local extension** of the microtubule lattice was achieved by bending microtubules using flow. This induces a local extension (as well as compaction) at the site of the bend. **Global extension** was achieved by attaching a single microtubule between two beads using optical tweezers. Moving the beads away from each other ensures extension of the microtubule lattice along its entire trapped length.

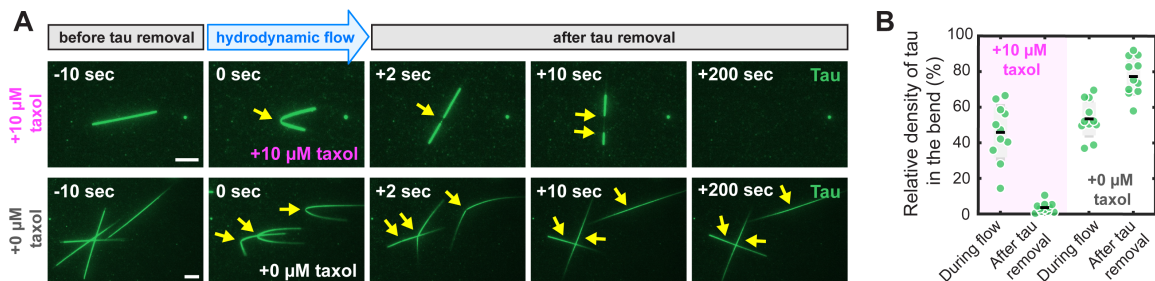


Figure 4.8: Local microtubule lattice extension induces tau envelope disassembly. **A.** Fluorescence micrographs of tau-mCherry envelopes on GMPCPP-capped GDP-lattice microtubules. Hydrodynamic flow was induced at $t=0$ sec. During flow, tau is removed either in presence of 10 μM taxol (top panels) or in absence of taxol (bottom panels). Yellow arrows indicate the location of the bend induced by the flow and the subsequent local decrease in tau density. Scale bars: 5 μm . **B.** Relative density of tau at the location of the MT bend, during and 200 sec after flow. With 10 μM taxol: density dropped to $45.9 \pm 15.9\%$ during flow and decreased to $3.6 \pm 2.8\%$ after flow. Without taxol: density dropped to $53.5 \pm 10.4\%$ during flow and recovered to $77.3 \pm 10.8\%$ after flow.

4.2. MICROTUBULE LATTICE SPACING GOVERNS TAU ENVELOPE FORMATION

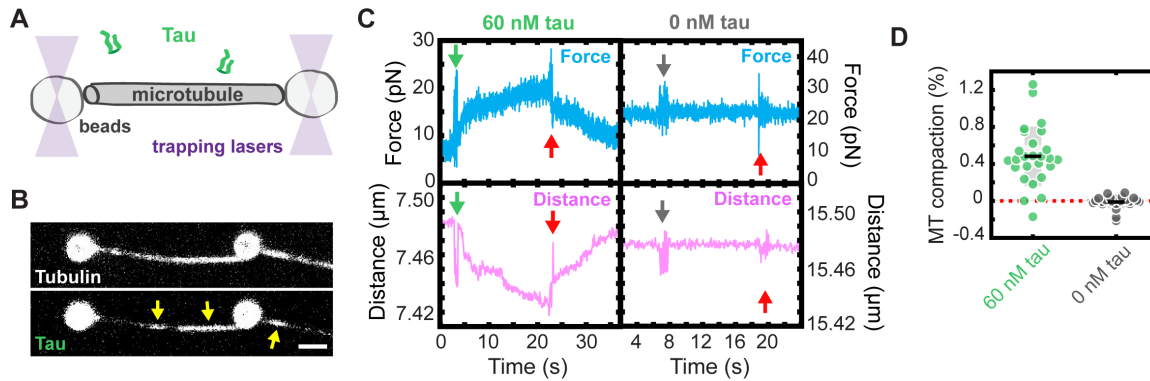


Figure 4.9: Microtubule lattice compaction can be measured in optical tweezers. **A.** Schematics of the optical tweezers assay. Biotin-HiLyte647-labeled taxol-stabilized microtubules are attached between two streptavidin-coated silica beads and 60 nM tau-mCherry is added to the measurement chamber. **B.** Fluorescence micrographs of a microtubule (top) suspended between two beads in the presence of 60 nM tau (bottom). Yellow arrows indicate the tau envelopes. Scale bar: 2 μm . **C.** Representative force-time (blue, top) and distance-time (pink, bottom) curves corresponding to a compaction event after addition of 60 nM tau-mCherry (left), or no compaction after addition of 0 nM tau-mCherry (right). "Distance" represents the distance between the beads, "force" is measured on the left bead in the x -direction. Time of tau addition is marked by a green arrow, and tau removal is marked by a grey arrow. **D.** Compaction of taxol-stabilized microtubules after addition of 60 nM tau-mCherry (green), or 0 nM tau-mCherry (grey). The compaction was measured by the decrease in relative distance. For 60 nM tau: $0.48 \pm 0.32\%$; 0 nM tau: $-0.01 \pm 0.07\%$.

For the **local extension** experiment we used GDP-microtubules that are in the compacted state and are stabilized by capping them with GMPCPP-grown extensions. We attached the microtubules to the coverslip surface at a single attachment point within the GDP-lattice, and added 60 nM tau to the microtubules to ensure full envelope coverage on the microtubules. We then induced a flow in the channel which causes the microtubules to bend at their single attachment site for the duration of the flow (see Fig. 4.8A). During the flow we removed tau from solution which should induce tau envelope disassembly and the assay buffer that was used to remove tau from solution was either in presence or absence of 10 μM taxol, which should further induce lattice extension. After inducing flow and removing tau from the measurement chamber, we observed a local decrease in tau density at the location where the microtubule was initially bent (see Fig. 4.8B). This local decrease can be attributed to a local fissure or break in the envelope. In absence of taxol, the envelopes prevailed after the brief period of flow and, interestingly, the local decrease in density increased to nearly the same density the envelope had before the brief period of flow, indicating that the fissure closed (see Fig. 4.8B). By contrast, in presence of 10 μM taxol, tau envelopes started disassembling and this disassembly happened from the boundaries of the microtubule lattice as well as from the fissured location, indicating that there was local damage in the envelope at the bent location which created a new "boundary-like" site from which taxol-induced extension could contribute to the disassembly of the tau envelope. These findings demonstrate that a local extension of the microtubule lattice, even if it was transient, can destabilize tau envelopes.

For the **global extension**, we used taxol-stabilized microtubules that were partly biotin-labelled and partly fluorescently-labelled. We attached a single microtubule between two streptavidin-coated beads that were optically trapped in an optical tweezers microscope (see Fig. 4.9A). We then moved the beads slowly apart to straighten out the microtubule and stopped when a force increase was observed. This ensured that the microtubule were in a straight but non-stretched position. The construct was then moved into a channel containing 60 nM tau where tau enve-

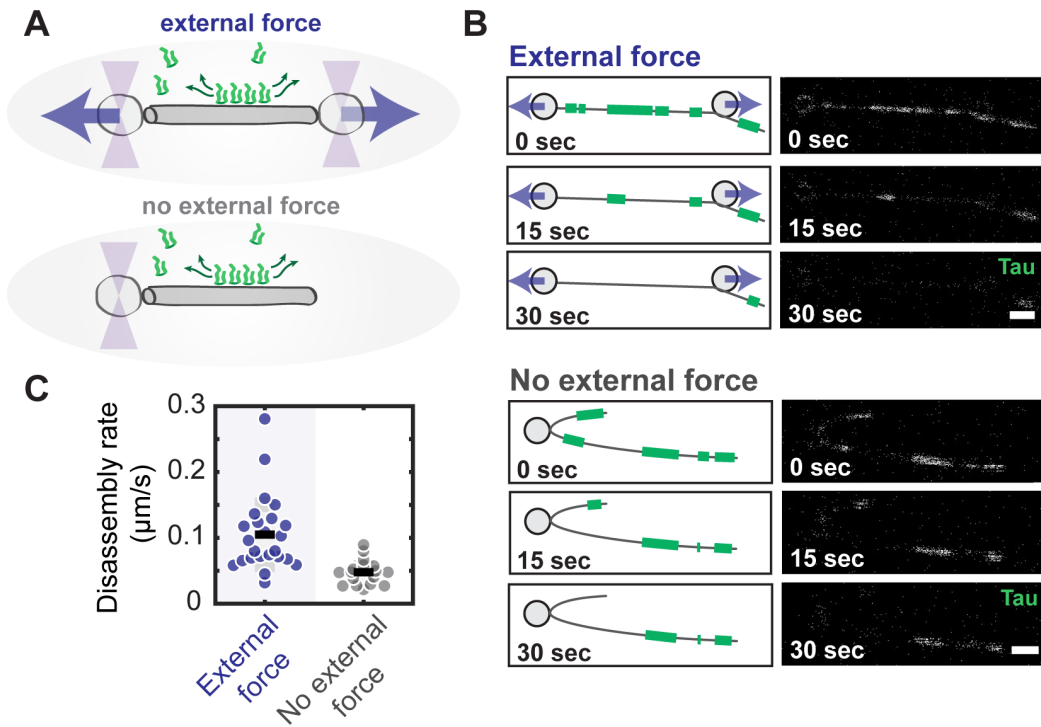


Figure 4.10: Global lattice extension induces tau envelope disassembly. **A.** Schematics of assay setup. External force is applied to the microtubule by moving the beads away from each other (top). To ensure that no external force is applied, the microtubule is attached to a single bead (bottom). **B.** Fluorescence micrographs of tau envelopes disassembling after removal of tau: in presence of external force on microtubule (top), or in absence of external force on microtubule. Sketches of the micrographs (left panels) indicate the size and positions of the tau envelopes (green lines). Scale bars: 2 μm . **C.** Disassembly rate of tau envelopes: In presence of external force: $0.11 \pm 0.06 \mu\text{m/s}$; no external force: $0.05 \pm 0.02 \mu\text{m/s}$.

lope formation could be observed (see Fig. 4.9B). The formation of tau envelopes induced a microtubule lattice compaction which we could observe by an increase in the force, accompanied by a decrease in the distance between the two beads (see Fig. 4.9C). By contrast, in a control experiment where we added buffer containing 0 nM tau to the measurement chamber, no force increase or distance decrease could be observed (see Fig. 4.9C). Quantifying this compaction by measuring the distance decrease after addition of 0 or 60 nM tau, we found that the microtubule compacted after the addition of tau, while no compaction was measured in our control experiment (see Fig. 4.9D), indicating that the compaction of the microtubule lattice can be measured using our optical trapping assay. The compaction measured using this setup can, however, not be directly be linked to the presence of a tau envelope and due to the large variance in tau envelope coverage, the measured compaction ($0.48 \pm 0.32\%$) is much lower compared to the measured compaction using our speckled MTs and FIESTA tracking ($3.2 \pm 1.1\%$).

To test whether global extension of the microtubule lattice induces tau envelope disassembly, we formed tau envelopes on a straight but non-stretched microtubule attached between two beads and removed tau from solution to trigger envelope disassembly. During tau envelope disassembly, we stretched the microtubule lattice by applying an external force to the microtubule, which was achieved by moving the beads apart from each other until we reached a set force of 40 pN (see Fig. 4.10A). In our control experiments, we did not apply an external force to the microtubule while removing tau from solution. We found that the disassembly rate of the tau envelopes increased more than two-fold when the microtubule was subject to external force that

4.2. MICROTUBULE LATTICE SPACING GOVERNS TAU ENVELOPE FORMATION

stretched the microtubule lattice compared to the envelope disassembly rate on microtubules in a relaxed non-stretched state (see Fig. 4.10B,C). Combined, we confirmed that extension of the microtubule lattice can induce tau envelope disassembly. These findings show that the presence of tau envelopes is sensitive to external factors that influence the underlying microtubule lattice.

4.2.3 COOPERATIVE ENVELOPE FORMATION IS A DIVERGENT PROPERTY WITHIN THE TAU-FAMILY

As described in the introduction, the tau family also includes MAP2 and MAP4, which are relatively well-preserved in their structure, and especially the microtubule-binding repeats, but differ in their projection domains (see Fig. 4.11A). To investigate whether envelope formation is conserved within the tau family, our collaborators in the lab of Richard McKenney¹ studied the shortest isoform of MAP2, MAP2c, and MAP4, both fluorescently tagged with GFP. They found that MAP2c formed envelopes similar to tau, and when mixing the two proteins in the channel, the two proteins cooperatively formed envelopes together, indicating that cooperative binding is possible between two members of the tau family despite their different flanking regions. Additionally, using our speckled microtubules in combination with FIESTA tracking software, we found that the MAP2c envelope formation induced a microtubule lattice compaction at almost identical extent to the tau-induced lattice compaction, that is $3.0 \pm 1.1\%$ within MAP2c envelopes where it was $3.2 \pm 1.1\%$ within tau envelopes. And similar to tau, this MAP2c compaction was not found outside the MAP2c envelopes (see Fig. 4.11B). MAP4, however, was not able to form envelopes and was excluded from envelopes formed by tau and MAP2c (Siahaan et al., 2022). Additionally, no microtubule lattice compaction was observed after addition of MAP4 to taxol-stabilized microtubules ($0.0 \pm 0.4\%$) (see Fig. 4.11B). Combined, these findings indicate that the cooperative binding mode is a divergent property within the tau-family, and different tau-family members can cooperatively form envelopes together while excluding members that do not possess the ability to form envelopes.

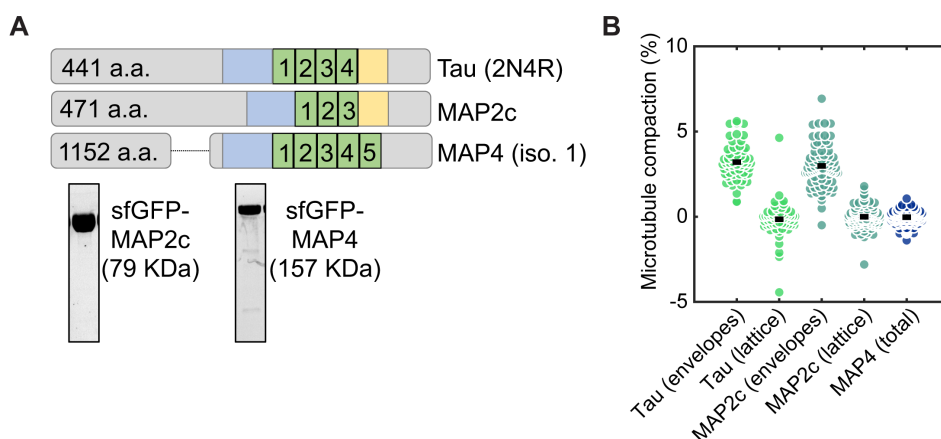


Figure 4.11: Tau and MAP2 compact the lattice while MAP4 does not. **A.** Schematics of the MAP proteins analyzed. Highlighted regions: MT binding region, green; proline-rich region, blue; pseudo-repeat, yellow. Below: Coomassie stained gels showing purity of the MAP2c and MAP4 proteins used. MAP preparation performed and analyzed by Ruernsen Tan⁶ and others from the laboratory of Richard McKenney¹. **B.** Compaction of the microtubule lattice measured on speckled microtubules after addition of MAPs. For tau, results can be found in Fig. 4.6C. MAP2c: compaction was $3.0 \pm 1.1\%$ in the envelopes and $0.0 \pm 0.6\%$ outside the envelopes; for MAP4: $0.0 \pm 0.4\%$ along the entire lattice.

