2020

**Tau Interdomain Relationships: Re-Examining The Role Of The Proline Rich Region In Tau's Structure And Function In Vitro**

Kristen May Mckibben  
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Abstract

Tau is an intrinsically disordered (IDP), microtubule-associated (MAP) protein that has a role in regulating microtubule dynamics. Despite intensive research, the molecular mechanisms of tau-mediated microtubule polymerization are poorly understood. In particular, although significant effort has poured into interactions between the microtubule protofilament and one of tau's regions, the microtubule-binding region (MTBR), the remaining tau domains and interactions with unincorporated soluble tubulin remain understudied. Here, we used single-molecule fluorescence to investigate the role of tau's N-terminal domain (NTD) and proline-rich region (PRR) in regulating interactions of tau with soluble tubulin. We assayed both full-length tau isoforms and truncated variants for their ability to bind soluble tubulin and stimulate microtubule polymerization. We found that tau's PRR is an independent tubulin-binding domain that has tubulin polymerization capacity. In contrast to the relatively weak interactions with tubulin mediated by sites distributed throughout tau's MTBR, resulting in heterogeneous tau:tubulin complexes, the PRR bound tubulin tightly and stoichiometrically. Moreover, we demonstrate that interactions between the PRR and MTBR are reduced by the NTD through a conserved conformational ensemble. On the basis of these results, we propose that tau's PRR can serve as a core tubulin-binding domain, whereas the MTBR enhances polymerization capacity by increasing the local tubulin concentration. Moreover, the NTD appears to negatively regulate tubulin-binding interactions of both of these domains. The findings of our study draw attention to a central role for the PRR in tau function and provide mechanistic insight into tau-mediated polymerization of tubulin. However, PRR binding was not observed in the recent cryo-EM structure of tau's MTBR bound to the microtubule protofilament (Nogales, 2018). Using an environmentally sensitive fluorophore acrylodan, we demonstrate similarities between tau's MTBR structure to soluble tubulin and the microtubule. Our preliminary work suggests PRR binding to tubulin is strongly mediated through the tubulin tails. In summary, this thesis provides mechanistic and structural insight into tau-mediated microtubule polymerization by (1) clarifying the role of tau's PRR thereby refining polymerization models (2) demonstrating a conserved ensemble between the NTD and the PRR/MTBR regions of functional significance, and (3) outlining preliminary groundwork for contrasting tau-microtubule and tau-tubulin interactions.

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Elizabeth E. Rhoades

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TAU INTERDOMAIN RELATIONSHIPS: RE-EXAMINING THE ROLE OF THE PROLINE RICH REGION IN TAU’S STRUCTURE AND FUNCTION IN VITRO

Kristen M. Mckibben

A DISSERTATION

in

Biochemistry and Molecular Biophysics

Presented to the Faculties of the University of Pennsylvania

in

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Degree of Doctor of Philosophy

2020

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TAU INTERDOMAIN RELATIONSHIPS: RE-EXAMINING THE ROLE OF THE PROLINE RICH REGION IN TAU’S STRUCTURE AND FUNCTION IN VITRO

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2020

Kristen M. McKibben

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DEDICATION

To all who don’t teach from books: Joshua Straquadine; my family Michael, Linda and Bernie M’Kibben, and my extended family; my mentors Dr. Desiree Gunning, Ms. Heather Cyr, Dr. Raji Joseph, and Dr. Tatiana Esipova; my friends Mikaela Leners, Chelsey Havick, Ashley Prochazka, Camilla Forsgren, Holly Maxwell, Patti Murphy, Martin Iwanicki, Dr. Ho Yee Joyce Fung, and Adriana Naomi Santiago Ruiz.

“To think I did all that
And may I say – not in a shy way”

- Frank Sinatra, *My Way*
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For others’ technical assistance throughout the years: the Chenoweth Lab, specifically Alex Kasznel and Sam Melton, for use of their fluorimeter and assistance; the Svitkina Lab for the use of their TEM microscope, and Changsong Yang for assistance with the microscope and insightful suggestions; the Baumgart lab, especially Jaclyn Robustelli, for materials and helpful discussions regarding cloning and TEM slide preparation; the Petersson lab, especially Jack Ferrie and Taylor Barret, for use of their fluorimeter, materials, and helpful discussions; and both the Matthews lab and Christianson lab for materials. Additionally, I would like to thank Ryan Kubanoff for genuine concern for departmental resources as well as all the departmental staff for doing the invisible things.
ABSTRACT

TAU INTERDOMAIN RELATIONSHIPS: RE-EXAMINING THE ROLE OF PRR IN TAU’S STRUCTURE AND FUNCTION IN VITRO

Kristen May M^oKibben

Elizabeth Rhoades

Tau is an intrinsically disordered (IDP), microtubule-associated (MAP) protein that has a role in regulating microtubule dynamics. Despite intensive research, the molecular mechanisms of tau-mediated microtubule polymerization are poorly understood. In particular, although significant effort has poured into interactions between the microtubule protofilament and one of tau’s regions, the microtubule-binding region (MTBR), the remaining tau domains and interactions with unincorporated soluble tubulin remain understudied. Here, we used single-molecule fluorescence to investigate the role of tau’s N-terminal domain (NTD) and proline-rich region (PRR) in regulating interactions of tau with soluble tubulin. We assayed both full-length tau isoforms and truncated variants for their ability to bind soluble tubulin and stimulate microtubule polymerization. We found that tau’s PRR is an independent tubulin-binding domain that has tubulin polymerization capacity. In contrast to the relatively weak interactions with tubulin mediated by sites distributed throughout tau’s MTBR, resulting in heterogeneous tau:tubulin complexes, the PRR bound tubulin tightly and stoichiometrically. Moreover, we demonstrate that interactions between the PRR and MTBR are reduced by the NTD
through a conserved conformational ensemble. On the basis of these results, we propose that tau’s PRR can serve as a core tubulin-binding domain, whereas the MTBR enhances polymerization capacity by increasing the local tubulin concentration. Moreover, the NTD appears to negatively regulate tubulin-binding interactions of both of these domains. The findings of our study draw attention to a central role for the PRR in tau function and provide mechanistic insight into tau-mediated polymerization of tubulin. However, PRR binding was not observed in the recent cryo-EM structure of tau’s MTBR bound to the microtubule protofilament (1). Using an environmentally sensitive fluorophore acrylodan, we demonstrate similarities between tau’s MTBR structure to soluble tubulin and the microtubule. Our preliminary work suggests PRR binding to tubulin is strongly mediated through the tubulin tails. In summary, this thesis provides mechanistic and structural insight into tau-mediated microtubule polymerization by (1) clarifying the role of tau’s PRR thereby refining polymerization models (2) demonstrating a conserved ensemble between the NTD and the PRR/MTBR regions of functional significance, and (3) outlining preliminary groundwork for contrasting tau-microtubule and tau-tubulin interactions.
# TABLE OF CONTENTS

DEDICATION ........................................................................................................ iii

ACKNOWLEDGMENT ............................................................................................ iv

ABSTRACT ............................................................................................................ vi

LIST OF ABBREVIATIONS ................................................................................... x

LIST OF FIGURES AND TABLES .......................................................................... xii

CHAPTER 1: Introduction....................................................................................... 1
  Tauopathies ........................................................................................................ 1
  Microtubules ...................................................................................................... 2
  Tubulin isotypes and the ‘tubulin code’ .............................................................. 3
  Tau-tubulin interactions and tau-mediated microtubule dynamics .................. 5
  Tau isoforms and domains .............................................................................. 9
  Intrinsically disordered proteins .................................................................... 12
  Experimental techniques as applied to disordered systems .......................... 21
  Scope of this work .......................................................................................... 38

CHAPTER 2: The N-terminal isoforms have a conserved ensemble independent of sequence ............................................................................................................ 41
  Introduction ..................................................................................................... 41
  The global ensemble of tau is conserved across N-terminal isoforms .......... 43
  Electrostatic interactions between domains drive ensemble formation ......... 49
  Discussion ...................................................................................................... 50

CHAPTER 3: Independent binding and polymerization of the proline rich region is negatively regulated by the N-terminal domain ......................................................... 52
  Introduction ..................................................................................................... 52
  Tau’s NTD negatively regulates tubulin binding ............................................. 55
  The PRR independently binds and polymerizes tubulin ............................... 61
  NTD negatively regulates the polymerization capacity of the PRR ............. 64
  PRR forms tight, saturable tau:tubulin complexes ........................................ 67
  Discussion ...................................................................................................... 73

CHAPTER 4: Biochemical and structural elucidation of the core binding sites to soluble tubulin ........................................................................................................... 80
  Introduction ..................................................................................................... 80
  Tau prefers GDP-tubulin within the lattice but is insensitive to nucleotide state in solution ................................................................. 81
  Structural features of tubulin- and MT-bound tau are similar but not identical 85
  PRR and MTBR-R’ share binding sites ............................................................ 87
  Disordered tubulin tails enhance tau binding ................................................. 89
  Discussion ...................................................................................................... 93
CHAPTER 5: Conclusions and Perspectives

(1) PRR versus MTBR binding: expanding functional tau binding sites to soluble tubulin

(2) PRR binding and NTD negative regulation: refining polymerization models

(3) NTD/PRR/MTBR conformational ensemble and combinatorial consequences of tau isoform to tau function

In summary: complexity in dynamic systems favors biological function

APPENDIX I: Experimental Procedures

i. Tubulin purification and handling

ii. Tau cloning, purification, and labeling

iii. FCS instrument and data analysis

iv. FRET instrument and analysis

v. Tubulin light scattering assay

vi. Electron microscopy imaging

vii. Microtubule pelleting assay

viii. Fluorescence anisotropy

ix. Tubulin tail digestion

x. MBP-TubB<sup>HB</sup> Purification

xi. TubB<sup>HB</sup> Purification

xii. CD Spectroscopy

xiii. Preparation and functionalization of imaging chambers

xiv. TIRF microscopy

xv. Microtubule tip tracking and fluorescence profile analysis

xvi. Acrylodan labeling

xvii. Acrylodan fluorescence

xviii. Analysis of acrylodan emission peak shifts

APPENDIX II: FCS Python code

BIBLIOGRAPHY
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>amyloid-beta protein</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per molecule (kHz)</td>
</tr>
<tr>
<td>cyro-EM</td>
<td>cyro-electron microscopy</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EBI</td>
<td>end binding protein 1</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>ETeff</td>
<td>energy transfer efficiency</td>
</tr>
<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GMPCPP</td>
<td>guanosine-5’-[(α,β)-methylene]triphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>IDP</td>
<td>intrinsically disordered protein</td>
</tr>
<tr>
<td>IDR</td>
<td>intrinsically disordered region</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>IR</td>
<td>inter-repeat region</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
</tr>
<tr>
<td>LIC</td>
<td>ligation independent cloning</td>
</tr>
<tr>
<td>LLPS</td>
<td>liquid-liquid phase separation</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization time of flight</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule associated protein</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MTBR</td>
<td>microtubule-binding region</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal Domain</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline solution</td>
</tr>
<tr>
<td>PEG-PLL</td>
<td>(ethylene glycol)poly(L-lysine)</td>
</tr>
<tr>
<td>PIE-FRET</td>
<td>pulsed interleaved excitation smFRET</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N,N’-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PRR</td>
<td>proline rich region</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-transitional modification</td>
</tr>
<tr>
<td>RMS_{exp}</td>
<td>root-mean-square distance from experiments</td>
</tr>
<tr>
<td>RMS_{RC}</td>
<td>root-mean-square distance from theory</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLiM</td>
<td>Short linear motif</td>
</tr>
<tr>
<td>smFRET</td>
<td>Single molecule Förster resonance energy transfer</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TEM</td>
<td>tunneling electron microscopy</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TIRF</td>
<td>total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>TubB2&lt;sup&gt;HB&lt;/sup&gt;</td>
<td>Helix bundle H9-H12 with IDP tail of bovine tubulin isotype 2</td>
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<tr>
<td>TubB4A&lt;sup&gt;HB&lt;/sup&gt;</td>
<td>Helix bundle H9-H12 with IDP tail of bovine tubulin isotype 4A</td>
</tr>
</tbody>
</table>
LIST OF FIGURES AND TABLES

CHAPTER 1: Introduction

- Figure 1.1 Schematic of tau domains and highlighted functions ........................................ 8
- Figure 1.2 Tau bound to microtubules and soluble tubulin ..................................................... 10
- Figure 1.3 Intrinsically disordered proteins and their ensembles ............................................. 15
- Figure 1.4 Network modeling as applied to tau-tubulin interactions ....................................... 20
- Figure 1.5 Structural experimental techniques ........................................................................... 28-29
- Figure 1.6 FCS and common complementary experimental techniques .................................... 32-33

CHAPTER 2: The N-terminal isoforms have a conserved ensemble independent of sequence

- Figure 2.1 Tau N-terminal constructs ....................................................................................... 44
- Figure 2.2 Tubulin binding of the N-terminal isoform .............................................................. 44
- Figure 2.3 SmFRET of tau N-terminal isoforms ....................................................................... 47
- Table 2.1 Summary of SmFRET biophysical data of tau N-terminal isoforms ......................... 48
- Table 2.2 Charge profile of individual tau domains .................................................................... 50

CHAPTER 3: Independent binding and polymerization of the proline rich region is regulated by the N-terminal domain

- Figure 3.1 Schematic of tau constructs ....................................................................................... 54
- Figure 3.2 Inhibition of tubulin binding by the NTD ................................................................. 56
- Figure 3.3 Regulation of the tau:tubulin complex heterogeneity by the NTD ......................... 57
- Figure 3.4 Impact of the NTD inserts N1 and N2 on binding ................................................... 59
- Figure 3.5 Impact of the NTD inserts N1 and N2 on polymerization ......................................... 61
- Figure 3.6 Independent polymerization capacity of the PRR, regulated by NTD ................. 63
- Figure 3.7 Impact of N-terminal inserts on the binding of PRR .............................................. 64
- Figure 3.8 PRR encourages the formation of straight microtubules ......................................... 67
- Figure 3.9 The PRR forms stoichiometric complexes with tubulin ......................................... 69
- Figure 3.10 P1 and P2 dependence of PRR binding ................................................................. 71
- Figure 3.11 Model for regulation of tau:tubulin interactions .................................................... 78

- Table 3.1 Tau-mediated polymerization .................................................................................... 65
- Table 3.2 Summary of FCS biophysical data of tau isoforms and constructs ......................... 72
- Table 3.3 Descriptive statistics of tau:tubulin .......................................................................... 73

CHAPTER 4: Biochemical and structural elucidation of tau’s core binding sites to tubulin

- Figure 4.1 2N4R tau preferentially binds microtubules composed of GDP-tubulin over GMPCPP-microtubules in vitro ............................................................. 84
- Figure 4.2 Structure of the tubulin- and MT-bound MTBR are similar .................................... 87
Figure 4.3: PRR and MTBR-R’ compete for soluble tubulin.................................89
Figure 4.4 Disordered tails of tubulin enhance tau binding ...............................91
Figure 4.5 PRR binds to helical bundles TUBB4A and TUBB2 .........................93

APPENDIX: Experimental Procedures

Figure A1.1 Filtering algorithm ..............................................................................111
Figure A1.2 Neither FCS mutations nor dye impact tubulin polymerization ....117
Figure A1.3 PRR-MTBR-R’ binding is not affected by pH .................................118
Figure A1.4 Labeling positions or common dye sizes do not affect binding ...121
Figure A1.5 Digestion of tubulin tails .................................................................122
Figure A1.6 Alignment of TubB helical bundles ...............................................123
Figure A1.7 Purified TUBB^{HB} are predominately helical ..............................125
Figure A1.8 Raw acrylodan data .......................................................................130
CHAPTER 1: Introduction

In 1975 the non-motor microtubule associated protein (MAP) tau was biophysically characterized as lacking a stable secondary or tertiary structure (2). Academic focus on tau increased upon its use as a biomarker for a class of neurodegenerative diseases known as tauopathies that include both Alzheimer’s and Parkinson’s disease (3). In 2018, an estimated 5.7 million Americans in 2018 suffered from Alzheimer’s disease, and this number is expected to rise (4); effective therapeutics to treat these diseases are critical not only for the suffers but also their caregivers whose uncompensated care totals an estimated $232 billion a year (4). Due to ongoing yet faltering efforts with other cellular targets for therapeutics, tau is now a primary candidate for pharmaceutical research. Despite renewed scientific vigor, remarkably basic scientific questions regarding tau’s functions within the cell remain unanswered or debated. Presented here is an expanded study on the oldest proposed function of tau – microtubule polymerization – and the regions that govern it including regulatory mechanisms and corresponding initial coarse-grained structural characterization. Combined, these studies provide additional mechanistic insight into tau-mediated polymerization of microtubules with implications for tau’s ability to decipher the tubulin ‘code.’