4.2.4 LATTICE SPACING GOVERNS MAP COOPERATIVITY IN VIVO

In living cells, microtubules are generally considered to be in the native compacted GDP-state, which leads us to assume that all tau bound to the microtubules is bound cooperatively. Therefore, the presence of cooperatively formed envelopes cannot be distinguished by the characteristic high- and low-density regions of tau as we observe *in vitro*. Using the knowledge that physically lengthening the microtubule lattice induces tau envelope disassembly, we wondered if we could use taxol in living cells to induce disassembly of tau envelopes, thereby revealing the envelope regions.

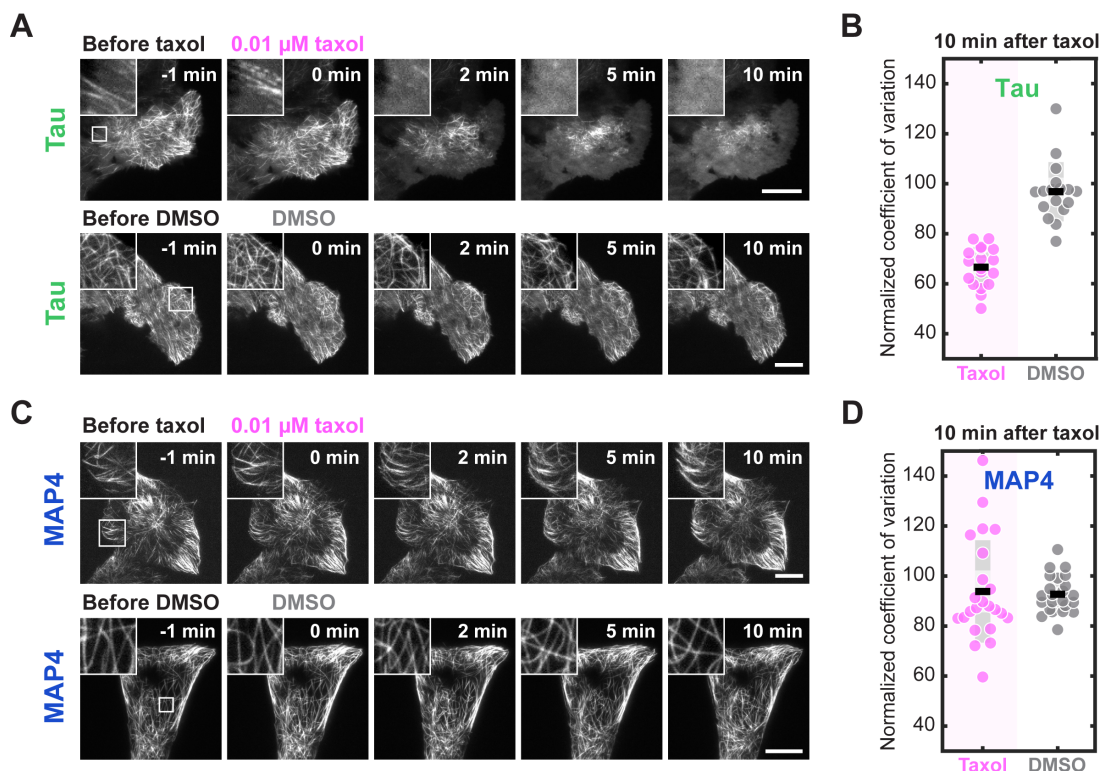


Figure 4.12: Taxol addition to cells induces tau displacement. **A.** Time lapse micrographs of U-2 OS cells expressing eGFP-tau treated with $0.01 \mu\text{M}$ taxol (top panels) or DMSO (bottom panels). Zoom-ins of the indicated region are provided in the top left corner of the original micrograph. **B.** Coefficient of variation (CoV) of eGFP-tau cells 10 minutes after taxol or DMSO treatment, normalized to the coefficient of variation before treatment (at $t=-1$ min). CoV was calculated over the whole cell. Normalized CoV after taxol: 66.6 ± 8.0 , after DMSO treatment: 96.8 ± 11.8 . **C.** Time lapse micrographs of U-2 OS cells expressing eGFP-MAP4 treated with $0.01 \mu\text{M}$ taxol (top panels) or DMSO (bottom panels). Zoom-ins of the indicated regions are provided in the top left corner of the original micrograph. **D.** CoV of eGFP-MAP4 cells 10 minutes after taxol or DMSO treatment, normalized to the coefficient of variation before treatment (at $t=-1$ min). CoV was calculated over the whole cell. Normalized CoV after taxol treatment: 93.8 ± 20.4 , after DMSO treatment: 92.7 ± 7.5 . All scale bars: $10 \mu\text{m}$. All experiments were performed and analyzed by Tereza Humhalova⁷.

Experiments performed by Tereza Humhalova⁷ from the lab of Lenka Libusova⁸ showed that addition of taxol to tau-overexpressing cells induced dissociation of tau from microtubules (see Fig. 4.12A). This dissociation of tau from the microtubules was quantified by the large change in coefficient of variation (CoV) of the tau signal. The CoV is a measure of the variance of the signal and should therefore give an indication of where the tau is located in the cell. When tau is

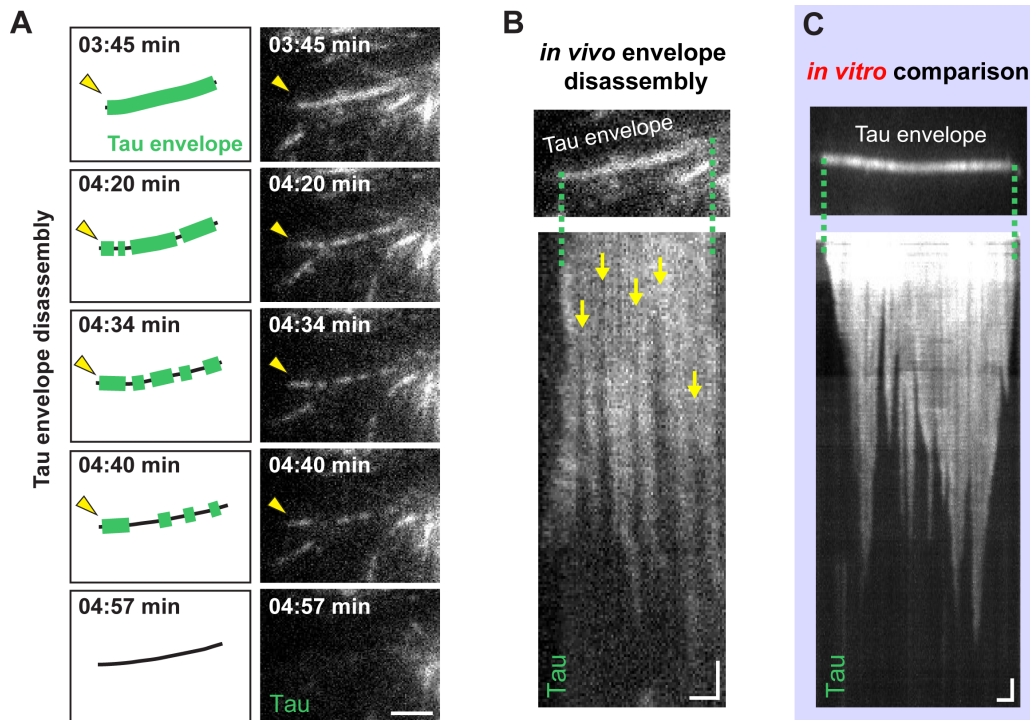


Figure 4.13: Taxol treatment induces tau envelope disassembly in vivo. **A.** Fluorescence micrographs of the eGFP-tau signal on a single microtubule (indicated by yellow arrowheads) after taxol treatment. Sketches of the micrographs (panels next to the original micrographs) indicate the size and locations of the patches of tau signal that appear on the microtubule lattice. Scale bars: 2 μ m. **B.** Fluorescence kymograph of the microtubule presented in **A** showing the fissures (yellow arrows) appearing in the eGFP-tau signal and the disassembly of the tau envelopes from their boundaries. Scale bars: horizontal 1 μ m, vertical 10s. **C.** Fluorescence kymograph of tau envelope disassembly in vitro showing striking resemblance with the in vivo observations in **B**. All experiments were performed by Tereza Humhalova⁷.

bound to the microtubules, we would measure a high CoV (large variance coming from brightly decorated microtubules and dim cytosolic background), when the protein is freely diffusing in the cytosol and not bound to the microtubules we would measure a low CoV. Quantifying the CoV after taxol treatment revealed a significant decrease in the CoV upon taxol treatment, while no decrease in the CoV was observed after DMSO (control) treatment (see Fig. 4.12B). Since non-cooperatively bound tau should not be affected by lattice extension induced by taxol addition, the dissociation of tau from the microtubules after taxol addition suggests that tau was bound cooperatively. Additional control experiments using MAP4-overexpressing cells showed that MAP4 was not affected by taxol addition and the MAP4 density along the microtubules remained unchanged after taxol addition, further quantified by the largely unchanged coefficient of variation (see Fig. 4.12C,D). These findings indicate that tau, but not MAP4, is affected by lattice extension, suggesting that tau is bound cooperatively to microtubules in living cells while MAP4 is not.

Strikingly, when taking a closer look at the manner of tau dissociation after taxol addition, the dissociation greatly resembled tau envelope disassembly as we observe in vitro (see Fig. 4.13A-C). We found that the tau signal that initially was uniform along the microtubule lattice started to display gaps in the signal and the remaining patches of tau signal decreased from their boundaries until all tau had disappeared from the microtubule (see Fig. 4.13A-C). These findings demonstrate the first visualization of tau envelopes in vivo and indicate that native GDP-microtubules in living cells can be fully enclosed by tau envelopes. Additionally, by artificially extending the

microtubule lattices using taxol we can induce tau envelope disassembly.

In summation, the work done for the Nature Chemical Biology paper of 2022 (attached in appendix [A.2](#)) was a great effort that included collaborations between four different labs. The highlights of the work show that tau and MAP2c, but not MAP4, can cooperatively form cohesive envelopes on the microtubule surface. This cooperative binding mode of tau and MAP2c induces a local compaction of the microtubule lattice that is required for envelope formation since reversal of the compaction induces disassembly of tau envelopes both in vitro and in vivo. These findings indicate that the cooperative binding mode of different MAPs can differentially affect the microtubule lattice spacing at specific location along the microtubule lattice, suggesting that MAPs can form distinct domains along the microtubule lattice that can differentially regulate access to the microtubule surface for other microtubule-associated proteins ([Siahaan et al., 2022](#)).

4.3 TAU PHOSPHORYLATION IMPEDES FUNCTIONALITY OF PROTECTIVE TAU ENVELOPES

Our work so far had answered a lot of questions about how tau envelopes regulate other microtubule-associated proteins, the question that still remained was how tau envelopes itself are regulated. Specifically, how does the cell control when an envelope should form and when it should not? When comparing the Nature Cell Biology papers of 2019 that were published back-to-back (Siahaan et al., 2019; Tan et al., 2019), there seemed to be a striking difference in the tau concentrations used for the in vitro TIRF assays. In Siahaan et al., tau concentrations ranged around 20-40 nM tau, whereas in the Tan et al. paper, concentrations ranged around 1-2 nM. It felt quite obvious that the sample preparations and buffer solutions used for the two papers could not attribute to the twenty-fold difference, so what could?

We realized early on that the difference in tau concentrations used for the two papers could be caused by the different expression systems of our two recombinant proteins. In the laboratory of Richard McKenney¹, tau was expressed in bacterial cells, whereas in our lab, tau was expressed in insect cells. The big difference between the used concentrations could therefore be due to post-translational modifications (PTMs), which the bacterial expressed tau would have minimal amounts of, while the insect cell expressed tau would have abundant amounts of. As mentioned in the introduction, tau is subject to many post-translational modification, but the most commonly described PTM of tau is phosphorylation, and (hyper)phosphorylation of tau has often been linked to various pathological downstream effects. We therefore wondered if tau phosphorylation could regulate tau envelope formation, and importantly, if it could influence tau envelope stability and functionality.

For this study, I worked together with Eva Lanska⁹ and Adela Karhanova¹⁰ from the laboratory of Zdenek Lansky² on the in vitro TIRF assays. Additionally, Romana Weisssova¹¹ from the laboratory of Martin Balastik¹² performed experiments in living cells, which will not be discussed in this thesis. The manuscript is currently in review at Nature Chemical Biology, and the manuscript including the materials and methods discussed in the following section can be found in appendix A.3.

4.3.1 TAU PHOSPHORYLATION AFFECTS TAU ENVELOPE FORMATION

To investigate whether tau phosphorylation affects tau envelope formation and stability, we expressed tau in insect cell and dephosphorylated half of the tau sample using an Alkaline phosphatase, while the other half was kept untreated. This yielded two samples with different degrees of phosphorylation that were analyzed using mass spectrometry. The insect cell expressed tau was phosphorylated at many different sites to different degrees (see Fig. 4.14A,B) and will

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¹²M. Balastik: Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic

4.3. TAU PHOSPHORYLATION IMPEDES ENVELOPE INTEGRITY AND FUNCTION

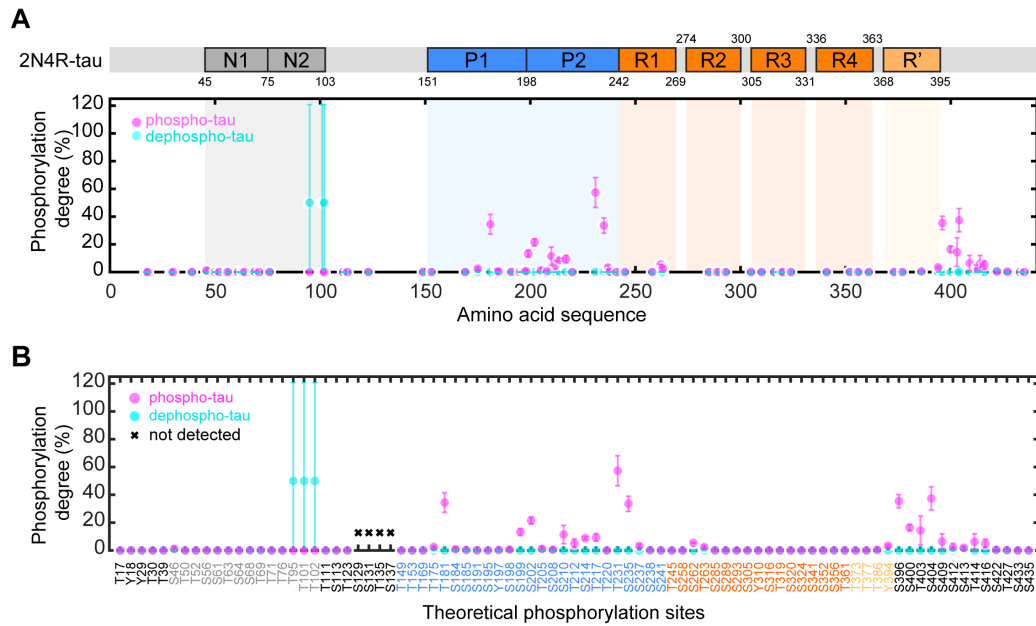


Figure 4.14: Phosphatase treatment reduces the phosphorylation degree of tau. **A.** Mass-spectrometry-determined degree of phosphorylation of phospho-tau (insect cell expressed tau, magenta) and dephospho-tau (phosphatase-treated insect cell expressed tau, cyan). Phosphorylation degree is presented as the mean \pm s.d. and displayed at the location of the phosphorylation site along the amino acid sequence of tau. The domains on the tau sequence are color-coded: N-terminal domains (N1, N2, grey), proline-rich domains (P1, P2, blue), microtubule-binding repeats (R1-R4, orange), and the domain pseudo-repeat (R', light orange). **B.** Phosphorylation degree of individual theoretical phosphorylation sites, same data and color-coding as **A.** If no peptides were detected for a specific phosphorylation sites, the site is marked with a black cross.

from now on be denoted as **phospho-tau**. The insect cell expressed tau dephosphorylated using phosphatase was nearly completely dephosphorylated at all phosphorylation sites after phosphatase treatment (see Fig. 4.14A,B) and will from now on be denoted as **dephospho-tau**. Note that the phospho-tau sample is mostly phosphorylated in the projection domains, and nearly no phosphorylation is found in the microtubule binding repeat domain. When then used our two tau samples and performed our general tau envelope formation assay. At all concentrations measured, we found a lower coverage of tau envelopes on microtubules after addition of phospho-tau compared to dephospho-tau (see Fig. 4.15A,B), indicating that phosphorylated tau has a lower propensity to form envelopes.

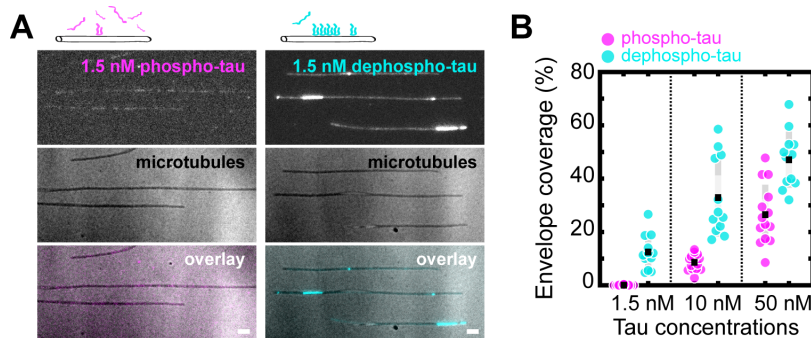


Figure 4.15: Phosphorylated tau less readily forms envelopes. **A.** Multichannel fluorescence micrographs of 1.5 nM phospho-tau (magenta, left), and 1.5 nM dephospho-tau (cyan, right) on MTs (IRM, middle). Scale bars: 2 μ m. **B.** Tau envelope coverage after 3 min incubation for 3 concentrations of phospho-tau (magenta), or dephospho-tau (cyan). All experiments were performed and analyzed by Eva Lanska⁹.

4.3. TAU PHOSPHORYLATION IMPEDES ENVELOPE INTEGRITY AND FUNCTION

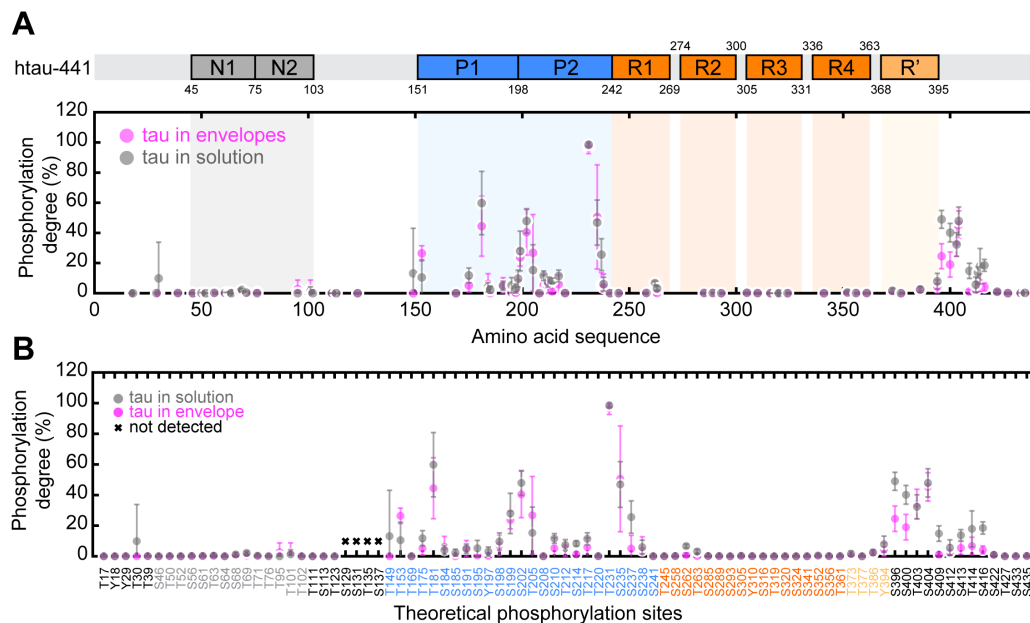


Figure 4.16: Phosphorylated tau participates in envelope formation. **A.** Mass-spectrometry-determined degree of phosphorylation of tau in envelopes (cooperatively binding tau from pellet, magenta) and tau in solution (high turnover tau from supernatant, light magenta). Phosphorylation degree is presented as the mean \pm s.d. and displayed at the location of the phosphorylation site along the amino acid sequence of tau. The domains on the tau sequence are color-coded: N-terminal domains (N1, N2, grey), proline-rich domains (P1, P2, blue), microtubule-binding repeats (R1-R4, orange), and the domain pseudo-repeat (R', light orange). **B.** Phosphorylation degree of individual theoretical phosphorylation sites, same data and color-coding as **A.** If no peptides were detected for a specific phosphorylation sites, the site is marked with a black cross.

We wanted to understand why phosphorylated tau is less capable of forming envelopes. The mass spectrometry analysis of the phospho-tau sample shows a large variation in the phosphorylation degree of the phospho-tau sample, indicating that we have a largely heterogenic sample. Therefore, we wondered if the lowered propensity of phospho-tau to form envelopes comes from the fact that we have less tau "capable" of forming envelopes, and the envelopes are "picking out" the envelope-competent tau molecules (with lower phosphorylation degree) to form the envelopes.

To study whether tau is able to bind cooperatively regardless of its phosphorylation degree we separated "envelope-competent" tau from "envelope-incompetent" tau. We accomplished this by forming phospho-tau envelopes on microtubules in a tube and spinning down the envelope-coated microtubules. By separating the pellet (containing the microtubules and tau envelopes) from the supernatant (containing the tau that did not participate in envelope formation), we now had a sample that (i) contained microtubules coated with the 'envelope-competent' tau (tau in envelopes), and (ii) a sample that contained mostly 'envelope-incompetent' tau that did not participate in the tau envelope formation (tau in solution). We analyzed the two samples using mass spectrometry and found that the envelope-competent tau contained phosphorylated tau with a phosphorylation degree roughly similar to the envelope-incompetent tau (see Fig. 4.16A,B). Overall there seems a slight increase in the phosphorylation degree in the envelope-incompetent tau sample, indicating that the phosphorylation degree influences the propensity of a single tau molecule to participate in envelope formation. However, in general, phosphorylation of tau does not completely inhibit tau from binding cooperatively.

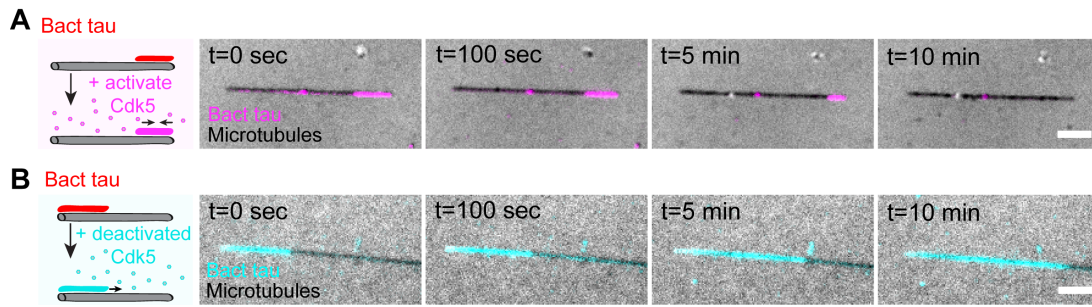


Figure 4.17: Tau phosphorylation destabilizes existing envelopes. **A.** Multichannel fluorescence micrographs of 15 nM Bact-tau (red in schematics) after treatment with active Cdk5 (Bact-tau in magenta). Microtubules (black) imaged using IRM. **B.** Multichannel fluorescence micrographs of 15 nM Bact-tau (red in schematics) after treatment with deactivated Cdk5 (Bact-tau in cyan). Microtubules (black) imaged using IRM. All scale bars: 2 μm . All experiments were performed and analyzed by Adela Karhanova¹⁰.

We next asked if tau phosphorylation can destabilize existing non-phosphorylated tau envelopes. Therefore, we prepared envelopes using bacterial-expressed tau which possesses a low phosphorylation degree and added active kinase (Cdk5/p35) to the channel while keeping tau in solution (see Fig. 4.17A). Within seconds after addition of the active Cdk5, we observed the disassembly of the tau envelopes, indicating that the kinase was actively phosphorylating the tau in the channel and/or in the envelope which reduced the propensity of tau to form envelopes (see Fig. 4.17B). In a control experiment we added deactivated Cdk5 to the bacterial tau envelopes and observed no disassembly of the envelopes. By contrast, we even observed growth of the tau envelope in the presence of deactivated Cdk5 (see Fig. 4.17A,B). Combined, these findings show that phosphorylation of tau can destabilize existing non-phosphorylated tau envelopes.

4.3.2 TAU PHOSPHORYLATION REDUCES ENVELOPE INTEGRITY

Knowing that phosphorylated tau molecules are capable of participating in tau envelope formation we wanted to understand the mechanism by which phosphorylation decreases the ability of tau to form envelopes. We hypothesized that the lowered propensity of phosphorylated tau to form envelopes can be explained by (a combination of) two mechanisms (i) a lowered affinity of phosphorylated tau to the microtubules (resulting in less binding and therefore lower envelope coverage); (ii) decreased cohesiveness of the phosphorylated envelopes, which would most likely be a result of reduced tau-tau interactions. We therefore studied the affinity of phospho- and dephospho-tau to microtubules in two different conditions: on GMPCPP-microtubules (see Fig. 4.18A), where tau cannot form envelopes due to the irreversible extension of the microtubule lattice (Tan et al., 2019; Siahaan et al., 2022), and on GDP-microtubules (see Fig. 4.18B,C), which have a compacted lattice and are therefore the preferential lattice for the cooperative binding mode of tau (Siahaan et al., 2022). We found that in all conditions, except for saturating conditions on GDP-microtubules, phospho-tau binds with lower densities on the microtubule lattice compared to dephospho-tau. Confirming previous findings (Lindwall and Cole, 1984; Biernat et al., 1993; Schneider et al., 1999; Cho and Johnson, 2003), and showing that (i) phosphorylated tau has a lowered affinity to microtubules compared to non-phosphorylated tau (see Fig. 4.18).

To test whether aside from the tau-microtubule interaction, tau phosphorylation additionally affects (ii) the tau-tau interaction, we formed envelopes using equal concentrations of phospho- and dephospho-tau (8 nM each) with different fluorescent tags so we can distinguish the popu-

4.3. TAU PHOSPHORYLATION IMPEDES ENVELOPE INTEGRITY AND FUNCTION

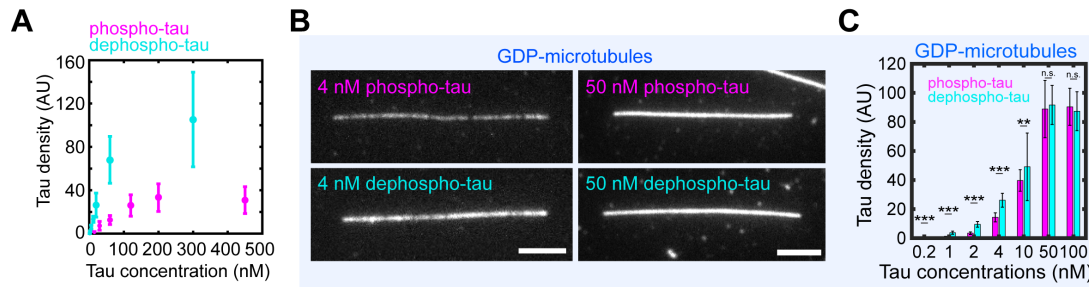


Figure 4.18: Phosphorylation decreases the affinity of tau for microtubules. **A.** Density of phospho-tau (magenta) or dephospho-tau (cyan) measured on GMPCPP-microtubules as a function of the concentration of phospho-tau or dephospho-tau in solution (phospho-tau: n=12 experiments; dephospho-tau: n=3 experiments). **B.** Fluorescence micrographs of 4 or 50 nM phospho-tau (magenta, top) or dephospho-tau (cyan, bottom) on glycerol-stabilized GDP-microtubules after 1 min incubation. Scale bars: 2 μ m. **C.** Concentration (nM) of phospho-tau (magenta) and dephospho-tau (cyan) plotted against the tau density (AU) on GDP-microtubules. Experiments performed and analyzed by Eva Lanska⁹ (4.18A) and Adela Karhanova¹⁰ (4.18B,C).

lations from one another. In agreement with previous findings, we observed signal of the phosphorylated tau sample within the envelope region, indicating that, indeed, phosphorylated tau is participating in tau envelope formation. Interestingly, when analyzing the ratio of phospho-tau inside the envelope region compared to outside the envelope region, we found that phosphorylated tau aside from a lowered affinity to the microtubule lattice (outside of the envelope region), had an even lower affinity to the envelope region, resulting in a lower ratio of phospho-tau in the envelopes compared to outside the envelopes (see Fig. 4.19A,B).