**Tauopathies**

Tau is primarily located in the axons of neurons within the brain and central nervous system (reviewed in (5)). In the diseased brain, tau forms proteinaceous plaques called paired helical filaments and neurofibrillary tangles – the particularly prominent
features of these diseases (6). The amyloid hypothesis suggests amyloid-beta (Aβ) was the underlying component of amyloidogenesis that is influenced by additional interactions with tau (7). However, tau alone is the predominate biomarker in other neurodegenerative diseases such as frontotemporal dementia and Parkinsonism linked to chromosome-17 (8). Consequently, tau has been implicated in both gain-in-toxicity as well as loss-of-function disease mechanisms (9). The former primarily arises from the amyloid hypothesis: hyperphosphorylation of tau leads to the sequestration of normal tau as well as other MAPs eventually leading to toxic and transmittable oligomers and large aggregated protein plaques that interfere with normal function (7). Less studied are loss-of-function disease mechanisms. Indeed, two of tau’s biological roles are regulating microtubule dynamics and cellular-transport primarily by modulating the progress of molecular motors along the axon (2, 10-13); arguably, healthy biological function within neurons is therefore dependent upon normal tau function.

**Microtubules**

Microtubules are a cornerstone of the cytoskeleton that provide structural support, and play a role in cellular organization, chromosomal segregation during mitosis, cellular transport, and cell force generation and movement (14, 15). Within neurons, they are nicknamed the ‘highway of the cell’ along which cellular cargo is transported (13). Microtubules are polymers of heterodimers of α and β tubulin, which are the focus of this thesis. Heterodimeric α/β tubulin subunits assemble into protofilaments in a guanosine-5’-triphosphate (GTP) hydrolysis dependent manner end-to-end such that each β subunit of one heterodimer is docked against the α subunit of next tubulin heterodimer subunit.
(16, 17). This polarity of growth is further indicated by (-) and (+) ends such that α(-) is the slow growing end, and β(+) is the positive growing end. Multiple protofilaments are then ‘zipped’ together to form a microtubule (18). Most in vivo microtubules consist of 13 protofilaments, although protofilament numbers vary in vivo (19) and are manipulated in vitro by MAPs (20, 21).

As previously alluded, microtubules undergo cycles of growth and shrinkage phases triggered by rescue and catastrophe events collectively referred to as ‘dynamic instability’ as first described by Mitchison and Kirschner (22). The characteristic parameters of these events are: growth rate, shrinkage rate, and catastrophe frequency and rescue frequency. Microtubules also ‘pause’ and there appears to be very little length change in the microtubule (22, 23). Simplistically, the growth happens predominately at the β (+) end by incorporating GTP-tubulin at the microtubule tip. Sufficiently high rates of incorporation lead to the ‘GTP-cap’ composed of multiple GTP-tubulin subunits (22, 24). Recent studies suggest incoming GTP-tubulin is incorporated as a tapered sheet that is zippered up into the hollow microtubule tube (18). As new tubulin heterodimers are incorporated, the previously incorporated subunit becomes hydrolytically competent eventually becoming GDP-tubulin. While GTP-tubulin is straight, GDP-tubulin is curved in solution and therefore introduces strain on the microtubule lattice (25-27). Once the GTP-cap is lost, microtubules peel away as curved protofilaments (28-30).

**Tubulin isotypes and the ‘tubulin code’**

As previously mentioned, α(-) and β(+) tubulin form one heterodimeric tubulin unit. As with all tubulins, they are highly conserved across species. In higher eukaryotes,
multiple tubulin gene families give rise to tubulin isotypes (31, 32). For example, within humans there are 8 α(-) and 9 β(+) tubulin isotypes (33, 34). Tubulin isotypes are 90% homologous in both amino acid sequence and structure, varying predominately in the intrinsically disordered C-terminal tails (35). Tubulin heterogeneity is generally referred to as a ‘mosaicism’, and is an active area of research (32). The precise tubulin isotype and its relative ratio of the tubulin pool vary across each organism and cell type with neurons generally having the highest complexity (33, 36). Interestingly, functional specialization in vivo and differences in microtubule dynamics in vitro have been demonstrated in a small set of systems (13, 37). Whether these functional differences come from the very subtle structural differences within the tubulin body (37) or other mechanisms involving the more variable tubulin tail, or a combination of both the tubulin body structure and the variable tail, is unknown. More concretely, mutations result in serious diseases, not all of which are neurological (32). For example, while mutations in the beta tubulin isotypes 2B and 3 (TubB2B and TubB3 respectively) and alpha tubulin isotype 1A (TubA1A) have similar neurodegenerative phenotypes (32), defects in beta tubulin isotype 8 (TubB8) uniquely and exclusively result in female sterility (38). Additionally, tubulin isotype expression is both temporally and spatially controlled (35, 36, 39). This has led to the proposition that the degree of mosaicism within the microtubule lattice may be precisely regulated determining a variety of cellular functions including axonal transport and cell differentiation (13, 32). Layered on top of the isotype are post-translational modifications (PTMs) to the C-terminal tail including: glutamylation, polyglutamation, tyrosination, glycylation, polyglycylation, and enzymatic digestion (40). Again, the PTMs are spatially
specific and impact a variety of microtubule and MAP associated functions (13, 32, 40). In particular, axons are rich in glutamylated tubulin which increases tau’s affinity for tubulin (41), however, as the chain length of subsequent polyglutamation increases tau binding decreases (42). In summary, the tubulin isotype mosaic, modifying PTMs, and the degree of strain or compaction along the microtubule lattice has been termed the ‘tubulin code’, and is an active area of research with implications in axonogenesis, axonal transport, cell signaling, and developmental biology.

**Tau-tubulin interactions and tau-mediated microtubule dynamics**

Tau belongs to a family of non-motor microtubule-associated proteins (MAPs) that generally function to modulate microtubule stability and dynamics, as well as regulate the transport cellular cargo. Of this group, tau is seen as a microtubule stabilizer (43). Specifically, tau does this by three major mechanisms: (1) suppression of catastrophe frequency, (2) reducing the length (in time) of both growing and shortening events, and (3) increased polymerization at both α(−) and β(+) microtubule polarities (2, 44-46). Tau’s location along the microtubule is debated with multiple diffusion models (47-49). Additionally, tau has been proposed to bind preferentially to either GDP-tubulin or GTP-tubulin, although both tau’s preferred binding partner (GDP-tubulin or GTP-tubulin) as well as the its discriminatory power between the two nucleotide states is debated (50-53). In some studies, GMPCPP-tubulin or GTPγS-tubulin was used instead of GTP-tubulin; both of these analogs mimic GTP-tubulin properties, but are not hydrolytically competent resulting in stable microtubules that have subtle structural and chemical differences from microtubules that incorporate GTP-tubulin. The use of these
synthetic stabilizers adds an additional layer of complexity regarding the overall structure of the microtubule and its influence on tau-tubulin interactions (50-53). Frequently invoked together are the diffusion model termed ‘kiss-and-hop’ in which tau samples the microtubule lattice briefly (47), and tau’s nucleotide preference for GDP-tubulin over GMPCPP-tubulin (50). Together these two theories lead to an overall enrichment of tau along the microtubule body, and consequently, a stabilizing effect to the microtubule. More recently, this view has been critiqued (51). One study with green fluorescently tagged tau showed that tau preferentially bound to the labile GTP-cap regions of the microtubule, and depletion of tau led to the loss of microtubule mass in the labile regions (54). These findings led the researchers to propose tau is not a bone-fide microtubule stabilizer, particularly in comparison to the stronger MAP6, but instead promotes the formation of labile/GTP-caps at the ends of microtubules (51). The definitions of tau’s function do not preclude tau from promoting labile ends but rather are largely in agreement as they highlight tau’s polymerization capacity. Arguably, the practical consequence of increasing mass within the labile ends (GTP-cap) is a decrease in the catastrophe frequency [tau-mediated mechanism of microtubule stabilization (1)]. Thus, the fundamental underlying mechanism remains unclear. This recent debate emphasizes the lack of molecular detail of tau-tubulin and tau-microtubule interactions and the ongoing efforts to clarify them.

Within tau, a particular region termed the microtubule-binding region (MTBR) binds to microtubules. These interactions are predominately electrostatic in nature (55-59). It contains four 18 amino acid repeats (R1-R4 in Figure 1.1) with flanking inter-
repeat regions (IR) (not depicted for simplicity) (55, 60). Both the repeats and the IRs strongly mediate the tau-tubulin and tau-microtubule interactions (59, 61). While R1-R2 dominate MTBR interactions with lattice-incorporated tubulin (1, 61), with R3 playing a key role in tau-tubulin interactions (62). See Figure 1.2 for a comparison of the two aforementioned structural models. Further work demonstrated the importance of R' and the C-terminal most half of P2 (amino acids 215-221) in enhancing microtubule affinity and targeting the MTBR to the microtubule lattice (63-65). Chemical shifts in NMR spectra of full-length tau have suggested transient association of both the P2 and R' regions to the both drug-stabilized microtubules and tubulin complexed with capping proteins derived from stathmins which lock two heterodimers together without polymerization capacity (65-67). More recently, part of PRR (amino acids 166-246) was shown to very weakly bind taxol-stabilized microtubules (65). The recent cryo-EM structure shows tau density from amino acids corresponding to the slightly conserved binding motif binding tightly in a straight line along the microtubule protofilament ridge (amino acids 256-267 within R1, specifically sequence VKSKIGSTENLK) (1). However, the tau construct used in this study was highly synthetic in which the R1 repeat was used in tandem four times thereby replacing tau’s other 3 repeats. As significant amounts of both biochemical and complementary structural evidence suggest there are variable affinities within the MTBR region assembling on the microtubule lattice in a variety of conformations proposed to be of functional biological importance (58, 63, 67), it is difficult to directly supplant the traditional picture of tau’s weak and heterogeneous interactions with this specific linear model. The low-resolution of the natural 4R
construct within the cryo-EM images – which was referenced but not modeled – speaks to this complexity.

Figure 1.1: Tau bound to microtubules and soluble tubulin

(A) Cryo-EM model of tau MTBR repeats modeled from R1 and R2 (red) bound to α (green) and β (blue) tubulin within the microtubule lattice. The C-terminal tails of the tubulin subunits are indicated by golden star. (B) Tau unbound and the conformational changes upon binding to soluble tubulin as determined by smFRET.

In better agreement are the regions of tubulin that tau binds, which are well reflected within the cryo-EM structure. Tau binding sites are located along helices 11 and 12 on both α(-) and β(+) tubulin (68). Tau:tubulin stoichiometry is 1 tau to 2 tubulin heterodimers (63, 69), and the interdimer interface between α(-) and β(+) of different heterodimers is favored over the intradimer interface (1). Other motor MAPs including kinesin-1 share these binding sites (1, 70), leading many to propose a variety of site-occlusion based models for tau’s interactions with other MAPs (12, 71). Additionally, tau
also binds to synthetic peptides of the intrinsically disordered tails of both α(-) and β(+) tubulin (68, 72, 73). In fact, these synthetic tails are sufficient to compete tau away from microtubules (45). Therefore, tau-binding sites are generally located to the C-terminal half of the tubulin subunits. Tau-tubulin and tau-microtubule interactions are both tau and tubulin protein concentration dependent (74), as well as salt (58) and pH dependent (75) reflecting tau’s heterogeneous, electrostatically driven intrinsically disordered character (76).

**Tau isoforms and domains**

Broadly, when tau is discussed it is in reference to the 6 major neuronal isoforms within humans arising from the alternative splicing of the single MAPT transcript (77). However, other isoforms arising from silenced exons within the human brain exist and vary across cell type and neuronal tissues (5, 56, 57, 77-85). Similar to the tubulin isotypes, tau isoforms are spatially and temporally controlled. Furthermore, the tau gene is both polymorphic containing two major haplotypes (86), and contains an exon-like intron (intron 9) that produces saithoin – a novel, nested protein within the tau transcript also associated with the onset of neurodegenerative disorders (87).

Focusing on the best studied human tau isoforms, the largest isoform is found in the peripheral nervous system (PNS), aptly named ‘big tau’, and contains exons 4A and 6 in addition to exons 2, 3, and 10 (Figure 1.1A) that are alternatively spliced in the human brain (Figure 1.1B) (5, 88). Few studies have been focused on exons 4A and 6 that produce additional sequences believed to control microtubule bundling and neurite extension respectively. Interestingly, exon 6 contains splice sites that result in truncations
that lack the MTBR altogether (85, 89). The longest tau isoform within the human adult brain is 2N4R (Figure 1.1B) in which exons 6 and 4A are spliced out leaving exons 2, 3 and 10. Using conventional tau nomenclature: exons 2 and 3 produce inserts 1 and 2 (N1 and N2 respectively) and exon 10 codes for repeat 2 (R2).

**Figure 1.2: Schematic of tau domains and highlighted functions**  
Adapted from Andreadis *Biochimica et Biophysica Acta* (2005)

(A) Schematic of ‘big tau’ protein domains with alternatively spliced regions labeled according to the governing exon. (B) Schematic of the 6 neuronal isoforms with traditional nomenclature on the right and the percent abundance in the adult human brain on the left. The fetal isoform 0N3R is indicated. The domains and corresponding residues that delineate them are marked: N-terminal domain (NTD) with N-terminal inserts (N1, N2), proline-rich region (PRR) with sub-regions (P1, P2), microtubule binding repeats (MTBR) with four imperfect repeat sequences (R1-R4) flanked by the pseudo-repeat R' and the C-terminus.