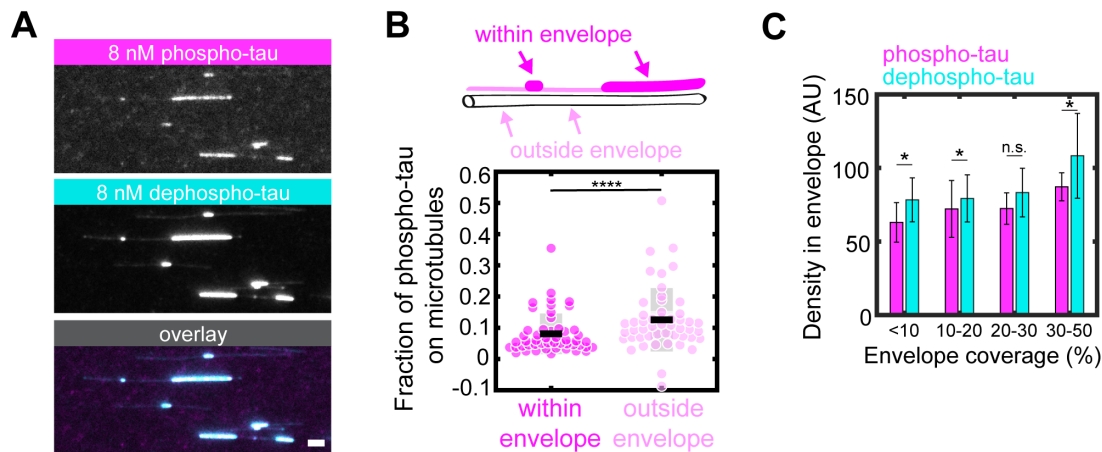


Figure 4.19: Tau phosphorylation affects tau-tau interactions. **A.** Multichannel fluorescence micrographs of 8 nM phospho-tau-mCherry (magenta, top panel) and 8 nM dephospho-tau-GFP (cyan, middle panel) incubated simultaneously (overlay, bottom panel) and imaged after 3 min incubation. Scale bar: 2 μ m. **B.** Fraction of the density of phospho-tau compared to the total density of all tau - within tau envelope region (magenta), or outside of tau envelope region (light pink). Schematic of regions considered as "within envelope" and "outside envelopes" above the plot. **C.** Density of tau within the envelope region for a given coverage range. All experiments were performed and analyzed by Adela Karhanova¹⁰ (4.19A,B) and Eva Lanska⁹ (4.19C).

Additionally, we formed envelopes on taxol-stabilized microtubules using either phospho- or dephospho-tau and studied the density of tau within the envelopes. Interestingly, we found that, similar to the GMPCPP- and GDP-microtubules (see Fig. 4.18), the density of tau within the envelopes on taxol-stabilized microtubules was likewise lower when envelopes were prepared from phospho-tau compared to dephospho-tau (see Fig. 4.19C), indicating that phosphorylated tau

4.3. TAU PHOSPHORYLATION IMPEDES ENVELOPE INTEGRITY AND FUNCTION

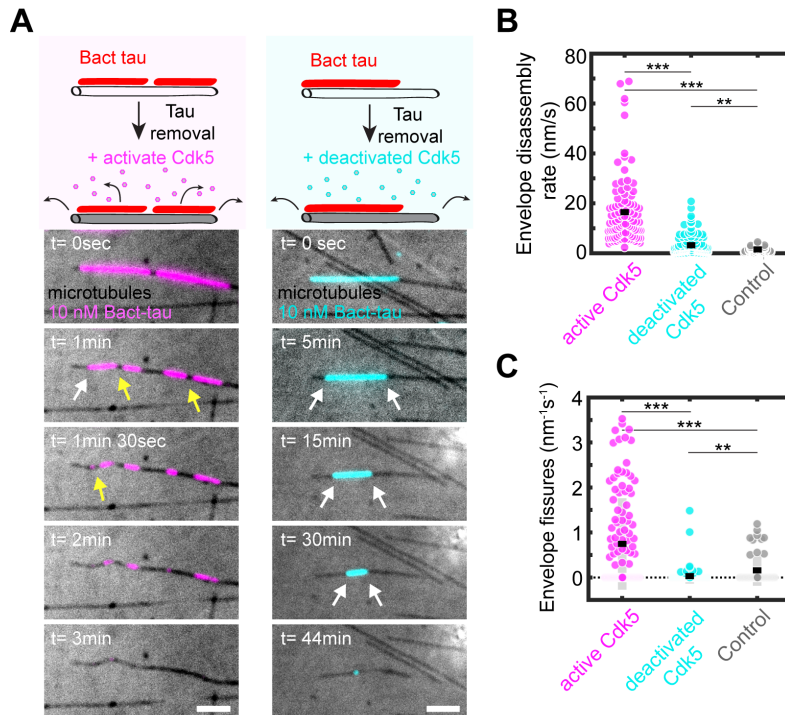


Figure 4.20: Tau phosphorylation compromises envelope stability. **A.** Fluorescence micrographs of removal of 10 nM Bact-tau (magenta or cyan) on microtubules (black) in presence of active Cdk5 (left panels) or deactivated Cdk5 (right panels). Envelopes disassemble from the boundaries (white arrows) with occasional fissures within envelope boundaries (yellow arrows). Note the different experimental timescales. Scale bars: 2 μm . **B.** Envelope disassembly rate after tau removal in presence of active Cdk5 (magenta), deactivated Cdk5 (cyan), or in absence of Cdk5 (control, grey). **C.** Number of fissure events within the envelope boundaries in presence of Cdk5 (magenta), deactivated Cdk5 (cyan), or in absence of kinase (grey). All experiments were performed and analyzed by Adela Karhanova¹⁰.

envelopes are prepared from a lower number of tau molecules, suggesting a less robust envelope structures which could be a result of (ii) weaker tau-tau interactions due to tau phosphorylation.

To further test if tau phosphorylation leads to weaker tau-tau interactions, we hypothesized that the stability and cohesiveness of the envelope structure is compromised when envelopes are prepared from phosphorylated tau. To test this hypothesis, we prepared tau envelopes from bacterial tau (with low phosphorylation degree) and studied the disassembly of the envelopes by removing tau from solution either in presence of active Cdk5 kinase; or in presence of deactivated Cdk5 kinase; or in absence of any kinase (control). We found that the disassembly rate of the tau envelopes was significantly faster when tau was removed in presence of active Cdk5 compared to the deactivated Cdk5 or the control experiments (see Fig. 4.20A,B), which indicates that the Cdk5 is actively phosphorylating tau in the channel. Interestingly, we also observed significantly more fission events within the boundaries of the envelopes when the disassembly was performed in presence of active Cdk5 (see Fig. 4.20A,C). These findings indicate that tau phosphorylation causes envelope **destabilization** and the increase in the number of fission events suggests that tau phosphorylation compromises the integrity and cohesiveness of the envelope structure. Combined, these findings suggest that our hypothesis is correct and tau phosphorylation does not only affect (i) the tau-microtubule interaction, but additionally affects (ii) the cooperative binding mode of tau which could be a result of weakened tau-tau interactions and causes a compromised structure of the phosphorylated tau envelopes.

4.3.3 TAU PHOSPHORYLATION IMPEDES ENVELOPE FUNCTIONALITY

Now that we know that tau phosphorylation negatively affects tau envelope formation and stability, we wanted to know if tau phosphorylation could, importantly, affect the functionality of the

4.3. TAU PHOSPHORYLATION IMPEDES ENVELOPE INTEGRITY AND FUNCTION

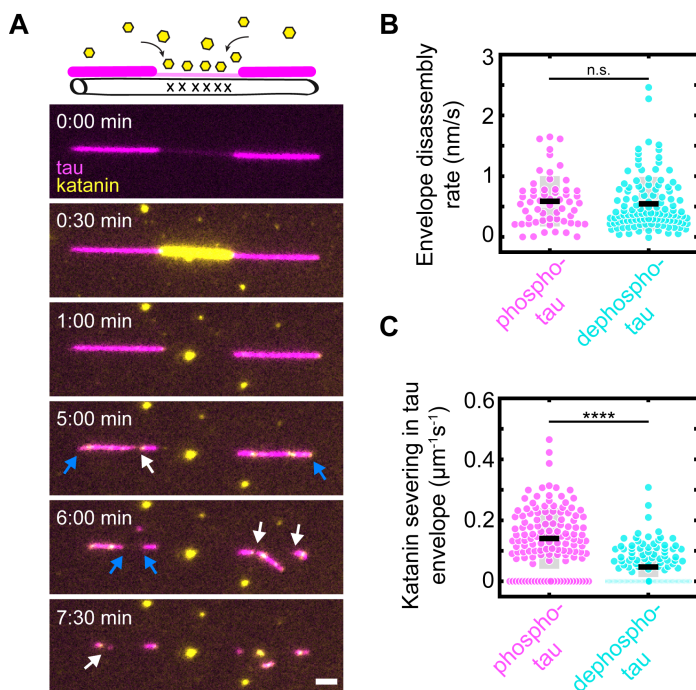


Figure 4.21: Tau phosphorylation decreases protective functionality of envelopes. **A.** Schematics and multichannel fluorescence micrographs showing katanin-mediated severing of a microtubule region not covered by a tau envelope (panel 2) and subsequently the disassembly of the microtubule regions protected by a tau envelope (panels 3-6). Katanin severing leading to disassembly of envelope-covered MT regions from the boundaries is indicated by blue arrows; occasional katanin severing within a tau envelope is indicated by white arrows. Scale bar: 2 μm . **B.** Envelope disassembly rate from the boundaries due to katanin severing of phospho-tau (magenta) or dephospho-tau envelopes (cyan). **C.** Katanin severing events within tau envelope boundaries in phospho-tau envelopes (magenta) and dephospho-tau envelopes (cyan). All experiments were performed and analyzed in collaboration with Adela Karhanova¹⁰.

tau envelopes. As we had demonstrated previously, tau envelopes protect the microtubule lattice against katanin-mediated severing (Siahaan et al., 2019) and we were interested to see if this protective functionality of tau would be affected by tau phosphorylation. To test the protective functionality of tau envelopes, we added katanin to pre-formed envelopes (either prepared from phospho- or from dephospho-tau) and studied the katanin-induced disassembly of the tau envelopes. We found that the rate of disassembly from the envelope boundaries seemed unaffected by tau phosphorylation (see Fig. 4.21A,B). However, katanin was able to penetrate the tau envelope structure more readily when envelopes were prepared with phosphorylated tau compared to dephosphorylated tau, and therefore an increased number of severing events was observed within the boundaries of the phospho-tau envelopes compared to dephospho-tau envelopes (see Fig. 4.21C). This data demonstrates that tau phosphorylation decreases the protective functionality of tau envelopes, which was substantiated by additional work from Romana Weissova¹¹ from the lab of Martin Balastik¹² in living cells (not shown in this thesis). Combining these findings with the lower density of tau found for the phosphorylated tau envelopes (see Fig. 4.19C), and the observation that we get more fission events in disassembling tau envelopes in presence of active Cdk5 (see Fig. 4.20C), it is plausible that the increased katanin activity within the boundaries of the tau envelopes is caused by the compromised and more gap-prone structure of the phosphorylated tau envelopes.

In summation, the work done for the tau phosphorylation story (attached in appendix A.3) was a great effort that was led by the laboratories of Zdenek Lansky² and Martin Balastik¹², with experiments performed by Vojtech Dostal¹³ (not discussed in this thesis) from the laboratory of Lenka Libusova⁸, and additional provided support by Veronique Henriot¹⁴ from the laboratory of Carsten Janke¹⁵. The highlights of the work show that tau phosphorylation impedes tau envelope formation and stability and most strikingly, the protective functionality of tau envelopes is nega-

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tively affected by tau phosphorylation, evidenced by decreased protection of phosphorylated tau envelopes against katanin-mediated severing (Siahaan et al. (in review)). Importantly, microtubule instability linked to tau hyperphosphorylation is often attributed to neurodegeneration, however, the exact mechanism by which this happened was never substantiated with additional results. Our findings that tau phosphorylation does not only cause envelope disassembly but also decreases the functionality of tau envelopes can explain how microtubule instability in neurodegeneration can be directly linked to tau phosphorylation, even before tau visibly leaves the microtubules.

Tau is a microtubule-associated protein that is preferentially found in the neuronal axons. In neurodegenerative diseases, tau malfunction and its displacement from axonal microtubules is often correlated with axonal degeneration (Kneynsberg et al., 2017). Tau protects microtubules from microtubule-severing enzymes such as katanin (Qiang et al., 2006) and can regulate motor-based transport on the microtubule lattice (Vershinin et al., 2007; Dixit et al., 2008), however, how tau carries out these regulatory microtubule-related functions remains ill-defined. In order to understand why tau displacement and malfunction is so catastrophic for healthy neurons, we have to first understand how tau carries out its regulatory functions in physiological conditions. This thesis describes the efforts and outcomes from the laboratory of Zdenek Lansky², in collaboration with many other laboratories around the world, to describe and study the fundamental interaction of tau binding to microtubules and the mechanism by which tau carries out its regulatory roles. Since 2019, this work has led to two major publications (Siahaan et al. (2019, 2022), attached in appendices A.1,A.2), and one manuscript that is currently in review at Nature Chemical Biology (Siahaan et al. (in review), attached in appendix A.3).

In collaboration with the laboratory of Richard McKenney¹, we discovered that tau can coexist on the microtubule lattice in two distinct phases: either as (i) single tau molecules rapidly diffusing along the microtubule lattice, and as (ii) cooperatively bound tau that forms a cohesive and protective coat around the microtubule lattice that we termed a tau "island", or tau "envelope" (Siahaan et al., 2019; Tan et al., 2019; Siahaan et al., 2022). Tau envelopes can differentially regulate the movement and functionality of other microtubule-associated proteins by selectively allowing access to the microtubule surface. This proposes an interesting functionality of the tau envelopes, where cooperative binding of tau can locally restrict cargo transport in a specific direction or by a certain type of molecular motor and can protect microtubules against the severing activity of microtubule-severing enzymes (Siahaan et al., 2019; Tan et al., 2019). Since particular sections of, for instance, nerve cells or neurons require different functions, these tau envelopes could play an important part in the proper functioning of these cells.