All of the major 6 isoforms found within the brain are divided into the projection domain (from the N-terminus to P1) and the binding domain (P2 to the C-terminus) with some studies further splitting tau into a third ‘hinge’ region primarily composed of the
proline rich region (PRR) and the C-terminal most region of the NTD (63, 88, 90). Based loosely on sequence charge characteristics and homology, tau is divided into 4 major regions: the highly negatively charged N-terminal domain (NTD), the proline rich region (PRR), the microtubule binding region (MTBR), and the C-terminal tail (63). The NTD along with P1 of the PRR regulates the spacing between microtubules through electrostatic screening between the two oppositely charged regions (11, 63, 74, 91, 92). The P2 region of the PRR is critical for tau-mediated tubulin polymerization and microtubule binding (67, 93-95), although the underlying mechanism is unclear (66, 93, 96). Additionally, within the PRR region are a large number of kinase and phosphatase sites thereby associating the PRR as a primary site for cellular partner recruitment and tau regulation (3). The MTBR contains 4 pseudo repeats spaced by interlinking regions called inter-repeats (IRs) each of which contain a weak binding site for tubulin (60, 61, 93). Following the MTBR is the C-terminus, which contains a 5th pseudo repeat termed R’ that has been shown to bind to tubulin and enhance formation of tau-tubulin ‘fuzzy’ complexes (58). Both the NTD and C-terminal most ends are also considered regulatory features that recruit other cellular partners.

Focusing on the human neuronal isoforms, the combinatorial presence of 0, 1 or 2 inserts and 3 or 4 repeats gives rise to the 6 isoforms termed: 2N4R, 2N3R 1N4R, 1N3R 0N4R, and 0N3R. These isoforms are developmentally regulated with varying distributions of isoforms across developmental stage, cell type and cellular location (5, 88). Most notably, 0N3R is the sole isoform in fetal brain tissue with the additional inserts and repeats only appearing later in life (56, 83). Interestingly however, the
emergence of additional tau isoforms is not correlated with temporal development of the neuronal tissue itself – ‘older’ tau isoform levels rise together across regions of the brain that mature at different rates such as the neocortex and the cerebellum in fetal rats (97). The MTBR isoforms (3R and 4R) have appreciable differences in both microtubule binding and polymerization capacity (61, 63). By comparison, the differences between NTD isoforms in microtubule binding, polymerization, and bundling are subtle (11, 63, 74, 91, 92), and consequently, it has been proposed the precise function of the NTD isoforms has not yet been discovered (3). Additional complexity comes from various disease states, in which aberrant tau isoform ratios drift from the healthy state. The best characterized of these is exon 10. The ratio of the 3R to 4R isoforms is tightly regulated at 50:50, and divergence from this ratio is a biomarker for Alzheimer’s disease although details and the underlying functional mechanism of these two isoforms remain unclear (98). Within the projection domain, early stages of neuronal apoptosis result in N-terminal truncations (99-101), and excessive levels of 2N isoforms are associated with gliopathy and spinal cord degeneration (102). Finally, tau transcription is also overall increased in those with Down’s syndrome (103). Altogether, these deviances from ‘normal’ tau transcriptional levels and their tight developmental and cell-dependent regulation, have led researchers to propose it is the ratio of tau isoforms that dictate healthy tau function rather than the precise isoform present (88).

**Intrinsically disordered proteins**

Tau belongs to a class of proteins that lack stable secondary structure and tertiary structure termed intrinsically disordered proteins (IDP). IDPs and smaller intrinsically
disordered regions (IDR) compose approximately a third of the proteome (104). On the extreme end of intrinsic disorder, some IDPs and IDRs randomly sample space only influenced by the peptide backbone itself and are unconstrained by inter-residue interactions (hydrogen bonds, pi-pi stacking, electrostatic interactions, etc) associated with higher order structures within structured proteins (105, 106). Within this thesis, I use the Gaussian polymer chain model that describes each amino acid as a randomly oriented monomer as a part of a polymeric chain that is bonded freely to the next monomeric unit. This Gaussian polymer chain samples space as a statistical distribution of states representing a variety of conformations within the polypeptide restrained only by the peptide bond length. (105, 106) Collectively the entire distribution of conformational states is termed a conformational ensemble. When the peptide bond length is the only restraining feature of this conformational ensemble, it is termed a random coil (106). A simplified cartoon version of a random coil is drawn in Figure 1.3A. However, not all conformational ensembles are random coils. Although conformational ensembles more broadly sample conformational space than the canonical well-folded domains α-helices and β-sheets; conformational ensembles can have conserved characteristics. Unlike structured proteins that have conserved and predictable motifs secondary and tertiary motifs, the driving characteristics of IDPs are primarily based on the primary amino acid sequences (107-110). Differing sequence characteristics – most importantly the fraction and distribution of positive and negative charges – determine the degree of compaction or expansion and segregate IDPs into different sub-classes (108, 111). More recently, the linear patterning of amino residues proved to be a strong predictor of IDP character
besides just their net composition within the sequence, and the suite of correctional theoretical considerations (pi-pi stacking from aromatics, steric restrictions from prolines, increased hydrophobic character, etc.) in IDP character prediction is rapidly expanding (111). These biophysical parameters thereby limit the random coil’s distribution of states within the conformational ensemble resulting in a less extended conformational ensemble that still broadly samples space but with additional biophysical constraints in comparison to the random coil (Figure 1.3B) (111). Additionally, transient secondary structures may form within the IDP itself (Figure 1.3C). This transition may driven by post-translational modification (112) or binding to a ligand (113) (discussed in subsequent paragraphs).

On top of IDP structural heterogeneity are inter-protein interactions. The disorder of IDPs or IDR s has known physiological roles of biological consequence including transcriptional regulation, cell signaling, and chaperone activity (112, 114, 115). The most frequently encountered examples are as ‘spacers’ between structured domains that remain unresolved in atomically resolved x-ray crystallography structures. Another related form of this function is a molecular cushion in which an IDP functions to space or ‘cushion’ interacting cellular partners such as tau’s N-terminal domain (NTD) and its role in microtubule bundling (63, 74, 91, 92). Beyond regulating space and dynamics, many IDPs contain binding sequences. These can collapse in a disorder-to-order transition upon binding (113).
**Figure 1.3: Intrinsically disordered proteins and their ensembles**

(A) A noodle diagram of a random coil protein. Alternating regions of red and blue are for added clarity. Black arrows indicate a single peptide bond and its orientation. (B) A noodle diagram of a conformational ensemble with the same length alternating red and blue regions from (A) to demonstrate relative restricted movement of the protein in comparison to a true random coil as drawn in (A). This may be due to several biophysical factors. For example, a hydrophobic pocket (shaded gray) may cause collapse in a particular region of the ensemble and favor a particular global architecture devoid of canonical secondary structure and still very heterogeneous and dynamic. (C) The formation of an alpha helix (red cylinder) in equilibrium with a conformational ensemble. (D) The binding of a ligand to a multivalent IDP. The binding of a ligand may not result in a conformational change within the IDP region it binds but will still bind specifically. This is termed a ‘fuzzy’ complex. Different binding valences with similar conformational ensembles can be grouped together into a ‘subclass’ (boxed). The binding of the ligand may cause conformational changes within the IDP. Each individual conformation with a specific bound ligand is a microstate. Altogether, the different binding valences and conformations grouped together are termed a global ensemble. It is important to remember each microstate and the unbound state are all in equilibrium with each other.

However, an ordered binding state is not a pre-requisite, and in many cases the disordered binding region and its transient interactions are favorable and biologically relevant. One common and widespread example is of a PTM to a disordered region resulting in
transient binding to a cellular partner during signal transduction (112). In this case, the binding interactions are stochastic, and the IDP contains a significant amount of structural heterogeneity on the surface of its bound partner. When these disordered interactions are specific but the structural disorder is still maintained, the heterogeneous structural conformations between the two bound proteins are collectively termed a ‘fuzzy’ complex (116) (Figure 1.3D).

The fundamental biophysical parameters of bond length, primary amino acid sequence, and electrostatic or hydrophobic interactions are the same within both classes of structured and intrinsically disordered proteins but the relative number and the corresponding net strength of each factor in governing net interresidue interactions varies between the two classes of proteins. For many IDPs and IDR, a ‘structure’-function description of one specific state is misleading in that these classes of proteins sample large conformational spaces. In some cases, this conformational ensemble persists in the bound state and may be multivalent. Prior work modeling multivalency of synthetic SH3 linked domains demonstrated binding configurations, specifically in transient nonequilibrium states, were influenced not only by the valency of the synthetic peptide but also by linker length and linker rigidity (117). Importantly, both the valency and IDR character of the linkers influenced early-stage binding dynamics. In this study, the authors introduced the idea of microstates in which one microstate represents a specific combination of the conformational state with one or more specifically occupied binding sites. Here, stoichiometry and occupancy are different – occupancy of sites 1 and 2 within in a trivalent system is different from occupancy of sites 2 and 3 resulting in two distinct
microstates (117). For both microstates, the stoichiometry is the same. In this model, all the binding affinities were assumed to be equal. To reiterate, a microstate is one specific conformation and occupancy of an IDP or IDR bound to its partner with the associated thermodynamic factors (Figure 1.3D). These microstates and the equilibrium between them are represented as a network that can be then modeled and predicted using ordinary differential equations (Figure 1.3D). Specific subsets of microstates were collected together into subclasses based on conserved conformational characteristics (117). A subclass is a set of microstates that have subtle differences in their precise conformation that results in energetic differences despite identical stoichiometry to the bound partner. Depending on the steric constraints of the given conformations, certain subclasses were enriched in comparison to other constrained subclasses. The dynamics between subclasses and the individual microstates represents a dynamic network (117). Inherent in their model is what I will term a global ensemble (Figure 1.3D).

A global ensemble is the probabilistic distribution of microstates – which may or may not be uniformly distributed into one subclass or modally distributed into multiple subclasses – that are in equilibrium with each other. Underlying all equilibrium are fundamental physical parameters of the energetic landscape. Therefore, a global ensemble can be influenced by perturbations such as binding of a novel ligand or environmental factors such as ionic activity. The altered energetic landscape would result in a ‘rerouting’ of populated microstates and larger subclasses. It is possible certain subclasses may have different biological functions. Therefore, previously unpopulated or underpopulated subclasses with a certain biological function would become populated
and a new emergent biological function would characterize the global ensemble. The same fundamental states would still be present within the system but a seemingly new emergent property would be evidenced.

Once again, tau serves as an example. Tau is an IDP with a conformational ensemble that deviates from a random coil and binds multiple tubulin dimers (62, 118, 119). Upon binding to tubulin, there are conformational changes throughout the protein (62, 118, 119). Previously, our lab demonstrated that the MTBR maintains an overall compact structure when bound to tubulin in solution, but that each individual repeat expands upon binding (119). It was hypothesized that the flanking repeats compacted in order to accommodate changes within the MTBR upon binding to a tubulin subunit with differences evidenced between the MTBR isoforms (119). Additionally, shifts in the ET_{eff} of two different FRET pairs (one spanning the N-terminus to the middle of the protein, and another spanning sites within the MTBR) occurred at different concentrations of tubulin, indicating a two-state conformational change within tau upon binding to tubulin (119). Additional work demonstrated that the degree of ‘fuzzy’-complex formation and the number of weak binding sites within the microtubule binding region (MTBR) and flanking pseudo-repeat R’ positively correlate with microtubule polymerization (58). Whether or not the precise occupancy at every potential binding site is influenced by or dictates the conformational ensemble is unknown. It is therefore important to consider both the occupancy and conformational ensemble when studying tau-tubulin interactions.

One way to represent the convoluted conformation and occupancy of tau-tubulin interactions is with the network global ensemble interpretation (Figure 1.4). For example,
while a tubulin dimer may be bound in the R1 repeat, the NTD may be expanded or compressed resulting into two separate microstates (Figure 1.4B) belonging to two separate subclasses (Figure 1.4C). If the two separate subclasses would each have five different microstates (considering five possible tubulin binding sites in the MTBR-R’) all of which are in equilibrium with each other (Figure 1.4C, bottom graphic). In practice, it serves as a theoretical, pictorial backdrop to avoid common incorrect implications that arise when discussing IDPs – that a single microstate is present with a specific valency and occupancy. Bluntly, the network picture of microstates and subclasses with the global ensemble serves only as a theoretical basis to emphasize the balance and heterogeneity of both the IDP conformation as well as the precise occupancy and stoichiometry. This is particularly advantageous when a particular microstate or subclass leads to a committed biological response – such as an aggregation-prone state – and therefore influence the overall global ensemble in a unique way to other subclasses or microstates regardless of the most populated microstate or subclass within the system. From an experimental standpoint (further discussed in section “Common experimental techniques”), distinguishing individually each microstate within globular proteins is challenging, if not impossible, let alone within the more conformationally heterogeneous IDPs. While this network representation has practical modeling and theoretical advantages, it is important to remember that this representation is more granular than conformational ensembles within IDPs whose ‘structures’ remain best captured by the noodle diagrams in Figure 1.3. Understanding the underlying biophysics of these tau-tubulin ensembles is directly applicable to understanding their biological function. For example, the propensity for the unbound versus bound state in tau-tubulin ensembles was altered by two tau disease point
mutants associated with Alzheimer’s disease, P301L and P322L. This study of point mutations impacting tau-tubulin assembly demonstrated that the equilibrium of states (or the global ensemble) within tau-tubulin assemblies can be altered by small changes to the energetic landscape (62).

**Figure 1.4: Network modeling as applied to tau-tubulin interactions**

(A) Cartoon of tau-tubulin binding. The MTBR-R' (red) binds to tubulin through distributed, weak interactions. The NTD expands upon tubulin binding. The PRR and C-terminus are colored orange and black respectively. The incoming tubulin dimers are bound at particular sites as indicated by a red asterisk (*). B) Example microstates in the first and second tubulin binding event to regions MTBR-R' with hypothetical expansion of the NTD distinguishing subclasses “E” and “C”. C) Possible microstates in the first tubulin-binding event separated into two subclasses “E1” and “C1”. Two hypothetical subclasses ‘C1’ and ‘E1’ represent a closed and open formation for the NTD, respectively. These two subclasses are in equilibrium with each other as is each microstate within each subclass.
Recently, IDRs and IDPs have come to the forefront of attention due to the discovery of liquid-liquid phase separation (LLPS) within cells (120). Phase separation, or the formation of biomolecular condensates, is the selective sequestration of macromolecular biomolecules such as RNA or protein into a liquid-like droplet (121). They are remarkably sensitive to environmental conditions, and can be triggered by both small molecules and macromolecules such as RNA (120, 122). These separate phases are proposed to drive the formation of membraneless bodies such as the nucleolus (123), and have several far-reaching implications for developmental biology, enzymology, cell signaling and mechanics. Indeed, the phase separation of tau nucleating bundled microtubules has recently been proposed as mechanism for microtubule initiation in axons (124). Comparing LLPS states to dissolved (un-separated) IDP microstates and global ensembles presents yet another concentration-driven layer to IDPs interactions. It also emphasizes the sensitivity of these proteins under a variety of experimental conditions.

**Common experimental techniques**

Experimentally probing the structural heterogeneity of IDP interactions is uniquely challenging. IDPs and IDR are conformational ensembles that represent a large distribution of conformations. Consequently, bulk structural experimental techniques that include averaging steps probe primarily either the average structure of the entire ensemble or the most frequently populated structure depending on the experimental design. For example, x-ray crystallography relies heavily on homogeneous and periodic structural characteristics to capture scattered electron densities (125). IDPs and IDR
sections generally do not have measurable electron densities, and are therefore unresolved in x-ray crystallography structures. Consequently IDP and IDRs are not easily probed using this approach. Nuclear magnetic resonance (NMR), circular dichroism (CD), and cyro-EM are better suited for heterogeneous structural information (76) although all of these techniques rely on averaging within the experimental design.