The ability of tau to bind cooperatively and form envelopes, combined with the distinct functionality that the cooperative binding-mode of tau has, explains previously contradicting or ill-explained findings. We therefore believe that the discovery of tau envelopes is a breakthrough in understanding the functionality of tau in both health and disease. For example, early studies showing the inhibition of kinesin by tau binding, found that tau's ability to inhibit kinesin was lost when performing the experiments on GMPCPP-stabilized microtubules (McVicker et al., 2014). Previously, this loss of inhibition was suggested to be due to different modes of interaction of either tau and/or kinesin on GMPCPP-stabilized microtubules, however, the exact mechanism could only be speculated. Our findings that tau does not form envelopes on GMPCPP-stabilized

microtubules due to the permanently extended state of the microtubule lattice, explains this previously mystifying finding. Additionally, the finding that tau dissociates from the microtubule lattice after addition of taxol (Samsonov et al., 2004; Ettinger et al., 2016), was an unexpected finding that was attributed to the sensitivity of tau to the MT geometry. Our findings confirm and explain that the artificial lattice extension induced by taxol disrupts the cooperative binding mode of tau and results in the disassembly of tau envelopes, thereby leading to the displacement of tau from microtubules as observed in these studies.

In order to understand the mechanism of tau envelope formation on a single molecule scale, we started mapping the regions of tau responsible for tau envelope formation. In our lab, we tested Δ N-tau (aa 242-441), which is a tau construct that lacks the N-terminus, and found that even at elevated Δ N-tau concentrations, this construct was not able to form envelopes, indicating that at least part of the N-terminus is necessary for tau to bind cooperatively and form envelopes (Siahaan et al., 2019). The lab of Richard McKenney¹ tried several other constructs including Δ C- Δ N-tau (MTBD alone), N-terminus alone, C-terminus alone, and the C- and N-terminus linked together while lacking the microtubule-binding domain ("bonsai" construct) (Tan et al., 2019). They found that none of the constructs showed a clear enrichment nor exclusion within enveloped regions prepared with full-length tau, while the isolated C-terminus and the bonsai construct showed a weak enrichment within the envelopes (Tan et al., 2019). Additionally, they studied the "mini-tau" construct (aa 151-395) which comprises the proline-rich domain, MTBD, and the additional pseudo-repeat domain (R') of tau. Mini-tau was able to form envelopes on its own and was further shown to have increased binding within tau envelopes, similar to full-length 2N4R-tau. Truncating the pseudo-repeat domain from mini-tau abolishes the cooperation within the envelopes (Tan et al., 2019). Combined, this data indicates that the pseudo-repeat domain is required for tau envelope formation, suggesting some type of tau-tau interaction between the projection domains of neighboring tau molecules. However, it could also point to an interaction of the projection domains with the microtubule. This additionally explains why phosphorylation within the projection domains can affect the cooperative binding of tau (Siahaan et al. (in review)).

The ability of "mini-tau" to form bind cooperatively gives an indication of the minimal sequence of tau that is necessary for tau envelope formation (Tan et al., 2019). This poses an interesting role for a specific PTM of tau: tau **truncation**. Earlier studies have shown that truncated tau has been found to initiate or accelerate the development of tangle pathology in neurodegenerative diseases (reviewed in Cotman et al. (2005)). However, the most studied truncation of tau is the caspase-cleavage at Asp421, which was found to seed filamentous aggregates in vitro, and induce toxic gains-of-function of the cleaved tau (Gamblin et al., 2003; Conze et al., 2022). The caspase-cleaved tau (aa 1-421), would according to the mini-tau findings still be able to form envelopes. Nonetheless, it will be interesting to study the propensity of the cleaved tau form envelopes, since a reduction in cooperative binding (and tau-tau interactions) could explain the pathological effects of caspase-cleavage of tau.

Using speckled microtubules, cooperative binding of tau to microtubules was measured to induce a 3.1% lattice compaction, and cooperative binding of MAP2c to microtubules induced a 3.0% lattice compaction (Siahaan et al., 2022). The lattice compaction by tau and MAP2c, and the absence of lattice compaction by MAP4, was further confirmed by work of our collaborator from Cambridge, Samuel E. Lacey¹, who performed cryo-EM analysis of the tubulin spacing

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within the lattice. He found consistent results with our findings where both tau and MAP2c binding resulted in $\pm 1\text{\AA}$ lattice compaction (around 2.4%) between tubulin monomers, whereas after addition of MAP4, no lattice compaction was measured (Siahaan et al., 2022). Alushin et al. showed that compaction of the microtubule lattice upon GTP hydrolysis induced a 2.4% compaction ($\pm 1\text{\AA}$ between tubulin monomers) (Alushin et al., 2014), which is strikingly similar to the measured compaction in our cryo-EM data and comparable to the measured compaction in our TIRF assay, indicating that our analysis techniques using FIESTA tracking software could be a valid method to measure microtubule lattice compaction or extension without the use of cryo-EM.

In our studies, we found that compaction of the microtubule lattice structure is one of the mechanisms by which tau binds cooperatively to the microtubule surface. However, tau molecules lose their ability to form tau envelopes when lacking (part of the) projection domains (Siahaan et al., 2019; Tan et al., 2019). This could either be caused by an inability to compact the microtubule lattice, or the inability of tau to bind cooperatively on microtubules, two mechanisms that are likely connected. Considering that tau-tau interactions underlie tau envelope formation, this suggests that the N-terminal region (mostly negatively charged) and the proline-rich domain (mostly positively charged), interact with the C-terminal tail (mix of negative and positive charges) of the neighboring tau molecule, which is not entirely unlikely based on the charge distributions of these regions. This begs the questions whether the different N-terminal inserts can affect tau cooperativity. Additionally, PTMs such as phosphorylation within the N-terminal insert region can greatly affect how different isoforms are regulated by effectors like kinases and phosphatases. In our spin-down experiment, we separate tau that is competent to form envelopes from tau that is incompetent to form envelopes. Strikingly, when taking a closer look at the mass-spectrometry defined phosphorylation degree, it appears that there are two phosphorylation sites within the N-terminal insert "N2" (which is not present in 0N- or 1N-isoforms), Thr95 and Thr101, where nearly all tau found phosphorylated at these sites end up in the pool of tau competent to form envelopes (Siahaan et al. (in review)). This suggests that phosphorylation of tau within the N2 insert can increase the affinity of tau to form envelopes. Considering that the N-terminal region of tau is already predominantly negatively charged, increasing that negative charge could be a factor in increasing tau-tau interactions. It would be interesting to study how PTMs of tau affect the distinct tau isoforms and, specifically, whether the number of N-terminal inserts are differentially regulated by tau PTMs such as phosphorylation.

The ability of tau to recognize the different nucleotide states of microtubules has been shown in a multitude of studies (Bechstedt and Brouhard, 2012; Ettinger et al., 2016; Duan et al., 2017; Castle et al., 2020). However, a clear explanation for this behavior remained elusive. Our finding that tau cooperativity is governed by the microtubule lattice spacing explains the preference of tau to bind the already compacted GDP-lattice. This additionally explains the increased localization of tau at regions on the microtubule with higher curvature as found in vivo (Samsonov et al., 2004; Ettinger et al., 2016). The high curvature, or bend, of the microtubule lattice gives rise to a lattice structure that can be assumed to be partly compacted on the inside of the bend where tau would prefer to bind. However, bending of the microtubule lattice would not only induce a partial compaction on the inside of the bend, the outside of the bend will adopt a partly extended conformation, giving rise to a microtubule structure mostly avoided by tau. This begs the question what the binding state of tau is within these regions of high curvature. Can we suggest that at the location of higher curvature, tau is only cooperatively bound on half of the lattice? And is the desire of tau to compact the microtubule lattice strong enough to straighten out these regions

of high curvature? In vitro, we often observed that cooperative binding of tau concomitantly related to straightening out of bends in the microtubule lattice (Siahaan et al., 2022). Therefore, it appears that a concentration of tau above the nucleation concentration for tau envelopes would be sufficient to induce microtubule lattice straightening. This leads us to wonder whether the high concentration of tau in axons contributes to the relatively straight shape of axonal MTs, that helps to prevent tangling of microtubules. It would be interesting to see whether compaction of the microtubule lattice is not facilitated by MAPs that are present in compartments of the neurons where MTs do not exhibit a preferably straight configuration, such as in the dendrites. MAP2 is a protein that is preferentially found in the dendrites (Meichsner et al., 1993), so our finding that MAP2c compacts the microtubule lattice would with this hypothesis be counter-intuitive (Siahaan et al., 2022). However, there are studies that suggest that the specific isoform that we worked with, MAP2c, is the only isoform of MAP2 that is located in the axons, where the rest of the isoforms is located solely in the dendrites (Tucker et al., 1988; Meichsner et al., 1993). Therefore, it could still be possible that not all MAP2 isoforms have similar functionality and in light of their localization could differentially affect the microtubule lattice structure.

Phosphorylation of tau greatly reduces tau's affinity to the microtubule lattice (Lindwall and Cole, 1984; Biernat et al., 1993; Schneider et al., 1999; Cho and Johnson, 2003) and further decreases the propensity of tau to form envelopes (Siahaan et al. (in review)). The N-terminal region was discussed above, but how are the other regions affected by tau phosphorylation? The proline-rich domain is the region that is most densely occupied with theoretical phosphorylation sites and it is therefore not surprising that this region showed the highest phosphorylation degree in our insect cell expressed tau sample. Considering the individual phosphorylation sites, it appears that there are no specific sites that completely abolish the ability of tau to form envelopes. However, the overall trend remains that phosphorylation nearly always decreases the ability of tau to bind cooperatively. The same trend is seen when considering the individual phosphorylation sites within the C-terminal tail, where the difference appears to be stronger. Within the microtubule-binding repeats we found nearly no phosphorylation, however, whenever there is a minor phosphorylation degree (i.e. at Ser262 and Thr263), the phosphorylated variant of tau appears to always end up in the pool of tau incompetent to form envelopes. This finding is rather unsurprising since it is expected that phosphorylation of tau within the microtubule-binding region greatly decreases the affinity of tau to microtubules, and these tau molecules are therefore much less likely to end up forming tau envelopes solely due to a decreased affinity to the microtubule surface. However, the decreased propensity of tau to form envelopes based on phosphorylation within regions other than the microtubule-binding domain seem to point towards a decreased tau-tau interaction, rather than decreased affinity to the microtubule surface. In our setup we are unable to see the direct effect of specific phosphorylation sites, and the heterogeneity of the samples and multitude of phosphorylation sites makes the data harder to interpret. However, the very low phosphorylation state within the microtubule binding repeats indicates that phosphorylation within the flanking regions is enough to not only decrease tau-tau interaction, but also lower the affinity to the microtubule surface.

Studying the effects of tau phosphorylation can be done using our in vitro TIRF assay, however, to pinpoint single phosphorylation sites to the loss of specific functions of tau, we need to use a different setup. In our most recent work, we used mass spectrometry to map the individual phosphorylation sites and study their phosphorylation degree (Siahaan et al. (in review)). This technique greatly improved our ability to understand our findings and we believe this technique will prove to be extremely useful in future work. Using different kinases or phospho-mimicking

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constructs of tau, we can use our spin-down assay to separate envelope-competent tau from envelope-incompetent tau and combine it with mass spectrometry analysis to directly assign specific phosphorylation sites to a decreased or increased ability of tau to form envelopes and to an enhanced or decreased functionality of the envelopes.

Phosphorylation of tau was shown to negatively affect tau envelope formation (Siahaan et al. (in review)). Nonetheless, our mass spectrometry analysis clearly indicates that phosphorylation does not completely inhibit tau from binding cooperatively. Therefore, independent of the phosphorylation state, single tau molecules can cooperatively bind on the microtubule lattice and participate in envelope formation. Additionally, envelopes prepared from phosphorylated tau were shown to have a decreased protective functionality against microtubule severing enzyme katanin. Considering that tau envelopes formed by phosphorylated tau had a lower density compared to envelopes formed by dephosphorylated tau, we can assume that phosphorylated tau envelopes have a more compromised (gap-prone) structure. This was further substantiated by the increased appearance of gaps, or fissures, within the tau envelope boundaries in presence of an active kinase. To deem phosphorylated tau envelopes as more gap-prone structures would explain why severing enzymes such as katanin are more likely to cut the microtubule lattice within the boundaries of the tau envelope. Therefore, the negative effects of tau phosphorylation appears to present an exponential rather than a linear deleterious effect. Additionally, these findings indicate that the negative effects of tau phosphorylation occur even before tau can visibly be seen leaving the microtubule lattice, which could explain deleterious effects of tau phosphorylation that can not be explained by a visible dissociation of tau from the microtubules.

Of all the PTMs of tau, tau phosphorylation is most often linked to the pathological effects in neurodegenerative diseases. However, aggregated tau from AD brains was additionally found to be increasingly acetylated and ubiquitinated, especially within the microtubule-binding repeats (Wesseling et al., 2020). This suggests that aside from phosphorylation, other tau PTMs could be involved in neurodegeneration as well. Tau acetylation has been found to inhibit tau functionality by impairing tau-MT interaction, inhibit clearance of tau by chaperone-mediated autophagy, and promote pathological tau aggregation (Min et al., 2010; Cohen et al., 2011; Caballero et al., 2021). Ubiquitination of tau has been implicated with neurodegenerative diseases due to its increase in tau aggregation and its impact on tau degradation (Petrucci et al., 2004). And as discussed above, tau truncation is linked to aggregation of tau into pathological filaments (Gamblin et al., 2003). Nonetheless, the exact mechanism by which each tau PTM influences the structure and function of tau, combined with the possible interplay between the PTMs, creates a complex system to unravel. Nonetheless, it would be of highly informative to investigate the effects of other PTMs on the propensity of tau to form envelopes, and explore how these PTMs contribute to the elusive early stages of neurodegeneration.

The first question we often get asked during talks or seminars is whether these tau envelopes exist in living cells. A valid question that in the first years we had no positive answer to even though we were convinced we were not looking at an artifact of working with an in vitro system. Tereza Humhalova⁷ removed a lot of our worries by visualizing the tau envelopes in vivo for the first time (Siahaan et al., 2022), however the lingering concern remained whether it could somehow be an artifact of taxol binding. Therefore, additional work of Romana Weissova¹¹ that showed tau envelope assembly in living cells (Siahaan et al. (in review)), proved that our hypotheses are correct and tau envelopes form in living cells. The efforts of Tereza Humhalova⁷ provides an assay that can be used to visualize tau envelopes by inducing their **disassembly**,

while the work of Romana Weissova¹¹ gives us a method to visualize the **assembly** and growth of tau envelopes. Both works therefore greatly complement each other and will be invaluable for our future research on tau envelopes.