Both NMR and CD use signal-averaging techniques. NMR perturbs the artificially aligned magnetic moments of individual nuclei within a molecule, and then measures the resulting electromagnetic frequency of their relaxation back to the aligned external magnetic moment (126). Therefore theoretically, each nucleus within the protein is measured individually. However, due to detection sensitivity, only sufficiently populated conformations are detected. Within the NMR field, extending the detection power of smaller populations is enhanced by literally using a bigger and stronger magnet, which increases the number of aligned particles prior to perturbation as well as increases signal strength and sensitivity. Additionally, all the detected frequencies are collected together. A particular nucleus that is experiencing a uniform distribution of different nuclear fields (chemical environments) will be measured as an averaged broad frequency. Thus, while NMR successfully collects heterogeneous and dynamic information from protein conformational ensembles, it is dependent on sufficient levels of a given conformation with distinctive frequencies to successfully distinguish different conformations. NMR has been frequently and successfully employed to probe tau’s solution structure and when bound to microtubules or stathmin-stabilized tubulin
oligomers (65, 67, 127). In these studies, NMR has shown transient secondary structures within two of tau’s regions, the MTBR and proline rich region (PRR) (67).

Similarly, CD averages a given signal within a bulk sample. In CD however, the observable is the polarity of absorbance of near-UV light (typically 260 nm to 180 nm) (128). This technique probes the amide-backbone of secondary structures such α-helices and β-sheets. The entire signal from all the conformations and populations within the sample are collected together at each wavelength representing a sum of the ellipticity of all the conformations weighted by relative populations and characteristic intensities. In comparison to NMR, CD is far less sensitive as each peptide bond absorbs within the same wavelength range and the all the measured polarities at each wavelength are measured and averaged together (128). CD is essentially a bulk measurement representative of the average of the entire conformational states. Thus, while NMR can experimentally resolve a bi-modal set of conformations by measuring two distinct peaks from a continuous distribution of conformations, CD cannot and the measured signal represents an average of the entire sample. However, additional computational and theoretical modeling of CD data or supplemental experiments can de-convolve separate populations within the sample.

Cyro-EM does capture individual conformations by taking thousands of EM images of immobilized particles orientated randomly on the detection grid. (129) However, in comparison to NMR and x-ray crystallography, the atomic resolution of these images is significantly lower (typically ~0.2 nm) (129). To overcome this resolution limit, thousands of EM images are classified and then summed together to form a more
accurate 3D image density. This 3D image density can then be modeled similar to an x-ray crystallography structure (129). In this experimental technique, the building of the classes prior to 3D structure determination is an averaging technique in which images are summed together according to their relative populations (129). Recently, cyro-EM was successfully employed to deliver near atomic-resolution of synthetic tau constructs bound to stabilized microtubules (Figure 1.2) (1). Unfortunately, many key tau regions surrounding tau-microtubule interactions are missing from this most recent structural advancement, such as the PRR and R’, highlighting the need for continued focus on the heterogeneous, largely unstructured, and dynamic tau-microtubule and tau-tubulin interactions.

Single-molecule techniques have a greater discriminatory power when examining individual conformational states within conformational ensembles in comparison to other techniques with averaging steps. Single molecule Förster resonance energy transfer spectroscopy (smFRET) uses the distance-dependent energy transfer between two dyes to measure the distance between two points (130-132). SmFRET techniques are diverse (133); for brevity I will discuss the technique used in this thesis – solution and intensity based intramolecular FRET. In this case, picomolar concentrations of a doubly labeled protein diffuse through a focused laser confocal volume (approximately a femtoliter in volume) (Figure 1.4A) (130). The probability that two molecules occupy the confocal volume is low, and therefore individual molecules are measured. The laser excites the donor dye that either fluoresces or transfers energy nonradiatively to the acceptor dye which in turn also fluoresces (Figure 1.5A) (131). The laser is focused and the incident
light on the sample is great; consequently, the sample is ‘flooded’ with excitation light. Furthermore, both the direct emission of the donor and the energy transfer between the dyes are fast (on the ns timescale). The fast timescale of FRET events and ample excitation light allow for many photophysical events to occur within the timescale of the experiment, which is limited by the ~ 1ms diffusion of most proteins. Therefore, photons from both the donor and acceptor fluorophores are measured although they do not occur simultaneously (Figure 1.5A). The efficiency of the energy transfer is distance dependent and can be calculated using the Förster resonance energy transfer equation (Eq. 4 in Appendix I.iiv). As the dyes come closer together, the probability of energy transfer increases and greater efficiencies are measured (Figure 1.5B, top and second panel). However, if the distance between the dyes is too great, the probability of energy transfer drops to zero (Figure 1.5B, third panel).

When a sufficiently large number of events are collected (typically in the tens of thousands), the individually calculated energy transfer efficiencies are plotted as a histogram. For IDPs, these histograms are frequently fit with one of a number of polymer models in order to convert the energy transfer efficiency distribution to a distance distribution (Figure 1.5B) (130, 131, 133). This thesis uses the Gaussian coil model (discussed in “intrinsically disordered proteins”). The range of distances that can be accurately determined is dependent primarily on the chosen dye pair. Within this thesis, I use FRET dye pair Alexa-Fluor 488 and Alexa-Fluor 594 allowing me to probe distances between 30 – 100 Å (134). SmFRET with the same dye pair has been previously used to characterize both a fragment of tau’s unbound and bound structure showing in which the
N and C termini become more distant upon binding to tubulin with variable compaction within each MTBR repeat (118, 119). With smFRET, individual distances are not averaged or detected together in bulk, although frequently the average root mean squared distance (RMS$_{exp}$) from the fitted histogram is reported along with the histogram distribution of energy efficiencies or converted distances (Figure 1.5B) (130). Although not frequently quantified, the breadth of the measured histogram may reflect the dynamics of conformational sampling by the protein – a broad peak may reflect slow (relative to the timescale of the measurement) sampling of distances between the two dyes (Figure 1.5B) (135, 136). For example, the relatively slow sampling of conformational states between two fluorophores would result in a broad histogram (Figure 1.5B, top panel) while more rigid and constrained regions such secondary structures may have a narrow histogram (Figure 1.5B, fourth panel) (135, 136). However, interpreting the breadth of peaks is convoluted; one important possibility is that different populations within the sample that are not distinguishable or stably populated (135, 136). For example, consider a transiently folded helix within an otherwise folded protein. Only if the folded and unfolded distances are sufficiently different and also stably populated, the two separate populations can be detected and quantified (Figure 1.5B, bottom panel) (136). Very broad peaks, however, make unique populations difficult to separate from heterogeneous or transient ones (132, 135, 136). Within smFRET, only the distances between each dye pair are measured, and residue-specific information is missing (Figure 1.5B). Therefore, 3D models are experimentally difficult to build with smFRET data alone. FRET constrained molecular dynamic simulations provide additional structural resolution (119).
The advantage of a single-molecule structural technique is in cases when there is a small population of a unique conformational population not characteristic of the bulk or average protein. Continuing with our transiently folded helix example, consider the case in which only 5% of the helix is unfolded and the transition between these two states is slow (on the ms timescale) (Figure 1.5C). Using smFRET and a variety of appropriately placed dye positions, the unfolded population would be detected although a full model of the structural protein would still be missing (Figure 1.5D). If the same structure was probed using NMR, the individual nuclei would give rise to a variety of frequencies, some clearly distinguishable and others more broad, reflecting their molecular movement and yielding an atomic model with a number of potential conformations within the IDR regions (Figure 1.5E). However, unless the population of the unfolded helix was sufficient, it would go undetected as a separate state within the protein global ensemble (Figure 1.5E) using NMR. If the same protein were measured using cryo-EM, the measurable densities would correspond to primarily to folded regions and a 3D model could be threaded with known homologous structures (Figure 1.5E). Here, although the unfolded population would be captured on the EM grid, it is unlikely the unfolded region would be preserved through the 2D class analysis due to both poor resolution from its IDP nature and its overall low population within the sample. For both cryo-EM and NMR, whether or not the small unfolded region is detected is dependent on the both the strength of the instrument’s detection and the percentage of the population each conformation comprises. This problem is amplified in IDP systems that contain a very large number of conformations and consequently the proportion of each individual
conformation is lower. Using smFRET overcomes the need for sufficient levels within each population to be detectable as a single-molecule technique (132, 133).

Legend on page 29.
(A) Doubly-labeled protein diffusing through a focused laser beam. The confocal volume indicated by a dashed black circle. When the protein diffuses through the confocal volume, the donor dye is excited which in turn excites the acceptor dye. Both fluorescence intensities of the donor and acceptor are recorded together. (B) A protein model with two dyes that are a FRET pair. Orange arrows represent a beta sheet, the red cylinder an alpha helix, and the black lines represent IDR regions. The energy transfer between the two dyes is distance dependent and can be calculated to give the energy transfer efficiencies (ET_eff) between the dyes (represented by dashed blue line). Due to molecular movement (indicated by the solid blue double arrow), the distance between these two dye pairs fluctuates. If sufficient FRET events are recorded, a histogram of the ET_eff emerges, and can be fit with models to give the corresponding distances (first panel). When the dyes are close together, the histogram shifts to higher ET_eff (second panel), but if the dyes too are far apart there will be no energy transfer, and the recorded fluorescence is only that of the donor (third panel). This lower peak centered on zero, in which no FRET is occurring, is termed the ‘zero-peak’. The width of the histogram may also reflect the degree of molecular movement between the two dyes resulting in narrower peaks for relatively immobile regions (fourth panel). When two different conformations exist between the dyes, then two peaks will be detected. This is assuming the two conformations have sufficiently different distances to be distinguishable (bottom panel). (C) Hypothetical example of a folded protein with an unstable helix that comprises 5% of the total protein population with molecular dynamics on the ms timescale. (D) Probing the system in (C) using smFRET. Using a few dyes positions, distances (black dashed lines) can be extracted but not residue specific information. Without additional experimental probes and molecular molding, the protein tertiary and secondary structure remains unknown (represented by gray protein cartoon). However, assuming appropriately placed dyes, the unstable helix would be detected (red dashed line). (E) Probing the system in (C) using NMR. Each labeled nuclei (here nitrogen) can be detected with a characteristic chemical shift (δ, ppm). Depending on the molecular movement and its timescale, the chemical shifts may be sharp and intense (light blue) or broadened (dark blue). With proper chemical labeling and pulse sequences, an entire molecular model of the protein can be built. The multiple lines for the IDRs represent the possible ensembles based on the detected broad peaks. However, small populations are generally below the detection limit of most NMR machines, and the unfolded helix would not be detected but hidden within the noise. (F) Probing the system in (C) using cryo-EM. Each individual conformation is detected on the EM grid. However, the 3D class average generally will not contain the IDRs or the disordered helix. Additional molecular detail comes from modeling homologous proteins into the 3D class average.
Finally, fluorescence correlation spectroscopy (FCS) can be used to detect the size of a fluorescently labeled species as it diffuses through a focused laser, again about a femtoliter in volume (130, 137). Similar to smFRET, the fluorescence intensity of a fluorescently tagged molecule is measured. In FCS, the fluctuations of that fluorescence intensity are autocorrelated and the resulting autocorrelation curve can be fit using the appropriate diffusion model (Figure 1.6A) (130, 137). Qualitatively, larger complexes shift the midpoint of the autocorrelation curve (G(τ)) to longer times and consequently have larger diffusion times. Additionally, the magnitude of the autocorrelation curve at time 0 is inversely correlated with the number of molecules, this corresponds to an increase in the number of molecules measured within the confocal volume as the amplitude decreases (Figure 1.6A). Quantitatively, biophysical parameters such as the number of molecules within the confocal volume, the average brightness or counts per molecule (CPM), and diffusion time can then be extracted (138). If proper standards are measured on a calibrated system, diffusion times can be converted to the molecule’s radius if it is assumed a sphere (most commonly the radius of interest is the hydrodynamic radius).

As FCS is sensitive to the molecule’s size, it can be used to determine ligand binding. Accurately fitting and quantifying changes in diffusion times between the unbound and bound species within the same sample requires a 8-fold increase in the mass ratio of the measured globular protein species due to mathematical and experimental restraints (a two-state diffusion model) (138). However, small changes in diffusion times
for the entire sample are easily measurable and can be considered in terms of their trends (a single-state diffusion model in which the average diffusion time of the entire sample is reported) (Figure 1.6C). These diffusion time averages are typically of several molecules at a time (in this thesis, ~10 to 20 molecules). This makes FCS an extremely useful technique for selectively measuring the diffusion times of protein-ligand complexes in a semi-single-molecule manner. As ligand is titrated into a solution of labeled protein, the diffusion time shifts to longer diffusion times (Figure 1.6B). Plotting the diffusion time ($\tau_D$) against the ligand concentration produces a binding curve (Figure 1.6B). However, as the size of the protein complex is being experimentally measured, conformational changes and ligand binding are convoluted and extracting binding parameters (such as the apparent binding affinity, $K_D$) may require additional or supplemental experimental information. Furthermore, the error in the number of molecules detected can vary between 20-30% due to photophysical damage to the fluorophore (138). Despite the low resolution of conformational information, FCS informs on the heterogeneity of multivalent ensembles as it detects changes is molecular size in a semi-single-molecule manner (58, 62, 130). As conformation and occupancy are convoluted, distinguishing separate microstates may not be possible. Instead, a spread of diffusion times and brightness within the ensemble are measured and are qualitatively distinguishable from homogeneously uniform samples (Figure 1.6C) (58). The spread of diffusion times indicates multiple different species within the sample that may indicate a larger network of microstates than within a homogeneous sample. A homogenous sample may still contain an equal number of microstates and subclasses as its heterogeneous counterpart,
but they would all be indistinguishable using FCS. Complementary structural techniques such as NMR or smFRET may inform on the conformational ensemble and the number of subclasses while calorimetry techniques to distinguish differential binding events may distinguish different occupancies.

Legend on page 33.
Figure 1.6: FCS and common complementary experimental techniques

(A) Singly-labeled protein diffusing through a focused laser beam. The confocal volume indicated by a dashed black circle. When the protein diffuses through the confocal volume, the dye is excited and the fluorescence intensities are recorded. The fluctuations in intensity are autocorrelated in time, resulting in an autocorrelation curve (G(τ)). The midpoint of the autocorrelation curve increases in time with increasing diffusion time while the amplitude at t=0 decreases with increasing number of molecules detected within the confocal volume. (B) As FCS is a measurement of size, it can be used to measure ligand binding. As unlabeled ligand is added to a labeled protein sample the overall diffusion time increases. The increasing diffusion times can be plotted as a function of ligand concentration to measure binding. (C) FCS is a semi-single molecule technique. Each measurement can be fit with a diffusion time and the corresponding spread reflects the degree of heterogeneity within the sample. Affinity, conformation, and stoichiometry are convoluted together as the size of the complex is measured as indicated by gray circle around model protein. (D) DLS measures the scattered light from an incident laser beam on a diffusing molecule. As this method does not use a fluorophore, the relative intensities of all the particles are reported. (E) AUC uses gravity to establish a concentration gradient as well as further distinguish heavier particles between similarly sized ones. This technique therefore allows for a continuous distribution of species to be determined and can potentially more accurately determine stoichiometry. (F) Fluorescence anisotropy is another method commonly used to determine binding. It uses polarized light and the resulting change in polarization from the fluorescence of a dye to calculate anisotropy. The anisotropy can then be plotted against ligand concentration. (G) SPR uses ligand immobilized on a gold film. The angle at which the light is resonant and reflected from the film is sensitive to the binding of incoming protein. This two-phase experiment in which incoming protein is flowed into the chamber and then subsequently buffer to remove the protein provides information on stoichiometry and kinetic information.

Similar to FCS, dynamic light scattering (DLS) and analytical ultracentrifugation (AUC) also measure the size of particles and provide the relative distributions of sized particles within the sample. For DLS no fluorescent labels are needed as it uses a collimated laser beam (a perfectly straight laser beam that theoretically does not diffuse or come to a focused point) (139). The light from the laser is scattered by particles and
observed; the relative intensities within the sample and particle size of the various species are calculated (Figure 1.6D) (139). In this case, there are additional photophysical characteristics that need to be considered. Examples include the surface of the scattering molecule and the angular dependence of the scattered light (termed anisotropy discussed at length later). These factors result in additional convoluting factors when analyzing DLS data (139). Although the fundamental photophysics are different, many theoretical and mathematical principles of FCS are derived from DLS and the two techniques face similar challenges. An advantage of DLS is that all species within the sample are quantified – not just the labeled protein of interest. However, this includes aggregates and dust. The reporting fluorescent label used in FCS allows for only the protein of interest and its ligand bound species to be analyzed.

Another fluorescent-independent diffusion-based technique is AUC. However, in AUC an additional force – gravity – is used to further discriminate particles based on weight and in part size (arises from drag) as the particle sediments (Figure 1.6E) (140). Due to the added force, a gradient across the sample is established, and therefore concentration of the species can be readily determined (140). This results in a continuous distribution of species characterized by their sedimentation co-efficient which is a determined by size and molecular weight (Figure 1.6E) (140). Fluorescent dyes can be added to further discriminate between species, and the measured fluorescence intensity can also be used to calculate concentration (140). One power of AUC is that stoichiometry can be determined accurately. The drawback is the length of time to acquire AUC data may be prohibitive for some systems. Interestingly, the sedimentation
co-efficient for tubulin changes throughout an AUC experiment (141). Whether this is due to instability of the protein or the concentration gradient is unknown. However, AUC with fluorescently labeled tau has been successfully used to characterize the stoichiometry of tau bound to soluble tubulin (142). However, AUC is a bulk measurement and single-molecule statistics are not quantified. This leaves an individual microstate’s detection dependent on its relative population within the sample.

As previously mentioned, buried within DLS data analysis are anisotropy values which can inform on the overall shape of the molecule. Anisotropy is the angular and directional dependence of a physical characteristic. However, many techniques to measure the anisotropy of physical properties exist and a thorough discussion of them all is beyond the scope of this thesis. Here, I will highlight one method in particular – fluorescent polarization (typically referred to as simply ‘anisotropy’) (Figure 1.6F) (143). In this method, polarized light is absorbed by fluorescent molecule which then emits the light along several axises as it tumbles through the solution (Figure 1.6F). Depending primarily on the fluorophore’s immobility and the diffusion of the labeled species, the emitted light may also be polarized along a separate angle from which it was absorbed (143). The ratio of fluorescent intensities along these axises are calculated. As ligand is added and the size of the molecule increases there is a corresponding change in anisotropy (Figure 1.6F) (143). Once again, this is a bulk technique, and it is difficult to capture statistics that look at the heterogeneity of microstates within the sample.

Unique to the above techniques, surface plasmon resonance (SPR) can provide kinetic parameters, concentrations, and affinities of protein-ligand interactions without
additional supplemental experimentation (Figure 1.6G) (144). Briefly, ligand molecules are immobilized on an electrically conductive surface whose index of refraction and resonance with the incoming polarized light changes upon binding of the analyte protein. If the degree of binding is plotted with time, the association and dissociation phases contain $k_{on}$ and $k_{off}$, respectively (Figure 1.6G) (144). Although an exceedingly powerful technique, SPR is both difficult and expensive as the conductive layer is traditionally made of gold and is exceptionally susceptible to experimental noise (144). Furthermore, the technique requires the immobilization of ligand on the surface that may or may not interfere with protein-protein interactions. SPR has been successfully used to study tau peptides interactions with proteins found in neurodegenerative diseases (145), but to the best of my knowledge, not tau-tubulin interactions. Again, SPR is not a single-molecule technique but a bulk film measurement. The potential resolution of different microstates comes from advanced theoretical and mathematical modeling from this information-rich technique (117). However, this has yet to be developed across a variety of systems and remains a primarily theoretical consideration for all the above techniques including SPR.

Finally, the above experimental techniques have varying restrictions on protein concentration, buffer conditions, and sample preparation. NMR, CD, cryo-EM, DLS, and AUC generally require milimolar concentrations or protein (126, 128, 129, 139, 140). While NMR, DLS, and AUC are amenable to varying buffer conditions (126, 139, 140), CD and cryo-EM are comparatively limited due to interference from various salts (128, 129). Furthermore, cryo-EM requires immobilization of the sample on the EM grid, and sample optimization is required to ensure protein-protein interactions are not altered.
While smFRET and FCS are not broadly restricted by buffer conditions, assuming they are not fluorescent, these techniques are limited to the picomolar and nanomolar protein concentration range (130). As previously discussed in “Intrinsically disordered proteins”, IDPs and IDRs are very environmentally sensitive and have concentration-dependent behaviors. Many structural and biochemical studies focusing on tau-microtubule and tau-tubulin interactions frequently used buffer BRB80 (63, 65, 67, 127). BRB80 contains the nonphysiological buffer component piperazine-N,N’-bis(2-ethanesulfonic acid) (PIPES) and was developed for purification of soluble tubulin from MAPs (146). Here I use a low ionic phosphate buffer that is more physiologically relevant; details in the screening of tau:tubulin interactions are discussed in Chapter 3 and Appendix I.

As I am interested in the broad heterogeneity of the system both in terms of conformation and occupancy rather than elucidating a specific representative structure (a specific microstate), I have chosen FCS and smFRET as my experimental techniques. Both smFRET and FCS are single-molecule techniques that uniquely provide statistical information on heterogeneous samples such as IDPs. Supplemental molecular detail from NMR or cyro-EM, and confirmation of stoichiometry from AUC or affinity data from calorimetry studies or SPR may further pinpoint differences between subclasses and microstates. However, no technique to date can distinguish the whole suite of biophysical parameters within a global ensemble. Furthermore, due to differing experimental conditions between the aforementioned techniques, the measured conformational ensembles may not be directly comparable between techniques, and observable
differences between techniques simply reflect heterogeneous, environmentally sensitive ensembles. This does not grant unique authority of one technique over another; instead a full sampling of the range of IDP interactions informs on the entirety of the IDP ensemble.

**Scope of this work**

Prior work within the field focused on tau-microtubule interactions, and utilized tau constructs predominately isolated by enzymatic digestion of tau bound to microtubules despite the demonstrated importance of the tubulin tails to tau binding and their enzymatic susceptibility (45, 63, 147). Given that soluble tubulin unincorporated into the microtubule lattice represents half of all cellular tubulin (148), understanding tau-tubulin interactions is critical for understanding the mass action of microtubule dynamics in the presence of tau. NMR studies of tau binding to stathmin-stabilized tubulin complexes have suggested specific regions in tau-tubulin binding interactions (65, 66). However, in all these cases, tau-tubulin interactions were studied in the presence of other stabilizing MAP constructs forming either artificially enforced tubulin interface surfaces between two heterodimers or possibly obstructing tau binding sites. Of the available tau-tubulin information, the NTD isoforms were not studied, rather the MTBR isoforms (119) – 2N4R and 2N3R – which combined represent only 7% of the cytosolic tau pool (88). As the ratio of tau isoforms present has been proposed to influence tau-mediated behavior, there is an obvious gap in the understanding of the NTD isoforms. The work presented here specifically addresses the impact of the tau NTD isoforms on tau-tubulin interactions. To reiterate, I chose smFRET since it is a single-molecule technique and a
**priori** knowledge of bimodal populations of conformations within the tau-tubulin ensembles is missing. One theory that the inserts themselves influence tau-tubulin interactions is implied within the literature (88, 91); if unique subpopulations exist between NTD isoforms, smFRET may be able to distinguish them while also collecting more broad coarse-grained information on the tau-tubulin conformational ensemble. Here, I used a variety of dye positions expanding several regions to probe the conformational ensembles of the NTD isoforms unbound and upon binding to soluble tubulin. I used smFRET-derived RMS$\text{exp}$ to identify a conserved, insert-independent conformational ensemble within the NTD/PRR/MTBR regions in tau’s unbound state (Chapter 2). Future studies using NMR for atomic resolution or advanced modeling from molecular dynamic simulations may distinguish governing interactions between these regions and possibly highlight more subtle differences between the isoforms.

To functionally probe these interactions between the NTD, PRR, and MTBR systematically in a domain-centric and isoform-dependent manner, I used FCS to measure binding of the NTD isoforms and tau fragments to soluble tubulin. Here, I quantify the increase in diffusion time as tubulin is added to fluorescently labeled tau to compare the variable binding between domains, as well as consider the varying degrees of heterogeneity in the sizes of tau-tubulin species formed – a convoluted reflection of both the affinity and degree of conformational heterogeneity within the tau-tubulin complexes. Primarily due to novel construct design, I reveal unprecedented tight and specific binding of the PRR region to soluble tubulin and demonstrated that PRR is capable of polymerizing tubulin independent of the MTBR (Chapter 3). Together, my
findings support an alternative model to other tau-mediated microtubule polymerization models. Additional studies of PRR-microtubule interactions are ongoing. Given the tight and specific interaction of PRR to tubulin, it is also a promising candidate for SPR measurements to quantify a variety of biophysical parameters including kinetics and confirmation of the FCS-determined stoichiometry.

Finally, with several collaborators, I sought to continue prior work comparing tau-tubulin and tau-microtubule interactions. We find there are differences in between tau’s binding preference for GDP-tubulin over GMPCPP-tubulin within microtubule lattice that cannot be recapitulated in solution (Chapter 4). This preliminary data suggests strain along the lattice may regulate tau binding. Structurally, we used the environmentally sensitive fluorophore acrylodan to probe the structure of the MTBR when bound to soluble tubulin and compared our results with the recent cryo-EM structure (1), and found the tau-tubulin and tau-microtubule interactions to be homologous throughout the MBTR. However, PRR binding to the microtubule lattice was not resolved in the cryo-EM structure (1). This led us to further investigate the role of tubulin tails to find a dominating role in tau-tubulin binding (Chapter 4). PRR binding is significantly reduced although not completely knocked down upon subtilisin digestion of the tubulin tails. This suggests PRR binding is mediated in part through both the tubulin tails and the tubulin body. Thus, tau-tubulin interactions may be capable of reading the tubulin ‘code’ in solution as well as on the lattice through these two binding sites on the tubulin body and the tails. Collectively, the work presented here opens many possibilities regarding
regulatory interactions of microtubule dynamics governed by tau’s interdomain interactions and their sampling of the tubulin binding sites.

CHAPTER 2: The N-terminal isoforms have a conserved ensemble independent of sequence

This chapter is adapted from McKibben, K.M. and Rhoades, E., “Independent tubulin binding and polymerization by tau’s proline rich region is regulated by its N-terminal domain” (2019) 294(50) 19381-19394 J. Biol. Chem. doi:10.1074/jbc.RA119.010172

Introduction

Software packages and algorithms that model IDP behavior predict the projection domain (NTD through P1) to be the most disordered region (149), and NMR spectra demonstrate the NTD samples large regions of space while the P1 region is more restricted (67). This large structural feature is thought to regulate binding to and spacing of microtubules primarily through electrostatics. There are two models with the NTD either acting as a ‘zipper’ by forming electrostatic contacts with the P1 or as an electrostatic polyelectrolyte brush that compresses/expands in response to osmotic pressure (11, 63, 74, 91, 92). It is additionally proposed to interact with other cellular partners such as signaling proteins (150) and the plasma membrane (151). Within the NTD, it contains two alternatively spliced inserts (N1 and N2). Isoform specific behavior have been demonstrated to tune microtubule bundling (11, 63, 74, 91, 92), tau aggregation propensity in a combinatorial manner with the MTBR isoforms (152), and perhaps tau’s dwell time on stabilized microtubules thereby impacting MAP motor
proteins (153). However, many of these comparative studies contrast 2N4R with 0N3R – a simultaneous change in the number of binding sites within the MTBR. To date there are only a few comparative studies of the NTD isoforms in which the MTBR isoform is not also varied. Prior work on the NTD isoforms focused primarily on the differences in bundling behavior, which scales well with the length of the N-terminal domain and is electrostatically driven (11, 74, 91, 92). Subtle differences between Kᵤ and polymerization kinetic parameters (specifically the time required to nucleate polymerization) indicate that the NTD isoforms also weakly influence tau-mediated polymerization kinetics (63). There are larger differences when the NTD is deleted, resulting in large and tight bundling of stabilized microtubules (63).

Despite demonstrated NTD isoform-dependent tau-microtubule interactions, most tau studies focus on the adult human 2N4R transcript in isolation which only comprises ~3% of the cytosolic tau pool (Chapter 1: Figure 1.1B) (88). To the best of my knowledge, no studies on tau-tubulin interactions varying only the NTD isoforms have been undertaken. It has been proposed the ratio of tau isoforms that dictate healthy tau function rather than the precise isoform present (88). Understanding individual isoform properties are critical to mapping emergent properties as a function of the various isoform properties and ratios. Before complex studies of microtubule dynamics with mixed tau isoform ratios can be undertaken, comparisons between the NTD isoforms are necessary for understanding the underlying components of the tau isoform library.
The global ensemble of tau is conserved across N-terminal isoforms

To probe the solution or unbound ensemble and the fully bound ensemble we first verified saturated tubulin binding using fluorescence correlation spectroscopy (FCS) using singly labeled tau constructs labeled at S433C (Fig. 2.1). These recombinant human isoforms are referred to as tau\textsubscript{2N}, tau\textsubscript{1N}, and tau\textsubscript{0N} respectively to differentiate the difference in isoforms from the difference between of labeling positions used throughout this study. I also designed an artificial NTD isoform in which the second insert N2 was present instead of N1 as in 1N4R to give tau\textsubscript{1N*}. In this way, we could probe the insert-specific properties of each isoform.

Prior work within the literature demonstrated small differences in the K\textsubscript{D} between the N-terminal isoforms tau\textsubscript{2N} and tau\textsubscript{1N} upon binding to taxol-stabilized microtubules (1.1 µM and 1.4 µM respectively) (63). Although not directly comparable, my FCS measurements of tubulin binding by all three physiological N-terminal isoform variants, tau\textsubscript{2N}, tau\textsubscript{1N} and tau\textsubscript{0N}, as well as by tau\textsubscript{1N*}, revealed their binding curves to be comparable (Fig. 2.2) and fully saturated at 10 µM tubulin. See Appendix I: Experimental Procedures for experimental details and Appendix II: Python Code for the analytical handling of the FCS curves.
Figure 2.1: Tau N-terminal constructs

The uppermost schematic is the longest tau isoform, tau\textsubscript{2N}. The domains and corresponding residues that delineate them are marked: N-terminal domain (NTD) with N-terminal inserts (N1, N2), proline-rich region (PRR) with sub-regions (P1, P2), microtubule binding repeats (MTBR) with four imperfect repeat sequences (R1-R4) flanked by the pseudo-repeat R' and the C-terminus. Below are the additional tau isoforms and truncated variants used in this study. All numbering of residues throughout the manuscript is based on tau\textsubscript{2N}. Nomenclature from (63) is in parenthesis for relevant constructs. The corresponding amino acids for each construct with deletions subscripted are given.