As microtubules in living cells are in the GDP-like compacted state, and we have confirmed that tau can bind cooperatively to microtubules in living cells, it is reasonable to assume that all tau bound to microtubules in living cells is bound cooperatively. When observing tau on microtubules in living cells there is no easy way to discern cooperatively bound tau from non-cooperatively bound tau, which could be the reason that tau envelopes, and the cooperative binding mode of tau, had not been observed previously in live cell experiments. As discussed above, we found two distinct ways to visualize the presence of tau envelopes in cells, and both are done by triggering disassembly of tau envelopes, either by a mild pH treatment (Siahaan et al. (in review)) or by extension of the lattice using taxol (Siahaan et al., 2022). It would be interesting to find a method how to visualize tau envelopes without having to trigger envelope disassembly. This would be greatly interesting especially when working with neurons, where the presence of tau envelopes has not (yet) been observed. Knowing that the residence time of tau within envelopes in vitro is highly increased when tau is bound cooperatively compared to non-cooperatively (Siahaan et al., 2019; Tan et al., 2019), suggests that the fluorescence recovery after photobleaching (FRAP) of the tau signal could differ between the two distinct binding modes. Additionally, the potentially closer proximity of single tau molecules within a tau envelope compared to outside an envelope might be inducing fluorescence resonance energy transfer (FRET) between neighboring tau molecules, which can then be used as a marker for tau envelope regions. Other markers for tau envelope regions would be microtubule-associated proteins, enzymes, or biomolecules that binds solely to enveloped regions, or solely to regions outside of the envelopes. Prime candidates would be prolyl-isomerases, kinases, or phosphatases that are known to interact with tau and could, like tau, have a higher residence time or distinct functionality or binding mode within tau envelopes.

Near-atomic resolution cryo-EM experiments showed that tau molecules bind along the protofilaments of microtubules (Kellogg et al., 2018), where a single microtubule-binding repeat of tau spans one α -tubulin and two halves of a β -tubulin at either end. The fact that a single tau molecule covers multiple inter-tubulin dimer spacings along the protofilament, could explain how tau can govern the spacing of tubulin along the microtubule lattice (Siahaan et al., 2022). Diffusive tau would not bind the microtubule long and strong enough to change the spacing, but cooperatively bound tau has a much higher residence time on the microtubule lattice, and importantly, tau molecules within an envelope are static (Siahaan et al., 2019; Tan et al., 2019). Therefore, the manner of tau binding to the microtubule lattice within an envelope can explain how tau is able to conform the lattice to its ideal structure. This compaction of the microtubule lattice by tau molecules is most likely restricted to a single protofilaments. However, compaction of the microtubule lattice along one protofilament will greatly influence neighboring protofilaments, likely resulting in strain within the microtubule lattice that would induce a compaction of the neighboring protofilaments as well, thereby increasing the affinity for new tau molecules to bind on the neighboring protofilaments. This would indicate that tau envelopes nucleate from small islands of tau on the microtubule surface that do not fully wrap around the entire microtubule lattice, however, a **through-the-lattice cooperativity** likely results in the tau island to grow not only along the protofilaments but perhaps with even greater affinity towards neighboring protofilaments, thereby enclosing the entire microtubule lattice and "enveloping" it with a cooperatively formed tau layer. It would be interesting to study how the seam of the microtubule,

often considered a weak link in the microtubule lattice due to the misalignment of the protofilaments, affects this through-the-lattice cooperativity.

How do single tau molecules bind to the microtubule lattice when they bind cooperatively? The findings that removal of the projection domains abolishes the ability of tau to bind cooperatively indicates that the cooperativity happens (partly) through the projection domains of tau. This suggests that neighboring tau molecules within the envelope "hold hands", meaning that the N-terminal projection domain of one tau molecule connects or somehow interacts with the C-terminal projection domain of the next tau molecule. We feel that this connection might be a requirement for tau to displace taxol from the microtubule lattice, thereby compacting the lattice. Such a close proximity of the neighboring projection domains has a high potential to show an intermolecular FRET signal. However, this is the exact opposite result from what Di Primio et al. found in 2017 when performing FRET experiments in cells using tau proteins labeled at the N- and C-termini with fluorophores, leading them to propose a paperclip-like conformation for tau bound to microtubules (Di Primio et al., 2017). However, upon closely reading their work, we noticed that a fluorescent taxane was used in these experiments to visualize the microtubules. As we now know that taxanes extend the microtubule lattice and disrupts tau envelope formation (Siahaan et al., 2022), we can assume that the binding of tau to microtubules in these experiments was non-cooperative. Therefore, it would be highly informative to repeat the same experiments in the absence of factors that can extend the microtubule lattice. Additionally, performing these experiments both in presence and in absence of taxanes could provide us with more information about the differences between the cooperative and non-cooperative binding modes of tau.

Kinesin-1 motor heads bind at the intratubulin–dimer interface between the α - and β -tubulin (Shigematsu et al., 2018), and this binding site overlaps with a weak binding site for tau (Kellogg et al., 2018). The overlap in binding was hypothesized to contribute to the inhibition of kinesin-1 motility in tau-rich areas, describing tau as a "roadblock" on the microtubule lattice. However, kinesin-1 has been shown to be recruited to microtubules decorated with MAP7, despite kinesin-1 and MAP7 competing for the same binding site (Ferro et al., 2022). Additionally, kinesin-1 has not only been shown to prefer GTP- over GDP-lattices, it was even found that binding of kinesin-1 induces an extension of the GDP-lattice by $\pm 1.6\%$ (Peet et al., 2018). Conversely, our findings show that tau envelope formation on taxol-stabilized microtubules induces a local microtubule lattice compaction (Siahaan et al., 2022), likely to the GDP-state, which would be highly unfavorable for kinesin-1 to bind to. The distinct preference of tau and kinesin-1 for differential lattice spacing can explain the inhibitory functions of tau in a more effective manner compared to investigating overlapping binding sites. These findings propose an interesting research field that focuses on studying how and if other microtubule-binding proteins govern the microtubule lattice spacing and could therefore be predicted to either repel or recruit one another to the microtubule surface.

As mentioned above, our work described that tau envelopes inhibit transport by kinesin-1 (Siahaan et al., 2019). Complementary work from a fellow PhD student from our lab, Verena Henrichs², showed that kinesin-1 motors are able to penetrate the tau envelope when the motor forms a complex with adaptor TRAK1 (Henrichs et al., 2020). The kinesin-TRAK complex had increased run lengths within the envelope covered regions, however at the cost of decreased velocity. Interestingly, the laboratory of Richard McKenney¹ showed that the retrograde-directed molecular motor dynein, when forming a complex with either dynactin and BicD (DDB com-

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plex), was able to completely pass through the tau envelopes, with considerable pausing events at the envelope boundary (Tan et al., 2019). Additionally, experimental work done by Jochen Krattenmacher⁵ showed that the superprocessive motor kinesin-8 (kip3) was allowed to pass through the enveloped regions (Siahaan et al., 2019). Kinesin-8 was found to pause at the boundary of a tau envelopes where it accumulated at high density until it started displacing tau while moving through the envelope region. Additionally, complementary work done by a fellow PhD student from the lab, Iliia Zhernov³, showed that kinesin-3 (kif14) was able to efficiently traverse microtubule regions covered by tau envelopes (Zhernov et al., 2020). Combined, these findings indicate that tau envelopes create regions along the microtubule lattice that are differentially accessible for different motor proteins. This suggests that the envelopes can directly regulate the bidirectional transport along the microtubule lattice by making the microtubule lattice selectively permissible for certain types of molecular motors, or for transport in a specific direction. This could additionally explain why overexpression of tau in living cells was shown to lead to a change in mitochondrial distribution, which was attributed to a preferential inhibition of tau to plus-end directed transport, causing minus-end directed transport to dominate (Ebneith et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Soundararajan and Bullock, 2014). Aside from differentially regulating molecular motor proteins, tau envelopes have been shown to protect microtubule against microtubule-severing enzyme katanin (Siahaan et al., 2019). Complementary work done by Ruernsen Tan⁶ from the laboratory of Richard McKenney¹ using microtubule-severing enzyme spastin revealed similar protective functionality of the tau envelopes with regards to the severing activity of spastin (Tan et al., 2019). These findings indicate that tau envelopes can affect both molecular motor movement as well as microtubule severing by constituting a selective barrier that makes the microtubule lattice differentially accessible for microtubule-binding proteins.

As discussed in the introduction, there are six main isoforms of tau that can be found in the human brain and CNS. Our work so far exclusively focused on the longest isoform of tau (2N4R). In the lab of Richard McKenney¹, two other isoforms were tested on their ability to form envelopes: the shortest isoform, 0N3R, and 2N3R. They found that both isoforms were able to constitute tau envelopes (Tan et al., 2019), and additionally, both isoforms were shown to be incorporated in 2N4R-tau envelopes. Finding that both the shortest and longest isoform of tau can bind cooperatively suggests that envelope formation is not grossly affected by alternative splicing. However, no isoforms have been tested that solely have one N-terminal insert (either 1N3R or 1N4R). Additionally, the propensity of each isoform to form envelopes still needs to be studied, and it would be interesting to test whether the number of N-terminal inserts, as well as the number of microtubule-binding repeats, affect the propensity of the isoform to bind cooperatively.

Missorting of tau is considered an early sign of neurodegeneration that does not only impair the physiological function of tau, it additionally enables tau to gain toxic functions (reviewed in Zempel and Mandelkow (2014)). It has been stated that distinct tau isoforms show differential localization within the neurons, leading to the assumption that the tau isoforms can have distinct functionality which could explain the toxic gain-of-function from tau missorting (Liu and Götz, 2013). Additionally, tau isoforms are developmentally regulated, with only the 0N3R-isoform expressed in the fetal brain (Bullmann et al., 2009), making it likely that the isoforms carry distinct functionality. While studying the different isoforms, the lab of Richard McKenney¹ additionally studied the functionality of the isoforms and found that the shortest isoform of tau

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(0N3R-tau), as well as the mini-tau construct described earlier, enabled progressively greater number of dynein-dynactin-BicD (DDB) complexes to pass through the envelope unimpeded compared to 2N4R-tau (Tan et al., 2019). Additionally, earlier studies found that 0N3R-tau has a greater ability to inhibit kinesin motility compared to 2N4R-tau (Seitz et al., 2002; Vershinin et al., 2007). Moreover, 4R-tau (all 3 isoforms) was shown to protect microtubules against katanin severing with greater ability compared to 3R-tau (all 3 isoforms) (Qiang et al., 2006). These seemingly conflicting findings cannot directly be explained by considering the cooperative binding mode of tau. However, assuming that single tau molecules forming a tau envelope occupy all available space along the protofilaments of the microtubule with their full MTBD, this would mean that there are more tau molecules present in a similar-length envelope when forming envelopes solely by 3R-tau compared to 4R-tau. Assuming that the off-rate of tau molecules within an envelope is relatively slow, this would mean that the structure of 0N3R-tau envelopes should be more robust compared to 2N4R-tau envelopes, and could therefore account for the stronger inhibition of kinesin-1. However, owing to the additional microtubule-binding repeat, the 2N4R-tau would have a higher affinity to the microtubule lattice and therefore a lower off-rate within the tau envelope compared to any 3R-tau. On the other hand, the different number of N-terminal inserts can also affect tau-tau interaction, therefore using a mix of all 3R- or 4R-tau isoforms could give a skewed result that potentially hides functional diversity between the different isoforms. Combined, these findings indicate that different isoforms of tau are differentially competent in regulating other microtubule-binding proteins suggesting distinct roles for certain isoforms in physiological conditions. These findings highlight the importance of studying all tau isoforms on their functional properties, and, assuming all isoforms can form tau envelopes, raises the question whether tau isoforms in neurons cooperate with one another and if mixing of the isoforms can compromise, or alternatively improve, the envelope structure and function.

As described above, the human brain and CNS contains six main tau isoforms. However, a series of larger tau proteins (ranging from 110 to 120 kDa in size), can be found in the peripheral nervous system (PNS) where exons 4a, 6, and 8 are exclusively transcribed which include a large insert in the N-terminal projection domain of tau. To avoid confusion when talking about the "longest isoform of tau" (which refers to the 2N4R isoform), this series of larger tau proteins is referred to as "**Big tau**". Due to the large interest in the tau protein in the scientific world, it is surprising that most of the work completely ignores the existence of Big tau. Therefore, a lot less is known about the bigger brother of the low molecular weight (LMW) isoforms. Functional distinctions between Big tau and LMW isoforms may involve differences in their impact on axonal transport and microtubule spacing due to their larger projection domain, as well as the lower propensity of Big tau to form toxic aggregates and fibrils (reviewed in Fischer and Baas (2020)). It would be interesting to test whether these larger tau isoforms can bind cooperatively on microtubules and if the Big tau envelopes possess any similarities in functionality compared to envelopes prepared from CNS-tau isoforms. Additionally, it is hypothesized that the longer projection domains of Big tau could alter the access of kinases and phosphatases to the tau structure, changing the rate at which Big tau can be phosphorylated and thereby reducing its propensity to aggregate and polymerize into filaments. These hypotheses could explain why tauopathies that prominently affect the brain do not have a comparable impact on the PNS, where only rare reports show tau tangles in peripheral neurons (reviewed in Fischer and Baas (2020)). These theories spark interest in studying the Big tau isoform, its ability to bind cooperatively on microtubules, and its regulation by tau phosphorylation.

Acetylation is a tubulin PTM that occurs in the microtubule lumen (Eshun-Wilson et al., 2019),

and might therefore not affect proteins that, like tau, associate with the outer surface of the microtubule. Overexpression of deacetylase HDAC6 and katanin in neurons and fibroblasts suggested that katanin-mediated severing is regulated by the acetylation state of microtubules and the binding of tau to microtubules (Sudo and Baas, 2010), where a decrease in acetylation (due to overexpression of HDAC6) lead to decreased severing by katanin. However, recent in vitro experiments using brain tubulin from mouse models showed that katanin-mediated severing was insensitive to tubulin acetylation, and tau binding to microtubules was likewise insensitive to tubulin acetylation (Genova et al., 2023). Therefore, it seems likely that the decrease in katanin interaction with microtubules in the in vivo experiments was a result from indirect effects of HDAC6 overexpression. HDAC6 is known to deacetylate tau as well as microtubules, and deacetylation of tau was shown to increase microtubule binding (Cohen et al., 2011). Therefore, overexpression of HDAC6 would lead to increased tau binding and therefore increased protection of microtubules against katanin-severing, as observed in vivo (Sudo and Baas, 2010), making tau deacetylation a likely cause of decreased katanin-mediated severing. These findings demonstrate the importance of performing in vitro experiments in combination with in vivo experiments. While in vivo experiments can give the big picture resulting from overexpression or inhibition of a particular protein or enzyme, in vitro experiments can help provide a direct causative result that can lead to more accurate hypotheses.