Figure 2.2: Tubulin binding of the N-terminal isoforms

The increase in the normalized diffusion time, $\tau_{\text{norm}}$, as a function of tubulin concentration reflects binding of fluorescently labeled tau to unlabeled tubulin. Data are presented as mean ± SD, n\geq3 independent measurements. See Appendix I.iii for details of data analysis. See Table 2 for numerical values for $\tau_D$ and $\tau_{\text{norm}}$ at 10 µM tubulin.
In solution, the N-terminus of tau makes relatively close contacts with both the MTBR and the C-terminus (118), which are lost when tau binds soluble tubulin (119). I used smFRET to assess how the N-terminal inserts impact tau’s solution conformational ensemble or bound state. Full-length tau isoforms were labeled with donor and acceptor fluorophores at sites spanning domains of interest (Fig. 2.3A). The mean energy transfer efficiencies ($ET_{eff}$) were converted to experimental root-mean-square (RMS$_{exp}$) distances using a Gaussian coil model (see Appendix I.iv for details). For constructs probing the C-terminus, tau$^{291-433}$, as well as the PRR, tau$^{149-244}$, all three isoforms gave rise to comparable RMS$_{exp}$ values (Fig. 2.3B and Table 2.1); this was expected, as the number of residues encompassed by the probes is the same for all three isoforms. The constructs probing the N-terminal domain (NTD), tau$^{17-149}$, also exhibited predicted behavior in that the presence of each N-terminal insert resulted in an increase in the RMS$_{exp}$ (Fig. 2.3B and Table 2.1).

Interestingly, constructs whose labels span the NTD through the PRR, tau$^{17-244}$, or the NTD through part of the MTBR, tau$^{17-291}$ had comparable $ET_{eff}$ histograms, and thus RMS$_{exp}$ values, in solution, despite an increase of up to 60 residues between isoforms (Fig. 2.3B and Table 1). Similarly, the RMS$_{exp}$ values for the constructs probing the entirety of the isoforms, tau$^{17-433}$, were also nearly equivalent; this is consistent with our observations of comparable RMS$_{exp}$ values for the subdomain constructs – tau$^{17-291}$ and tau$^{291-433}$ – which make up tau$^{17-433}$ (Fig. 2.3B and Table 2.1). The similar inter-domain distances suggest homologous conformational ensembles between isoforms. Moreover, the ensembles for tau$^{17-244}$ and tau$^{17-291}$ are significantly more compact than
expected for a random coil. To illustrate, the RMS$_{\text{exp}}$ values of the tau$_{17-291}$ isoforms are of similar magnitude to those of tau$_{149-244}$ despite being 120 to 180 residues longer (Fig. 2.3B and Table 2.1). Furthermore, the dimensions of tau$_{149-244}$ are close to values predicted for a random coil of an equivalent number of residues (gray dashed line in Fig. 2.3B,C; values calculated in Table 2.1), such that despite encompassing this very expanded region, tau$_{17-244}$ and tau$_{17-291}$ constructs are very compact. Upon binding to tubulin, deviations from scaling behavior were diminished, and all constructs yielded RMS$_{\text{exp}}$ values that scale with the number of residues in a manner consistent with an extended, random structure (Fig. 2.3C and Table 2.1) (1, 67, 119).
Figure 2.3: SmFRET of tau N-terminal isoforms

(A) Schematic of reference construct tau\textsubscript{2N} with residues labeled for smFRET measurements indicated. (B) SmFRET histograms of tau\textsubscript{2N}, tau\textsubscript{1N}, tau\textsubscript{1N*} and tau\textsubscript{0N} in the absence (dark, left axis) and presence (light, right axis) of 10 µM tubulin. Labeling positions are indicated at the top of each column. The histograms are fit to a sum of Gaussian distributions to determine the mean ET\textsubscript{eff} as detailed in the Appendix I.iv. Measurements were carried out in phosphate buffer pH 7.4 at 20° C. (C) The root-mean-square distances (RMS) between labeling positions calculated from a Gaussian chain model (RMS\textsubscript{exp}) are plotted in the absence (left) and presence (right) of 10 µM tubulin (154). Shaded regions on this plot indicate RMS\textsubscript{exp} that are too large to be determined accurately by the Alexa 488-Alexa 594 fluorophore pair. For reference, the RMS calculated for a random coil (RMS\textsubscript{RC}) as in Reference (155) is indicated by the gray dashed line. Data are presented as mean ± SD, n≥3 independent measurements. See Table 2.1 for numerical values of ET\textsubscript{eff} ± SD, RMS\textsubscript{exp} ± SD and RMS\textsubscript{RC} for each construct.
Table 2.1: Summary of SmFRET biophysical data of tau N-terminal isoforms

All numbering throughout the manuscript is based on tau2N. Unless otherwise noted, all constructs contain C291S and C322S mutations. \textit{Labels} identifies the residues mutated to cysteines for site-specific labeling. Mean ET_{eff} values from fits to histograms as shown in Fig. 2.2 from measurements in the absence and presence of 10 \muM tubulin. Values are mean ± SD for ≥3 independent measurements of 20-40 pM tau in phosphate buffer pH 7.4 at 20 °C. RMS_{exp} calculated from mean ET_{eff} values as described in the Appendix I.iv and RMS_{RC} calculated from the theoretical random coil model for the number of residues noted (155).

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<th># residues</th>
<th>ET_{eff} - tubulin</th>
<th>+ tubulin</th>
<th>RMS_{exp} (Å) - tubulin</th>
<th>+ tubulin</th>
<th>RMS_{RC} (Å)</th>
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<td>75</td>
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<td></td>
<td>291 433</td>
<td>143</td>
<td>0.42 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>69 ± 1</td>
<td>84 ± 2</td>
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<td>89 ± 1</td>
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<td>0.14 ± 0.02</td>
<td>66 ± 2</td>
<td>117 ± 10</td>
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<td>0.46 ± 0.01</td>
<td>0.30 ± 0.02</td>
<td>65 ± 1</td>
<td>83 ± 2</td>
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</table>
Electrostatic interactions between domains drive ensemble formation

The comparable ET_{eff} values for tau^{17-244} and tau^{17-291} constructs, irrespective of the number of N-terminal inserts, suggested conserved interactions within the NTD/PRR/MTBR that were not strongly dependent upon sequence. To test this, I deleted the N1 insert from tau_{2N} to create tau_{1N*} (Fig. 2.1). As expected, the ET_{eff} values and, consequently, the calculated RMS_{exp} values for tau^{17-149}, tau^{149-244} and tau^{291-433} constructs of tau_{1N*} were all comparable to those of the physiological isoforms (Fig. 2.2B and Table 2.1). Consistent with our hypothesis, smFRET measurements of tau^{17-244}, tau^{17-291} and tau^{17-433} constructs of tau_{1N*}, gave rise to RMS_{exp} values comparable to those measured for the same constructs of tau_{0N}, tau_{1N} and tau_{2N} (Fig. 2.3B and Table 2.1). As a whole, my data demonstrate that the conserved ensemble within the NTD/PRR/MTBR are not insert dependent, but arise from a more general interaction mechanism within these domains. Both the MTBR and the PRR carry a positive net mean charge while the NTD that carries a negative net mean charge regardless specific isoform (Table 2.3B and Table 2.1).
Table 2.2: Charge profile of individual tau domains

The *net charge* is the number of positively charged residues minus the number of negatively charged residues for each domain listed, calculated at pH 7.4 using Sequence Manipulation Suite (156). The *mean net charge* is the *net charge* divided by the number of residues for each domain.

<table>
<thead>
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<th>net charge</th>
<th>mean net charge</th>
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<td>NTD₁N⁻</td>
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<tr>
<td>C-terminus</td>
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<td>-3</td>
<td>-0.07</td>
</tr>
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</table>

One feasible explanation is that attractive electrostatic interactions between the NTD and the PRR/MTBR result in a compact ensemble that is largely independent of the inserts themselves. These interactions between the negatively charged NTD and the positively charged PRR/MTBR are effectively neutralized upon binding to tubulin, another negatively charged particle, causing the tau ensemble to open up becoming a Gaussian coil.

**Discussion**

Presented here to the best of my knowledge, is the first comparative structural study of the N-terminal isoforms bound to soluble tubulin. Prior NMR studies of full-length tau to stabilized microtubules, have shown the NTD does not strongly interact with microtubules and retains a heterogeneous conformation in solution that makes transient long range contacts with other domains (67). Prior work from our lab on 2N4R and 2N3R
similarly demonstrated long-range contacts between the NTD and the other tau regions that are disrupted upon binding to soluble tubulin (119). In both studies, only the 2N N-terminal isoform was studied. In this work, I used smFRET to compare the N-terminal isoforms global interdomain interactions in an insert-specific manner. Demonstrated here is the conserved global ensemble between the NTD and the PRR/MTBR across all N-terminal isoforms in an insert-independent manner driven primarily by electrostatics. I find the NTD of tau_{2N} deviates equally from the predicted random coil sampling as the MTBR in solution; the only region that scales as predicted is the PRR, indicating the majority of tau’s regions are compacted under my experimental conditions. It is only upon binding to soluble tubulin does the NTD, as well as all the other probed regions, reproduce predicted random coil ensembles (149). Subtle structural differences between the isoforms do exist; however, it is important to remember the coarse-grained nature of this structural information, the anticipated difference of losing/gaining 30 amino acids per insert, and the wide distribution of possible states being averaged within the reported RMS_{exp}. This compact global ensemble between the NTD/PRR/MTBR is remarkably unchanged upon the addition or removal of the NTD inserts N1 and N2 despite a 20% increase/decrease in amino acid length with each insert. This suggests an overall conserved, electrostatically based mechanism driving the formation of this conformational ensemble with potentially a corresponding biological function. As these interdomain interactions are disrupted upon binding to tubulin, it is possible the NTD may regulate tau-tubulin interactions indirectly by either generally screening the negatively charged incoming tubulin – effectively guarding both the binding regions and flanking regions – or may transiently sample the regions within the PRR/MTBR thereby
temporarily weakening the tau-tubulin binding interaction indirectly. Speculatively, the combination of NTD with MTBR isoform (for example, 2N4R and 0N3R) would have altered interactions between the NTD and the MTBR, and the corresponding global ensemble would be more expanded or heterogeneous within 0N3R as both domains would have weakened screening capacity. If certain regions with stronger interactions between the NTD and MTBR exist, then a combinatorial effect on the global ensemble would also be possible.

CHAPTER 3: Independent binding and polymerization of the proline rich region is negatively regulated by the N-terminal domain

This chapter is adapted from McKibben, K.M. and Rhoades, E., “Independent tubulin binding and polymerization by tau’s proline rich region is regulated by its N-terminal domain” (2019) 294(50) 19381-19394 J. Biol. Chem. doi:10.1074/jbc.RA119.010172

Introduction

The conservation of the conformational ensembles across N-terminal isoforms suggests a functional origin. This led me to examine the impact of the N-terminal inserts on tau binding to soluble tubulin. Despite intense interest in tau, the molecular details of its numerous proposed functions remain relatively obscure. This is in part due to the challenges that arise from its lack of stable structure (157), and that it does not form well-defined stoichiometric complexes with tubulin (58). To illustrate, it was demonstrated more than 20 years ago that P2 (63, 64, 93) greatly enhanced tau binding to microtubules
and its ability to polymerize tubulin (59), yet this region of tau is not observed in a recent structure of microtubule-bound tau (1). It may be that P2 enhances binding indirectly through interactions with the MTBR (93), or that bound P2 is too disordered and dynamic on the microtubule surface to be resolved by EM. These apparently diverging observations, and the need to reconcile them, highlights the requirement for studies of tau function that look beyond the MTBR.

Here, I investigate the role of the NTD and PRR in regulating tau’s interactions with soluble tubulin. FCS of the full-length and tau variants were used to monitor binding in tau:tubulin assembles. We found that in the absence of tubulin, the NTD interacts with the PRR and MTBR through a conserved conformational ensemble. The NTD negatively regulates binding to soluble tubulin and subsequently slows polymerization. Strikingly, we find that the isolated PRR is capable of both stoichiometric binding to, and polymerization of, soluble tubulin. The presence of the NTD dramatically reduces the binding and polymerization capacity of the PRR. Based on our results, we propose a model where the PRR serves as a core tubulin binding domain of tau, with both binding and polymerization capacity enhanced by the MTBR and R', and reduced by the NTD.
Figure 3.1: Schematic of tau constructs

The uppermost schematic is the longest tau isoform, tau\(_{2\text{N}}\). The domains and corresponding residues that delineate them are marked: N-terminal domain (NTD) with N-terminal inserts (N1, N2), proline-rich region (PRR) with sub-regions (P1, P2), microtubule binding repeats (MTBR) with four imperfect repeat sequences (R1-R4) flanked by the pseudo-repeat R’ and the C-terminus. Below are the additional tau isoforms and truncated variants used in this study. All numbering of residues throughout the thesis is based on tau\(_{2\text{N}}\). Nomenclature from (63) is in parenthesis for relevant constructs. The corresponding amino acids for each construct with deletions subscripted are given.
Tau’s NTD negatively regulates tubulin binding

Tubulin binding was assessed by FCS under non-polymerizing conditions. Fluorescently labeled tau was measured in the presence of increasing concentrations of tubulin. Both the longest full-length isoform, tau2N, and an NTD deletion fragment, tauΔN (amino acids 148 to 441), bound tubulin as seen by an increase in the normalized diffusion time, \( \tau_{\text{norm}} \), with increasing tubulin concentration (Fig. 3.2 and Table 3.2). However, there are significant differences in the binding curves; tau2N reached its maximum \( \tau_{\text{norm}} \) at ~2.5 \( \mu \)M tubulin, while the \( \tau_{\text{norm}} \) for tauΔN continued to increase. At 10 \( \mu \)M tubulin, the \( \tau_{\text{norm}} \) of tauΔN was more than 2x larger than that of tau2N (Fig. 3.2). This effect was specific to the NTD. Binding by a C-terminal deletion construct, tauΔC (amino acids 1 to 395), resembled that of tau2N (Fig. 3.2) while a combined N-terminal and C-terminal deletion construct, PRR-MTBR-R' (amino acids 148 to 395) behaved like tauΔN (Fig. 3.2). These measurements suggest that the NTD of tau reduces or negatively regulates its binding to soluble tubulin, while the C-terminus does not have a significant role.
Figure 3.2: Inhibition of tubulin binding by the NTD

The increase in the normalized diffusion time, $\tau_{\text{norm}}$, as a function of tubulin concentration reflects binding of fluorescently labeled tau to unlabeled tubulin. All measurements were carried out in phosphate buffer pH 7.4 at 20° C. Data are presented as mean ± SD, $n \geq 3$ independent measurements. See Appendix A1.iii for details of data analysis. See Table 3 for numerical values for $\tau_D$ and $\tau_{\text{norm}}$ at 10 µM tubulin, as well as the labeling position for each construct.

The $\tau_{\text{norm}}$ values measured at 10 µM tubulin for constructs lacking the NTD are significantly greater than those including this domain, ~2.7 ms as compared to ~1 ms, with a larger spread in the diffusion times sampled (Fig. 3.3). It was demonstrated tau forms fuzzy complexes with soluble tubulin consisting of multiple, weakly-associated tubulin dimers that positively correlate the rate of tau-mediated polymerization of tau fragments (58). Using a similar approach as described in that work, we analyzed the individual autocorrelation curves of tau$_{2N}$ and PRR-MTBR-R' taken in the presence of 10 µM tubulin in order to assess the heterogeneity of the tau-tubulin complexes (Fig. 3.3; details of analysis are in the Appendix I.iii). From this analysis, it was apparent that not
only were tau:tubulin complexes formed by PRR-MTBR-R' on average larger (median diffusion time, $\tau_{\text{median}} = 2.02$ ms) than those formed by tau$_{2N}$ ($\tau_{\text{median}} = 1.29$) but that PRR-MTBR-R':tubulin complexes also had the largest spread in diffusion times (1.26 to 2.89 ms). These complexes persisted at 300 mM KCl, indicating they were not only present in low salt buffer (Table 3.2). Analysis of the average brightness, counts per molecule (CPM), of the diffusing species demonstrated that while tau$_{2N}$ typically consisted of a single tau molecule, the PRR-MTBR-R':tubulin complexes, especially the larger ones, may have included several tau molecules (Fig. 3.3 and Table 3.3).

**Figure 3.3: Regulation of the tau:tubulin complex heterogeneity by the NTD**

(A) Individual autocorrelation curves (gray dots) are plotted for tau$_{2N}$ (right) and PRR-MTBR-R' (left) in the presence of 10 $\mu$M tubulin. Averaged curves are shown with colored dots and fits of the averaged curves to Eq. 2 are in black. Data plotted represent all collected curves from independent triplicate measurements measured on different days. (B) The autocorrelation curves from (A) were individually fit to obtain a distribution of $\tau_D$ and CPM (kHz) values. When the NTD is absent, larger tau:tubulin complexes form as seen by the larger values of $\tau_D$ containing additional tau molecules as seen by the increase in CPM (kHz). Measurements were carried out in phosphate buffer pH 7.4 at 20° C. See Table 3.3 for labeling positions of constructs.
As observed for tau\textsubscript{2N}, analysis of the individual autocorrelation curves of tau\textsubscript{1N}, tau\textsubscript{1N*} and tau\textsubscript{0N} demonstrates that these isoforms also form smaller tau:tubulin complexes than PRR-MTBR-R' (Fig. 3.4C and Table 3.3), containing only a single tau (Fig. 3.4D and Table 3.3). Analysis of diffusion times and brightnesses of the complexes suggest that the NTD limits both: (1) the average number of tubulin dimers bound to a single tau molecule; and (2) the average number of tau bound to a single tubulin dimer. I explicitly tested for sequence specificity in the NTD’s regulation of tubulin binding by scrambling the sequence of the N1/N2 inserts, while retaining a distribution of charged and hydrophobic residues similar to the N1/N2 wild-type sequence, tau\textsubscript{2Nscr} (Fig. 3.4A). As predicted, both the tubulin binding curve as well as the heterogeneity analysis of tau\textsubscript{2Nscr} are comparable to tau\textsubscript{2N} (Fig. 3.4A, Tables 3.2 and 3.3). Along with my smFRET measurements, these data support a model whereby regulation of tubulin binding by the NTD is sequence and insert independent, likely arising from general electrostatic interactions between the NTD and the PRR/MTBR.
Figure 3.4: Impact of the NTD inserts N1 and N2 on binding

(A) To test the sequence-dependence of the WT N-terminal inserts, we scrambled the insert sequences (tau$_{2N_{scr}}$) while preserving the IDP character of the region by equivalent mixing of hydrophobic and charges residues (111). (B) The increase in the normalized diffusion time, $\tau_{\text{norm}}$, as a function of tubulin concentration reflects binding of fluorescently labeled tau to unlabeled tubulin. Data are presented as mean ± SD, n≥3 independent measurements. Measurements were carried out in phosphate buffer pH 7.4 at 20 °C. See Appendix I.iii for details of data analysis. See Table 3.3 for numerical values for $\tau_{D}$ and $\tau_{\text{norm}}$ at 10 µM tubulin. Average tau$_{2N}$ from Fig. 3.2 are replotted for comparison. Scrambling the sequences of the N-terminal inserts does not change the binding behavior. (C) The autocorrelation curves for tau$_{2N}$, tau$_{1N}$, tau$_{1N*}$, tau$_{0N}$, tau$_{2N_{scr}}$, and PRR-MTBR-R' in the presence of 10 µM tubulin were individually fit to obtain a distribution of $\tau_{\text{norm}}$ values. Each individual $\tau_{D}$ was converted to $\tau_{\text{norm}}$ by the average $\tau_{D}$ of each independent measurement (Table 3.3). When the NTD is absent, larger tau-tubulin complexes form as seen by the larger values of $\tau_{\text{norm}}$. (D) The corresponding distributions of CPM (kHz) from the fits of autocorrelation curves in (B) of tau$_{2N}$, tau$_{1N}$, tau$_{1N*}$, tau$_{0N}$, tau$_{2N_{scr}}$, and PRR-MTBR-R' in the presence of 10 µM tubulin. When the NTD is absent, an increase in the number of tau molecules associated to the tau:tubulin assemblies is reflected in the increase in CPM. Overlays in both (B) and (C) are lognormal distributions. See Table 3.2 for labeling positions of constructs and Table 3.3 for descriptive statistics of distributions. Measurements were carried out in phosphate buffer pH 7.4 at 20° C. See Appendix I.iii for details of data analysis.
In order to determine a relationship between tubulin binding and polymerization of these constructs, the polymerization capacity of each construct was quantified. PRR-MTBR-R' had the fastest polymerization half-time ($t_{1/2}=52 \pm 7$ s) while the full-length isoforms, including tau$_{1N^*}$ were all slower (Fig. 3.5). This observation is in good agreement with prior work, where deletion of the NTD from various constructs led to small increases in polymerization rates (63). Interestingly, tau$_{2N}$ was the slowest ($t_{1/2}=137 \pm 9$ s), lagging behind tau$_{1N}$, tau$_{1N^*}$ and tau$_{0N}$ ($t_{1/2}=85 \pm 5$ s, 88 $\pm$ 13 s and 76 $\pm$ 10 s, respectively and within error of each other) (Fig. 3.5 and Table 3.1). Thus, while the presence of any part of the NTD has the largest effect on binding and polymerization, the presence of both native inserts may enhance the inhibitory interactions between the NTD and PRR/MTBR. Minor differences in multiple microtubule dynamic parameters have been noted previously for tau$_{2N}$ and tau$_{0N}$, both in vivo and in vitro (64, 158).
Figure 3.5: Impact of the NTD inserts N1 and N2 on polymerization

Tubulin polymerization as measured by scattered light at 340 nm as a function of time. Measurements were made in phosphate buffer pH 6.9 with 1 mM GTP at 37 °C with 5 µM tau and 10 µM tubulin. See Table 3.1 for fit parameters. Data are presented as mean ± SD following normalization, n=3 independent measurements. See Appendix I.vii for details of data analysis. Arrows indicate depolymerization at 4 °C.

The PRR independently binds and polymerizes tubulin

The reduced binding of NTD containing constructs (Figs. 3.2-4) coupled with the conserved conformational ensembles in the NTD/PRR/MTBR constructs observed in the smFRET measurements (Chapter 2), led me to hypothesize that the NTD may regulate tubulin binding though interactions with the PRR or MTBR. To investigate this hypothesis, I created constructs corresponding to these domains and measured binding by FCS as well as tau-mediated polymerization. Although the MTBR (amino acids 244-372) associates with microtubules in the context of the full-length protein or in constructs containing P2 (1, 60), the isolated domain bound only weakly to soluble tubulin (Fig.
3.6A) and was not capable of polymerizing tubulin (Fig. 3.6B). The addition of R', MTBR-R' (amino acids 244-395), enhanced binding (Fig. 3.6A) but still did not yield a construct that promoted efficient polymerization (Fig. 3.6B). Early studies demonstrated that the MTBR-R' (63) or even peptides corresponding to the individual MTBR repeats (159) had weak polymerization capacity, although 5 to 10-fold more tau was required than the 10 µM used here.

Strikingly – and surprisingly – the isolated PRR (amino acids 148-244), bound tubulin tightly when compared to the MTBR and MTBR-R' measured under the same conditions (Figs. 3.6A,B). Similarly, PRR exhibited a higher affinity for taxol-stabilized microtubules in comparison to MTBR-R' (Fig. 3.6C). While P2 has been identified as enhancing binding and accelerating polymerization in vitro when coupled to the MTBR (63, 64, 93, 95) independent polymerization capacity for PRR has not been reported previously. Rather, the opposite conclusion was reached by one study – namely, that the PRR is not capable of strong, independent assembly of microtubules (93). More recent work demonstrated binding of a PRR-like construct (amino acids 166-246) to both stathmin-complexed tubulin and taxol-stabilized microtubules with 1:2 stoichiometry (65). However, the polymerization capacity of this construct was not tested.
Figure 3.6: Independent polymerization capacity of the PRR, regulated by the NTD

(A) Binding of tau constructs to tubulin as measured by an increase in $\tau_{\text{norm}}$ as a function of tubulin concentration. Data are presented as mean ± SD, n≥3 independent measurements. Measurements were carried out in phosphate buffer pH 7.4 at 20°C. See Table 3.2 for labeling positions of constructs and Table 3.3 for descriptive statistics of distributions. See Appendix Iii for details of data analysis. The PRR data is fit with the Hill Equation (orange line; Eq. 1) yielding n=1.7 ± 0.2 and with an apparent $K_D$ ≈ 900 nM. (B) Tubulin polymerization as measured by scattered light at 340 nm as a function of time. Measurements were made in phosphate buffer pH 6.9 with 1 mM GTP at 37°C with 10 µM tau and 10 µM tubulin. See Table 3.1 for fit parameters. Data are presented as mean ± SD following normalization, n=3 independent measurements. Arrows indicate depolymerization at 4°C. (C) Taxol-stabilized microtubules were re-suspended in phosphate buffer pH 6.9 and then incubated at room temperature for 10 minutes with 1 µM PRR or MTBR-R’ labeled with Alexa Fluor 488 prior to separation of bound and free tau by ultracentrifugation to pellet microtubule-associated tau. Coomassie (right) and Alexa Fluor 488 fluorescence (left) images supernatant (S) and Pellet (P) were analyzed to yield ~70% MTBR-R’ and ~95% PRR bound to microtubules. The tau-only loading control is also shown. The band at approximately 50 kDa is the composite unresolved bands of α and β monomer tubulin subunits; the bands at ~25 kDa and 18 kDa are MTBR-R’ and PRR, respectively.
NTD negatively regulates the polymerization capacity of the PRR

Our observation that the PRR binds to and polymerizes tubulin independently of the MTBR (Fig. 3.6A), combined with the slower polymerization rate of tau constructs including the NTD (tau$_{2N}$, tau$_{1N}$, tau$_{1N^*}$ and tau$_{0N}$) relative to PRR-MTBR-R' (Figs. 3.5), motivated us to determine the impact of the NTD on interactions of the PRR with tubulin. Tau$_{2N}$ was truncated after the PRR at amino acid 244 (2N-PRR), and binding to soluble tubulin and polymerization capacity were measured. The presence of the NTD dramatically reduced binding (Fig. 3.6A) as well as significantly diminished tubulin polymerization capability (Fig. 3.6B). Truncated constructs based on tau$_{0N}$, 0N-PRR, and tau$_{1N^*}$, 1N*-PRR, showed similar binding behavior (Fig. 3.7).

![Figure 3.7: Impact of N-terminal inserts on the binding of PRR](image)

The $\tau_{\text{norm}}$ of tau constructs 1N*-PRR and 0N-PRR are plotted against increasing tubulin concentration. Tau constructs PRR and 2N-PRR are re-plotted from Fig. 3.6A for comparison. Data are presented as mean ± SD, $n$≥3 independent measurements. Measurements were carried out in phosphate buffer pH 7.4 at 20 °C. See Appendix I.iii for details of data analysis. See Table 3 for numerical values for $\tau_D$ and $\tau_{\text{norm}}$ at 10 µM tubulin.
Collectively, these results led us to propose that the binding and – by extension – polymerization capacities of tau are regulated by interactions between the NTD and the PRR, evident by the conserved ensembles observed with smFRET for this domain (tau^{17-244} in Chapter 2). Because the conserved ensembles extend into the MTBR (tau^{17-291} in Chapter 2), we tested this idea by making a construct lacking the PRR (2N-MTBR-R' amino acids 1 to 148 fused to 245 to 395). This construct also did not demonstrate appreciable binding to tubulin (Fig. 3.6A), while that same construct lacking the NTD (MTBR-R') clearly did (Fig. 3.6A). As may be expected based on its weak binding to soluble tubulin, 2N-MTBR-R' was also not polymerization competent (Fig. 3.6B). As a whole, these results strongly support a functional, regulatory role for the compact, albeit disordered, NTD/PRR/MTBR ensembles observed by smFRET in Chapter 2.

**Table 3.1: Tau-mediated polymerization**
Polymerization half-times (t_{1/2}) for tau constructs shown in Figs. 5 and 6. Values listed for t_{1/2} are mean ± SD for n = 3 independent measurements. NA indicate constructs tested but that did not measurably polymerize underneath assay conditions. The tubulin concentration was held constant at 10 µM across all polymerization experiments with the tau concentration varied according to the table below. Polymerizations were carried out in phosphate buffer pH 6.9 with 1 mM GTP at 37 °C.

<table>
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<td>5</td>
</tr>
<tr>
<td>tau_{2N}</td>
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<td>5</td>
</tr>
<tr>
<td>tau_{1N}</td>
<td>85 ± 5</td>
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</tbody>
</table>
The formation of bone-fide microtubules was confirmed using tunneling electron microscopy (Fig. 3.8). Mixtures of PRR, 2N-MTBR-R’, and PRR-MTBR-R’ of completed polymerization reactions from Fig. 3.5 and 3.6 were incubated with taxol prior to imaging. Using this more sensitive technique, we were able to observe small amounts of microtubule-like structures formed by 2N-MTBR-R’. This is in agreement with older in vitro studies that observed tau-mediated polymerization of the MTBR region repeats at much higher concentrations (159). It should be noted, however, these structures do not represent the majority of the TEM grid nor do they resemble canonical microtubules. Also in agreement with previous studies, PRR-MTBR-R’ forms large bundles of microtubules. The varying morphology of PRR and 2N-MTBR-R’ warrants further study; currently, the data speculatively suggests PRR is responsible for tau-tubulin interactions which favor straight unbundled, microtubules.
**Figure 3.8: PRR encourages the formation of straight microtubules**

TEM images of microtubules formed by tau-tubulin polymerization reactions as in Figs. 3.5 and 3.6: (A) PRR; the region outlined in white in the left-hand panel is shown at higher-magnification in the center left panel. (B) tubulin-only control (C) 2N-MTBR-R’; representative blank field with 2N-MTBR-R’ present (left) and example of microtubule-like structures also infrequently found (right) (D) PRR-MTBR-R’ formed microtubule bundles.