A multitude of studies in independently generated tau KO mice have shown that tau KO does not lead to any gross deficits or loss of viability (Harada et al., 1994; Dawson et al., 2001; Fujio et al., 2007; Tan et al., 2018). The lack of phenotypes has mostly been attributed to upregulation of other MAPs that could functionally compensate for the loss of tau (Harada et al., 1994; Ma et al., 2014). MAPs that have been shown to be upregulated after tau KO are MAP1a/1b and MAP2 (Harada et al., 1994; Ma et al., 2014; Liu et al., 2019). Our study on tau and MAP2 envelopes can explain this compensatory role of MAP2 for tau, since we have found that MAP2 can form envelopes that similarly compact the microtubule lattice and similarly regulate other microtubule-binding proteins (Siahaan et al., 2022). This begs the question whether MAP1a and MAP1b could be compensating for tau functionality in a similar way, making MAP1a and MAP1b likely candidates to study for envelope-competent MAPs and interesting to study using our in vitro TIRF assay.

Like MAP1a/1b, there are other MAPs that, based on various findings, are likely candidates for envelope-competent MAPs. One of these candidates is the end-binding protein EB3, which was found to partially compact GMPCPP-microtubules and induce a MT lattice twist (Zhang et al., 2018). Intriguingly, their findings showed that there were two main classes of results, the first class showed no microtubule compaction corresponding to weak EB3 decoration on the microtubule. The second class had partially compacted microtubule lattices with higher EB3 density on the microtubules. This concentration-dependent compaction of the microtubule lattice appears strikingly similar to our observed findings using tau (Siahaan et al., 2022), suggesting that EB3 could be a candidate to study for cooperative binding and potential envelope formation. These findings suggest that, depending on the number of bound proteins, EB3 can cause compaction of the GMPCPP-microtubule structure (Zhang et al., 2018), which is likely mediated by GMPCPP hydrolysis. The hypothesis that EB3 can induce GMPCPP hydrolysis is, however, plausible since multiple EB orthologs have been found to increase the frequency of microtubule catastrophes which led to the hypothesis that EB proteins are "maturation factors" that increase the rate of GTP hydrolysis in the lattice leading to smaller stabilizing caps (Bieling et al., 2007; Maurer et al., 2014; Zhang et al., 2015). These findings indicate that the inter-tubulin dimer

spacing and the structure of the microtubule lattice is not only determined by the nucleotide state of tubulin, but also by microtubule-binding proteins. It is intriguing that EB3 is able to compact GMPCPP-microtubules which we assumed to be nearly non-hydrolyzable, raising the question whether EB3 is able to increase GMPCPP hydrolysis or if the compaction of the lattice is independent of the hydrolysis state of the GTP-analog. This additionally raises the question whether the compaction of the microtubule lattice due to tau envelope formation could increase GTP hydrolysis, and thereby increase catastrophe rate of microtubules. Therefore, it would be interesting to study the effect of tau envelope formation on microtubule dynamicity.

Aside from the compaction of the microtubule lattice, EB3 binding was found to induce a microtubule twist on the microtubule lattice, which was observed regardless of the number of EB3 bound and appeared independent on the presence of compaction of the microtubule lattice (Zhang et al., 2018). This indicates that a potential cooperative binding mode of EB3 is not necessary to induce the MT twist. The ability of EB3 to induce a MT twist can be attributed to the binding mode of EB3 to four tubulin dimers across two PFs. Therefore, it is unlikely that MAPs, such as tau, that bind along a single protofilament are sensitive to PF skew and therefore are unlikely to induce MT twist. This would, however, have to be tested with high-resolution techniques.

In contrast to MAPs such as EB3, there likely are MAPs that, just like MAP4, do not form envelopes. Why do some MAPs have the ability to bind cooperatively while others do not? The inability of some MAPs to bind cooperatively could be studied from an evolutionary point of view, or from a structural point of view, and either way this is a matter that will need a lot more research. Considering the cross-talk between envelope formation and microtubule lattice spacing, one hypothesis to explain the inability of some MAPs to form envelopes is that these MAPs are not able to compact the lattice. Assuming that these MAPs still prefer a compacted lattice over an extended one, these MAPs would preferentially bind to the compacted lattice of the tau/MAP2 envelopes on taxol-stabilized microtubules. By contrast, we found that MAP4 was excluded from tau/MAP2 envelopes (Siahaan et al., 2022), suggesting that MAP4 does not prefer a compacted lattice, or MAP4 is excluded from tau/MAP2 envelopes due to its inability to undergo cooperative binding with the envelope-competent MAPs. Another hypothesis is that MAP4 and some other MAPs prefer an extended lattice over a compacted lattice, which would explain the absence of MAP4 in tau/MAP2 enveloped regions. This preference for a certain lattice spacing would be straightforward to test using our TIRF assay and could give an indication of the working mechanism of envelope-incompetent MAPs.

One candidate to be an envelope-incompetent MAP is MAP7, which has been shown to be excluded from tau-rich areas on taxol-stabilized microtubules, that greatly resembled tau envelopes (Monroy et al., 2020). This could indicate that MAP7 lacks the ability to bind cooperatively with tau and is therefore excluded from tau-rich areas. Conversely, giving the finding that MAP7 recruits kinesin-1 to the microtubules (Ferro et al., 2022) and kinesin-1 was shown to prefer an extended lattice (Peet et al., 2018), it is likely that MAP7 likewise prefers an extended lattice. It would be intriguing to study whether MAP7 is able to bind cooperatively and, instead of only preferring an extended lattice, is able to actively extend the lattice by binding to the microtubule surface, thereby recruiting kinesin. This regulation of the microtubule lattice spacing adds another layer of complexity to the "MAP code" that describes the regulatory roles of MAPs on microtubule-related processes (Monroy et al., 2020), and suggests that distinct MAPs can differentially regulate the spacing of the microtubule lattice, thereby either attracting or repelling

specific microtubule-binding proteins to the microtubule surface.

The tubulin code suggests that **PTMs** of tubulin as well as different tubulin isotypes, create a molecular pattern or "code" on individual microtubules that microtubule-associated proteins can "read". For the cooperative binding mode of tau, the effect of different PTMs have been tested, showing that tau appears differentially affected by tubulin PTMs (Genova et al., 2023). Tau binding, and tau envelope formation, has been shown to be insensitive to tubulin acetylation, likely due to this PTM occurring on the inside of the MT lumen where tau does not bind. Tubulin polyglutamylation, however, was shown to increase the affinity of tau to microtubules, and induced increased tau envelope formation (Genova et al., 2023), indicating that tau binding and tau envelope formation is differentially regulated by tubulin PTMs. On the other hand, the effect of distinct tubulin isotypes on tau binding and tau envelope formation has not yet been studied. Owing to the finding that taxol binds differentially to different tubulin isotypes and is not able to extend the $\alpha 1 \beta 3$ isotypes while it is able to extend the $\alpha 1 \beta 4$ isotypes (Chew and Cross, 2023), indicates that different tubulin isotypes show distinct interactions with binding partners. However, since both isotypes appear to be compacted in absence of taxol, this suggests that in physiological conditions, these isotypes will grow microtubules of similar structures. Therefore, it is unlikely that tau will show a preference for either $\alpha 1 \beta 3$ - or $\alpha 1 \beta 4$ -tubulin in physiological conditions, but this remains to be tested.

A multitude of studies have demonstrated that tau can undergo liquid-liquid phase separation (LLPS) in vitro under a wide range of experimental conditions (Hernández-Vega et al., 2017; Zhang et al., 2017; Wegmann et al., 2018; Ambadipudi et al., 2019; Lin et al., 2020). LLPS, also called condensation, is a process by which macromolecules, such as proteins or nucleic acids, condense into a dense phase that can coexist with a dilute phase and resembles a liquid droplet both in physical appearance as well as in biological features (reviewed in Alberti et al. (2019)). Features that qualify compartments as liquid droplets, and not as aggregates, include:

- (1) Ability to **undergo fusion** with other droplets or fission.
- (2) Ability to ensure rapid **diffusion of contents** and exchange of components within the cytoplasm.
- (3) Ability to **deform**, for instance by flow.
- (4) "**Wetting**" ability on a surface.

Under conditions of molecular crowding (using molecular crowders dextran, PEG, Ficoll, or Heparin), tau was shown to form droplets that exhibited liquid-like features including fusion with other drops, rapid fluorescence recovery after photo-bleaching (FRAP), wetting features on glass surface, and fission (Hernández-Vega et al., 2017; Lin et al., 2020). During early work with tau envelopes, we wondered whether tau envelope formation is a form of liquid-liquid phase separation. Considering the features that qualify compartments as liquid drops, tau envelopes can undergo fission during envelope disassembly and fusion with other envelopes during assembly (Siahaan et al., 2019; Tan et al., 2019). Tau molecules can exchange with tau in solution, however the position of individual tau molecules in the tau envelopes are static (Siahaan et al., 2019; Tan et al., 2019). Additionally, we did not observe deformation of tau envelopes for instance by flow, nor "wetting" ability of tau envelopes on a surface. The lack of these latter two features indicates that tau envelope formation is not a form of LLPS.

Assuming that a structure would be formed through LLPS generally entails that this structure can grow indefinitely in three dimensions by the addition of more tau. Early on, we found that tau

envelopes do not grow indefinitely, and the tau density in the envelopes saturate above a certain concentration of tau regardless of the amount of tau added to the measurement chamber (Siahaan et al., 2019). This inability of tau envelopes to grow in the third dimension further indicates that tau envelope formation is not a process of LLPS. What is more, the fact that there is a maximum number of tau molecules that can form a tau envelope, suggests that interaction of individual tau molecules with the microtubule lattice is a requirement for tau envelope formation, leading to a finite number of tau molecules that can make up an envelope that is limited by the number of microtubule-binding sites for tau. Additionally, we found a significant difference in the densities of tau within the tau envelopes when using different concentration of tau in solution (Siahaan et al., 2019). This raises the question if a single microtubule binding repeat of tau is sufficient for tau binding to the microtubule within a tau envelope, or whether all four repeats have to bind. Based on the cryo-EM structure of tau bound to microtubules (Kellogg et al., 2018), it seems likely that tau binds with all four repeats, however more in-depth analysis has to be done to confirm this. Fluorescence microscopy would most likely not be sufficient to explore the number of tau repeats binding to the microtubule lattice within an envelope, but based on fluorescence intensity it could give some indication whether one repeat per tau molecule would be enough to bind cooperatively on the microtubule lattice. This would additionally explain the observed differences in the tau densities that are measured within the envelopes and suggests that increased concentrations of tau could lead to fewer binding repeats of tau interacting with the microtubule lattice when forming a tau envelope.

Us and others have shown, using different techniques, that tau can bind cooperatively on the microtubule lattice in vitro as well as in living cells (Siahaan et al., 2019; Tan et al., 2019; Siahaan et al., 2022). The lingering question that remains is how the cell regulates when a tau envelope should form. One regulatory mechanism we have shown is through phosphorylation of tau (Siahaan et al. (in review)), however, tau is subject to many different PTMs and it would be essential to study other PTMs of tau on how they affect the cooperative binding mode of tau. Aside from tau PTMs, another mechanism shown to affect tau envelope formation is through PTMs of tubulin that decorate the microtubule lattice (Genova et al., 2023). Since removal of the tubulin C-terminal tails (CTTs) has been shown to diminish tau envelope formation by reducing tau binding to the microtubule (Tan et al., 2019), it is likely that PTMs on the tubulin CTT would affect tau binding. The fact that it has been shown that polyglutamylation reduces tau envelope formation supports this theory (Genova et al., 2023), however the effect of, for instance, polyglycylation and (de)tyrosination of the CTT remain to be tested. Other factors that would be interesting to study in future work is the effect of cell stress on tau envelopes, which could include inducing oxidative stress, different levels of extracellular ATP, varying pH, or introducing different types of ions into the cellular environment. In general, a lot of work still needs to be done to study how tau envelopes are regulated in a cellular environment.

The work of our lab and the lab of Richard McKenney¹ on tau envelopes is considered the first work on the cooperative binding mode of tau and the dynamics and function of cooperatively bound tau (Siahaan et al., 2019; Tan et al., 2019). However, our work does not appear to be the first observation of the cooperative binding mode of tau. The first observation of tau envelopes likely goes back as far as 2003, when Makrides et al. found that tau binds to microtubule in two distinct binding modes that can co-exist on microtubules (Makrides et al., 2003). These experiments were performed on taxol-stabilized microtubules and imaged using AFM. One binding mode was described as single tau monomers with low organization, while the second binding mode was described as a high-organization single layer of tau oligomers that encircle the micro-

tubule lattice. These findings highly suggests that the researchers were looking at microtubules partially covered by tau envelopes. Since experiments were performed using AFM and data was presented as topography images, the results are difficult to compare to our TIRF microscopy images where the distinct binding modes can directly be visualized. What makes it even more likely that the researchers were visualizing cooperative binding of tau comes from the observation that they observed tau-dependent changes in the MT structure (Makrides et al., 2003), which was not extensively described but could have been the compaction of the microtubule lattice due to tau envelope formation. However, these structural changes were not directly assigned to the location of the tau oligomers.

The tau protein is implicated in a wide range of neurodegenerative diseases, collectively called tauopathies (reviewed in Lee et al. (2001)). In many of these diseases, certain types of highly phosphorylated tau aggregates or tangles are found in the brain of the patients (reviewed in Weseling et al. (2020)). These tangles of tau are made up of tau molecules which self-assemble into long filaments that bundle together to form tangles. The cooperative binding mode of tau is a mode in which tau self-associates or self-assembles on the microtubule lattice to form an envelope. However, the self-assembled tau molecules within the envelopes remain dynamic, since individual tau molecules can turn over with tau in solution, and tau envelopes grow and shrink by the addition or removal of tau molecules from the envelope boundaries (Siahaan et al., 2019; Tan et al., 2019). This raises the question whether self-association into tau tangles is a natural form of tau and when and how it becomes a pathological form that is not able to disassemble anymore. Additionally, it makes us wonder whether tau envelope formation is in any way related to the tau tangles and whether studying the tau envelopes and the cooperative binding mode can help us understand the shift towards pathological self-assembly of tau.

Taxanes, including paclitaxel (taxol) and docetaxel, are widely-used chemotherapy agents for treating a broad range of diseases including many types of cancer (reviewed in Verweij et al. (1994)). Although very active clinically, the usage of taxanes in chemotherapy often comes with severe side effects attributed to taxane neurotoxicity that are still poorly understood. One side effect, called taxane-induced peripheral neuropathy, affects up to 97% of paclitaxel-treated patients and becomes chronic in over 60% of the cases (reviewed in da Costa et al. (2020)). Our in vivo data shows that addition of taxol to cells overexpressing tau causes the disassembly of tau envelopes (Siahaan et al., 2022). Additionally, Qiang et al. found that tau does not stabilize microtubules in the literal sense of the word, it rather helps microtubules to grow and stay dynamic (Qiang et al., 2018). Therefore, the use of microtubule-stabilizing agents as medical treatment would not only lead to potential loss of tau interaction with microtubules, the stabilized microtubules would additionally lose their dynamic properties with tau's function not being properly replaced. It will be interesting to evaluate to what extent taxane-induced alterations in tau and other envelope-competent MAPs interacting with microtubules contribute to taxane neurotoxicity.

This thesis aims to describe the work that was done by the author of this thesis in the laboratory of Zdenek Lanksy² under supervision of Marcus Braun⁴. The research presented in this thesis describes the cooperative binding mode of tau and the regulatory roles of this binding mode in protecting microtubules against severing enzymes such as katanin. The conclusions of the research are listed based on the outlined goals from chapter 2:

- **Characterize the distinct tau binding modes on microtubules**

Using in vitro reconstitution and TIRF microscopy, we found that tau envelopes assemble through a cooperative process by the addition of tau molecules to the boundaries of the envelope. Removal of tau from solution results in the disassembly of tau envelopes, which occurs primarily from the envelope boundaries with occasional fission events within the boundaries of the envelope. Single molecule experiments revealed that, while tau molecules outside the envelope regions diffuse on the microtubule lattice, tau molecules bound in the envelope are stationary but can exchange with tau molecules in solution. Combined, these findings describe the dynamic properties of the cooperative binding mode of tau on microtubules.

- **Explore the mechanism of tau envelope formation**

By tracking single tubulin subunits within the microtubules lattice, we found that tau envelope formation induces a local microtubule lattice compaction that replaces taxol from the microtubule lattice. Reversal of this compaction, either locally by bending microtubules or globally by stretching microtubules optically trapped between two beads, we observed the disassembly of tau envelopes, indicating that microtubule lattice compaction governs the cooperative binding mode of tau. We additionally found that cooperativity is a divergent property of the tau family, where MAP2 shares the ability to form envelopes and compact the microtubule lattice, while MAP4 does not bind cooperatively and also does not compact the microtubule lattice. Additionally, tau and MAP2 can cooperate and form an envelope together that excludes MAP4. These findings suggest that MAPs can form distinct domains along the microtubule lattice that can locally alter the microtubule lattice spacing, thereby regulating access of other microtubule-binding proteins to the microtubule surface.

- **Investigate the regulatory roles of tau envelopes**

In vitro reconstitution and TIRF microscopy revealed that tau envelopes block transport by molecular motor kinesin-1, while allowing molecular motors can pass through. These findings show that the cooperative binding mode of tau creates a selectively permissible barrier on the microtubule lattice that can directly regulate transport by specific molecular

motors or transport in a certain direction. Additionally, tau envelopes protect microtubules from the severing activity of microtubule-severing enzyme katanin. Combined these findings suggest that tau and other MAP envelopes create functionally distinct segments along microtubules, that divide the microtubule surface in separate domains that can differentially regulate the interaction of other microtubule-binding proteins with the microtubule lattice.

- **Study the effect of tau phosphorylation on tau envelope integrity and function**

Using TIRF microscopy combined with mass spectrometry analysis of phosphorylation state, we found that tau phosphorylation decreases tau envelope formation and destabilizes existing tau envelopes. Envelopes prepared from phosphorylated tau additionally had a compromised, more gap-prone structure, indicating that tau phosphorylation reduces tau envelope integrity. Additionally, we found that tau phosphorylation impedes the functionality of the tau envelopes, leading to reduced protection against microtubule-severing enzyme katanin. These findings establish a link between tau phosphorylation and decreased microtubule stability, which can provide insights into potential mechanisms contributing to neurodegenerative diseases.

In conclusion, this thesis describes the cooperative binding mode of tau proteins, forming adaptable protective envelopes on microtubules that locally alter the microtubule lattice spacing. Besides protection of microtubules against severing enzymes, these tau envelopes can influence intracellular transport and regulate the interaction of other microtubule-associated proteins with the microtubule surface. By studying the fundamental binding properties of tau to microtubules, these insights not only contribute to the understanding of cellular transport and microtubule stability in relation with tau, but also improve our understanding on how tau and other MAPs organize the complex cytoskeletal processes and organization needed for cells to carry out their many functions.

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LIST OF ABBREVIATIONS

MTs Microtubules	8
PF protofilament	8
GTP guanosine triphosphate	9
GDP guanosine diphosphate	9
cryo-EM cryo-electron microscopy	10
EB end-binding proteins	10
PTMs post-translational modifications	10
GMPCPP guanylyl-(α,β)-methylene-diphosphonate	11
DCX doublecortin	12
CTT C-terminal tail	13
MAPs microtubule-associated proteins	14
DCLK1 doublecortin-like kinase-1	17
AD Alzheimer's disease	21
NFTs neurofibrillary tangles	21
PHF paired helical filament	21
SF straight filament	21
FTDP-17 frontotemporal dementia with parkinsonism linked to chromosome 17	22
PP2A protein phosphatase 2A	23
Cdk5 cyclin-dependent kinase 5	23
ATP adenosine triphosphate	24
Aβ amyloid- β	24
TTL6 tubulin tyrosine ligase-like enzyme 6	25
KO knockout	25
IDP intrinsically disordered protein	25
MTBR microtubule-binding region	25
MTBD microtubule-binding domain	25
R' " pseudo-repeat domain "	25
FRET fluorescence resonance energy transfer	26
EPR electron paramagnetic resonance	26

MAPT microtubule-associated protein tau	27
aa amino acid	27
CNS central nervous system	27
MAPT microtubule-associated protein tau	27
UTR untranslated region	28
AIS axon initial segment	28
Ser serine	29
Thr threonine	29
Tyr tyrosine	29
Pi phosphate group	29
MARK Microtubule Affinity Regulating Kinase	30
SAD Synapses of Amphids Defective	30
GSK3β glycogen synthase kinase-3 β	30
PPIase peptidyl-prolyl cis-trans isomerase	31
PDPK Proline-directed serine/threonine protein kinases	32
non-PDPK Non-proline-directed serine/threonine protein kinases	32
TPK Tyrosine protein kinases	32
TTBK1/2 tau-tubulin kinase 1/2	32
DYRK1A dual-specificity tyrosine phosphorylation-regulated kinase 1A	32
SFK Src family kinases	32
Lck lymphocyte-specific protein tyrosine kinase	32
PKA protein kinase A	32
PKI protein kinase inhibitor	32
PPP Phosphoprotein phosphatase	32
PTP Protein tyrosine phosphatase	33
SiR-tubulin silicon rhodamine-labeled tubulin	34
NMR Nuclear magnetic resonance	34
TIRF total internal reflection microscopy	35
AFM atomic force microscopy	36
MTOC microtubule organizing center	39
CoV coefficient of variation	57
FRAP fluorescence recovery after photobleaching	73
DDB dynein-dynaction-BicD	76
PNS peripheral nervous system	76
LMW low molecular weight	76
LLPS liquid-liquid phase separation	79

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Appendices

PUBLICATIONS RELATED TO THESIS A

A.1 KINETICALLY DISTINCT PHASES OF TAU ON MICROTUBULES REGULATE KINESIN MOTORS AND SEVERING ENZYMES

Siahaan, V.*, Krattenmacher, J.*, Hyman, A.A., Diez, S., Hernández-Vega, A., Lansky, Z., Braun, M. Kinetically distinct phases of tau on microtubules regulate kinesin motors and severing enzymes. *Nat Cell Biol* 21, 1086–1092 (2019).

*These authors contributed equally.

Contributions as stated in the article:

A.H.-V. and M.B. first observed the islands and initiated the project; A.H.-V., A.A.H., S.D., Z.L. and M.B. conceived the experiments; A.H.-V. generated the tau(Δ N)–mGFP construct; V.S., J.K., A.H.-V. and M.B. generated the proteins, performed and analysed the experiments and V.S., J.K., S.D., Z.L. and M.B. wrote the manuscript. All authors discussed the results and commented on the manuscript.

In detail: V.S. optimized the methods and performed all experiments of figures 1 and 2, part of figure 3 (specifically, multiple repeats from 3a and 3b were performed by V.S.), part of figure 4 (specifically 4a and 4b were optimized and performed by V.S.), and all experiments of figure 5. All experiments were performed using TIRF microscopy. All of the analysis was optimized and carried out by J.K., with help from V.S. All analysis was done using ImageJ, Excel, and Matlab. Tau-GFP, tau-mCherry, and katanin-GFP constructs were generated by V.S. with help from J.K. The manuscript was written by V.S., J.K., S.D., Z.L. and M.B.

A.2 MICROTUBULE LATTICE SPACING GOVERNS COHESIVE ENVELOPE FORMATION OF TAU FAMILY PROTEINS

Siahaan, V.*, Tan, R.*, Humhalova, T. Libusova, L., Lacey, S. E., Tan, T., Dacy, M., Ori-McKenney, K. M., McKenney, R. J., Braun, M., Lansky, Z. Microtubule lattice spacing governs cohesive envelope formation of tau family proteins. *Nat Chem Biol* 18, 1224–1235 (2022).

*These authors contributed equally.

Contributions as stated in the article:

The manuscript was conceptualized by R.T., K.M.O.M., R.J.M., M.B., and Z.L.; methods were developed by V.S., T.H., L.L., S.E.L., R.J.M., M.B., and Z.L.; recombinant proteins were generated by V.S., R.T., T.T., and M.D.; TIRF experiments were performed by V.S., R.T., and R.J.M.; optical tweezers experiments by V.S.; cryo-EM experiments by S.E.L.; live-cell experiments by T.H.; data were formally analyzed by V.S., R.T., T.H., S.E.L., K.M.O.M., and R.J.M.; the manuscript was written by V.S., L.L., K.M.O.M., R.J.M., M.B., Z.L., with reviewing and editing by T.T. and M.D.; the project was supervised by K.M.O.M., R.J.M., M.B., and Z.L.; funding was acquired by V.S., T.H., L.L., K.M.O.M., R.J.M., M.B., and Z.L.

In detail: V.S. optimized the methods and performed all experiments visualized in figure 1 and 2. These experiments include work using both TIRF microscopy and optical trapping. Additionally, V.S. optimized the methods and performed the experiments represented in figure 3e. All analysis for figures 1 (all), 2 (all), and 3e were carried out by V.S. using ImageJ, Excel, FIESTA tracking software, and Matlab. Analysis for the in vivo data presented in figure 4 was carried out by T.H. with help from V.S. Visualization for figures 1 (all), 2 (all), 3e, and 4 (all) was carried out by V.S. using Matlab and Illustrator. Tau-GFP and tau-mCherry constructs were generated by V.S. The manuscript was written by V.S., L.L., K.M.O.M., R.J.M., M.B., Z.L. Additionally, funding was acquired by V.S. from the Charles University Grant Schemes: GAUK no. 373821, and START reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935.

A.3 TAU PHOSPHORYLATION IMPEDES FUNCTIONALITY OF PROTECTIVE TAU ENVELOPES

Siahaan V.*, Weissova, R.*, Lanska, E., Karhanova, A., Dostal, V., Henriot, V., Janke, C., Libusova, L., Braun, M., Balastik, M., Lansky, Z. (in submission at Nature Chemical Biology)

*These authors contributed equally.

Contributions as stated in the article:

The manuscript was conceptualized by M.Br., M.Ba., Z.L.; methods were developed by V.S., R.W., E. L., A.K., V.D.; TIRF in vitro experiments were performed by V.S., E. L., A.K.; tau lysate preparation and experiments were performed by R.W., E.L.; live-cell experiments were performed by R.W., V.D.; FRAP experiments were performed by V.S., R.W.; data were analyzed by V.S., R.W., E.L., A.K., V.D.; resources were provided by V.H., C.J.; the manuscript was written by V.S., M.Br., Z.L., with reviewing and editing by R.W., M.Ba.; visualization was done by V.S.; the project was supervised by L.L., M.Br., M.Ba., Z.L.; funding was acquired by V.S., R.W., V.D., L.L., M.Br., M.Ba., and Z.L.

In detail: For all in vitro TIRF experiments, development of the methods and optimization of the analysis of the data for figures 1, 2 and 4a-c was done by V.S. with help from E.L., A.K., M.Br. and Z.L. All in vitro TIRF experiments and its repetitions for figure S1h, and some of the repetitions for figure 4a-c and S4a,b,c were performed by V.S. Sample preparation and data analysis of the Mass Spectrometry results (figures 1a,b, S1a,b,d-g, 2a,b and S2a,b) were optimized and performed by V.S. using Excel and Matlab. All FRAP experiments (figure 3a-c, S3g-i) were optimized by V.S. and R.W., all repetitions were performed by V.S. using spinning disk. The manuscript was written by V.S., M.Br., and Z.L. Visualization of all figures was done by V.S. using Matlab and Illustrator. Additionally, funding was acquired by V.S. from the Charles University Grant Schemes: GAUK no. 373821, and START reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935.

PUBLICATIONS UNRELATED TO THESIS

B

B.1 ANILLIN PROPELS MYOSIN-INDEPENDENT CONSTRICTION OF ACTIN RINGS

Kučera, O., **Siahaan, V.**, Janda, D., Dijkstra, S. H., Pilátová, E., Zatecka, E., Diez, S., Braun, M., Lansky, Z. Anillin propels myosin-independent constriction of actin rings. *Nat Commun* **12**, 4595 (2021).

Contributions as stated in the article:

Conceptualisation, M.B. and Z.L.; Methodology, O.K., M.B. and Z.L.; Investigation, O.K., V.S., D.J., S.H.D. and E.P.; Formal analysis, O.K., D.J., E.P. and V.S.; Data curation, O.K., D.J., V.S. and S.H.D.; Validation, O.K.; Resources, O.K., E.Z. and S.D.; Writing, O.K., M.B. and Z.L.; Visualisation, O.K.; Supervision, M.B. and Z.L.; Funding acquisition, M.B. and Z.L.

In detail: V.S. optimized and performed part of the TIRF experiments visualized in figure 1 (specifically figure 1k and all repetitions) and 4 (specifically data for figure 4h and all repetitions), and all experiments visualized in figure 3. All experiments done by V.S. were performed using TIRF microscopy. Additionally, part of the analysis for these experiments were done by V.S. using ImageJ and Excel.