**PRR forms tight, saturable tau:tubulin complexes**

Furthermore, unlike constructs where the PRR is coupled with the MTBR and/or R’, such as PRR-MTBR or PRR-MTBR-R’, the PRR demonstrated saturable binding and did not form large tau:tubulin complexes (Figs. 3.3 and 3.9). As a consequence, unlike the binding curves of the PRR-MTBR and PRR-MTBR-R’, the PRR binding curves can be meaningfully fit with Hill equation:

$$\tau_{\text{norm}}^\text{max} = \tau_{\text{norm}} + \tau_{\text{norm}} \frac{[\text{tub}]}{K_D + [\text{tub}]}$$  
Eq. 1

where $\tau_{\text{norm}}^\text{max}$ is the normalized diffusion time for tau:tubulin measured at 10 $\mu$M tubulin, $n$ is the Hill coefficient and reflects the extent of cooperativity, $K_D$ is the apparent
dissociation constant and [tub] is the concentration of tubulin dimer. Fitting the PRR:tubulin binding curve to the Hill equation yields $n=1.7 \pm 0.2$ and with an apparent $K_D \approx 900$ nM (Fig. 3.6A). In our previous work, the engineered protein construct RB3, which binds tubulin with 1:2 RB3:tubulin dimer stoichiometry was used to determine the expected $\tau_D$ of a 1:2 protein:tubulin dimer complex (58, 160). Here, the $\tau_D$ measured for the PRR at 10 $\mu$M tubulin ($0.82 \pm 0.03$ ms) is consistent with a 1:2 tau:tubulin dimer stoichiometry. This observation, coupled with the cooperativity seen in Hill equation fit, strongly supports the presence of two tubulin-dimer binding sites in the PRR. This apparent specificity suggests that formation of tau:tubulin fuzzy complexes arises primarily from the collective binding properties of the PRR and MTBR-R'. Additional biophysical studies such as NMR to determine kinetic parameters or AUC confirm the stoichiometry via a complementary method may be beneficial for future studies.
Figure 3.9: The PRR forms stoichiometric complexes with tubulin

(A) Individual autocorrelation curves (gray dots) are plotted for PRR (upper) and PRR-MTBR-R' (lower) in the absence (left) or presence (right) of 10 µM tubulin. PRR-MTBR-R' was replotted from Fig. 3.3 for comparison. Averaged curves are shown with blue dots and fits of the averaged curves to Eq. 2 are in black. Measurements were made with 20 nM PRR or PRR-MTBR-R' and 10 µM tubulin in phosphate buffer pH 7.4 at 20 °C. (B) The autocorrelation curves for PRR and PRR-MTBR-R' were individually fit to obtain a distribution of $\tau_{\text{norm}}$ values at each tubulin concentration. Each individual $\tau_D$ was converted to $\tau_{\text{norm}}$ by the average $\tau_D$ of each independent measurement (Table 3.3). Unlike PRR-MTBR-R' which forms tubulin-concentration dependent large complexes at tubulin concentrations exceeding ~1 µM tubulin, PRR binding saturates and does not form large complexes. Data plotted represent all collected curves from independent triplicate measurements on measured on different days. See Appendix I.iii for details of data analysis.
Tight binding of the PRR to tubulin required the presence of both proline rich regions; fragments corresponding to P1 (amino acids 148 to 198) or P2 (amino acids 199 to 244) bound tubulin only weakly (Fig. 3.10A). Interestingly, a prior study noted that while a P2-MTBR-R' construct lacked microtubule bundling capacity, the addition of P1 to this construct conferred this ability (63), reflecting a similar enhancement in the interaction with stabilized microtubules that we find with soluble tubulin. One reason independent function of the PRR has been overlooked may be in part due to the widespread use of the K16 fragment consisting of P2 and the 4R MTBR (amino acids 198 to 372, P2-MTBR) (63). By FCS, the P2-MTBR construct binds to tubulin, however, it does not bind as many tubulin dimers at high tubulin concentrations as PRR-MTBR (Fig. 3.10B). Thus, while the isolated P1 does not bind tubulin strongly (Fig. 3.10B), it does enhance binding and contribute to tau function.
Figure 3.10: P1 and P2 dependence of PRR binding

(A) Binding of tau constructs to tubulin as measured by an increase in $\tau_{\text{norm}}$ as a function of tubulin concentration. Data are presented as mean ± SD, n≥3 independent measurements. The PRR data is fit with the Hill Equation (orange line; Eq. 1) yielding $n=1.7 ± 0.2$ and with an apparent $K_D \approx 900$ nM. P1 and P2 bind only weakly, but are comparable or stronger than, MTBR. (B) The $\tau_{\text{norm}}$ of tau constructs PRR-MTBR and P2-MTBR measured by FCS are plotted against increasing tubulin concentration. Data are presented as mean ± SD, n≥3 independent measurements. Measurements were carried out in phosphate buffer pH 7.4 at 20 °C. See Appendix I.iii for details of data analysis. See Table 3 for numerical values for $\tau_D$ and $\tau_{\text{norm}}$ at 10 µM tubulin.
Table 3.2: Summary of FCS biophysical data of tau isoforms and constructs

All numbering throughout the manuscript is based on tau_{2N}. Unless otherwise noted, all constructs contain C291S and C322S mutations. C_{FCS} is the residue number mutated to cysteine for labeling for FCS measurements. Diffusion times (\(\tau_D\)) of tau constructs in the absence and presence of 10 \(\mu\)M tubulin. CPM_{norm} is the average CPM of labeled tau in the presence of tubulin divided by CPM of labeled tau without tubulin. Values are mean ± SD for \(n \geq 3\) independent measurements. Each measurement is the fitted average of multiple FCS curves from 15-25 nM tau incubated with 10 \(\mu\)M tubulin in phosphate buffer pH 7.4 at 20 °C. (*) Indicates measurements with 300 mM KCl. The \(\tau_{norm}\) is calculated as described in the Appendix I.iii.

<table>
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<tr>
<th>Construct</th>
<th>C_{FCS}</th>
<th>(\tau_D) (ms)</th>
<th>(\tau_{norm}) (ms)</th>
<th>CPM (kHz)</th>
<th>CPM_{norm} (kHz)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>- tub + tub</td>
<td>+ tub</td>
<td>- tub + tub + tub</td>
<td></td>
</tr>
<tr>
<td>tau_{2N}</td>
<td>433</td>
<td>0.80 ± 0.03</td>
<td>1.54 ± 0.09</td>
<td>0.92 ± 0.09</td>
<td>11 ± 4</td>
</tr>
<tr>
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<tr>
<td>tau_{1N}</td>
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<td>12 ± 1</td>
</tr>
<tr>
<td>tau_{0N}</td>
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<td>1.57 ± 0.12</td>
<td>1.00 ± 0.12</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>tau_{2Nscr}</td>
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<td>0.78 ± 0.01</td>
<td>1.49 ± 0.09</td>
<td>0.92 ± 0.09</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>tau_{1N*}</td>
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<td>0.96 ± 0.14</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>tau_{0N}</td>
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<td>0.79 ± 0.02</td>
<td>1.57 ± 0.12</td>
<td>1.00 ± 0.12</td>
<td>12 ± 1</td>
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<tr>
<td>tau_{2Nscr}</td>
<td>433</td>
<td>0.78 ± 0.01</td>
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<tr>
<td>MTBR-R'</td>
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<td>0.52 ± 0.01</td>
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<td>9 ± 2</td>
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<tr>
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<td>0.23 ± 0.01</td>
<td>8 ± 1</td>
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<tr>
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<td>0.71 ± 0.01</td>
<td>0.40 ± 0.01</td>
<td>8 ± 1</td>
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<tr>
<td>0N-MTBR-R'</td>
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<td>0.72 ± 0.01</td>
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<td>-0.01 ± 0.02</td>
<td>8 ± 1</td>
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<tr>
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<td>0.37 ± 0.01</td>
<td>0.16 ± 0.01</td>
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<tr>
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<td>11 ± 2</td>
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<tr>
<td>P2-MTBR</td>
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<td>1.37 ± 0.18</td>
<td>1.27 ± 0.18</td>
<td>10 ± 1</td>
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<tr>
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<td>0.67 ± 0.06</td>
<td>0.89 ± 0.13</td>
<td>0.33 ± 0.13</td>
<td>11 ± 2</td>
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Table 3.3: Descriptive statistics of tau:tubulin
Resulting correlation curves from 15-25 nM tau was incubated with 10 µM tubulin in phosphate pH 7.4 at 20 °C were analyzed individually rather than averaged and fit to describe the distribution within each dataset. Statistics of the diffusion times from select tau constructs incubated with 10 µM tubulin without and with our filtering algorithm. IQR and SD stand for interquartile range and standard deviation respectively. See Appendix I.iii for details.

<table>
<thead>
<tr>
<th>construct</th>
<th>pre-filtering</th>
<th></th>
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<th>post-filtering</th>
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<td>τ₀ (ms)</td>
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<td>mean ± SD</td>
<td>IQR</td>
<td># curves</td>
<td>median</td>
<td>mean ± SD</td>
<td>IQR</td>
</tr>
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<td>PRR-MTBR-R'</td>
<td>2.24</td>
<td>2.52 ± 1.41</td>
<td>0.99</td>
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<td>2.02</td>
<td>2.06 ± 0.42</td>
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<td>1.35</td>
<td>1.43 ± 0.34</td>
<td>0.29</td>
<td>419</td>
<td>1.29</td>
<td>1.31 ± 0.12</td>
<td>0.23</td>
<td>327</td>
</tr>
<tr>
<td>tau₂₂Nscr</td>
<td>1.48</td>
<td>1.79 ± 1.11</td>
<td>0.37</td>
<td>392</td>
<td>1.42</td>
<td>1.44 ± 0.15</td>
<td>0.20</td>
<td>301</td>
</tr>
<tr>
<td>tau₁₁N</td>
<td>1.49</td>
<td>1.76 ± 0.96</td>
<td>0.59</td>
<td>578</td>
<td>1.31</td>
<td>1.33 ± 0.19</td>
<td>0.27</td>
<td>348</td>
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<tr>
<td>tau₁₁N*</td>
<td>1.55</td>
<td>1.73 ± 1.31</td>
<td>0.40</td>
<td>424</td>
<td>1.50</td>
<td>1.51 ± 0.20</td>
<td>0.34</td>
<td>383</td>
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<tr>
<td>tau₀₀N</td>
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<td>1.80 ± 0.86</td>
<td>0.47</td>
<td>434</td>
<td>1.55</td>
<td>1.57 ± 0.25</td>
<td>0.36</td>
<td>378</td>
</tr>
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</table>

Discussion

Since it was first isolated over 40 years ago (2), studies of tau have primarily focused on the MTBR (1, 61, 63, 67, 72). My current study examines two domains of tau that have been the subject of significantly less scrutiny: the NTD and the PRR. My observation that the isolated PRR has the capacity to bind tubulin cooperatively and polymerize microtubules in vitro, and that this function is negatively regulated by the NTD, draws attention to the importance of these two domains in understanding tau function.

The NTD has previously been shown to regulate the interaction of tau with microtubules(63). Although not directly comparable to my results presented here with soluble tubulin, prior work found that removal of the NTD increases the affinity of tau for
microtubules (63). Moreover, in that work they observed that an NTD-lacking tau fragment, was capable of bundling taxol-stabilized microtubules, whereas the comparable construct including the NTD was not (63). This suggests a more promiscuous binding interaction for stabilized microtubules in the absence of the NTD, consistent with our observation for soluble tubulin (Fig. 3.2). Isoform dependent and electrostatic sensitive regulation of microtubule bundling by the NTD is also cited in several more recent papers (discussed in Chapter 2) (74, 91).

While I do not quantify the impact of the NTD on the affinity of tau for soluble tubulin, I do find an inhibitory effect of the NTD in binding to soluble tubulin (Figs. 3.2-4). This inhibition seems to be due to the NTD as a whole, rather than resulting from the absence or presence of a specific insert within the domain, as only small differences in binding are observed for the tau\textsubscript{0N}, tau\textsubscript{1N}, tau\textsubscript{1N*} and tau\textsubscript{2N} variants when compared to a variant lacking the NTD (Fig. 3.4). Insight into why the inserts do not have a significant effect in regulating binding is gained from our smFRET measurements which show that the relative dimensions corresponding to the NTD-PRR (tau\textsuperscript{17-244}) or NTD-MTBR (tau\textsuperscript{17-291}) are independent of the number of inserts (Chapter 2). This suggests that conserved long-range interactions and/or conformational features of the NTD are important for regulating interactions with tubulin, more so than the inserts themselves (Chapter 2). Given that the NTD also significantly reduces the size and heterogeneity of ‘fuzzy’ tau-tubulin complexes (Figs. 3.3 and 3.4, and Table 3.3) (58), it follows that the NTD may dynamically shield the weak tubulin binding sites distributed throughout the MTBR and R' (Fig. 3.11). My results suggest this screening is a general function of the NTD that
serves as an initial regulatory gate to tau-mediated polymerization, and is largely independent of the individual N-terminal inserts.

Prior work from the Rhoades lab demonstrated a positive correlation between the rate of tau-mediated tubulin polymerization and the size of tau:tubulin complexes by systematically varying the number of tubulin binding sites in tau fragments (58). I find that this observation broadly holds for the full-length isoforms and fragments studied here in that PRR-MTBR-R' forms the largest tau:tubulin complexes and has shortest polymerization lag-time (Fig. 3.5). My current work refines that model to include controlling access to binding sites, and not simply the number of binding sites, as further regulating the kinetics of tubulin polymerization. Consistent with this model are the smFRET data that show that the interactions between the NTD and PRR/MTBR observed in solution are lost upon tubulin binding (Chapter 2).

Our observation of assembly of tubulin by the isolated PRR was unexpected, as to the best of my knowledge, there are no prior reports of this in the literature. NMR chemical shifts suggesting binding were measured in the PRR of longer tau fragments in the presence of MAP-stabilized tubulin constructs (65, 67). We also find tight, saturable stoichiometric binding of 1:2 tau:tubulin dimers. Notably, PRR residues were not observed in the recent cryo-EM structure of microtubule-bound tau (1). However, the high-resolution structures shown in that study were obtained with tau fragments lacking P1, which our results suggest is critical for tight binding of the PRR (Fig. 3.9). It may also be that the PRR binds to a region unresolved within the structure, such as the intrinsically disordered tubulin tails. Alternatively, it may be that the PRR mediates
binding at low tau:tubulin ratios, such as the conditions of our experiments, but that it is not associated with the microtubule lattice at the high tau:tubulin ratios required for cryo-EM reconstructions.

The stoichiometric binding to soluble tubulin of the PRR provides a striking contrast with the dynamic, heterogeneous ‘fuzzy’ tau-tubulin complexes formed when the MTBR and R' were present in the constructs. In particular, tight and specific binding of the PRR may offer an attractive target for therapeutic targeting relative to the comparatively weak binding by the MTBR-R' (161). Interestingly, both P2 and R' were identified relatively early as sequences important for productive tau-mediated polymerization (67, 94). The ‘jaws’ model proposed that targeting of tau to the microtubule lattice is through these regions, while the MTBR played a catalytic role in assembly (64, 94, 95). Specifically, the introduction of either P2 or R' to the three-repeat MTBR fragment enhanced binding to taxol-stabilized microtubules almost 10-fold, as well as decreasing the polymerization lag-time by a factor of two (63). The presence of both P2 and R' further enhanced the binding affinity and polymerization rate.

However, in my study, the PRR is the only isolated domain which demonstrates any significant tubulin polymerization capacity; this is not seen for MTBR nor MTBR-R' (Fig. 3.6B). This leads me to propose a variation to that model (Fig. 3.11). In my model, the PRR serves as the core tubulin binding domain, binding to two tubulin dimers in a critical step towards initiating polymerization. Multiple weak tubulin binding sites in the MTBR and R' allow for increasing the local concentration of tubulin, resulting in accelerated microtubule growth. The ubiquitous screening by the NTD of both the PRR
and the MTBR serves as an initial gating that controls the size of these tau:tubulin ensembles and, consequently, tubulin assembly. To clarify, within the global ensemble, multiple microstates may exist, and tubulin may be bound to the MTBR-R’ prior to the PRR. Additionally, conformational changes within the tau – specifically the NTD may alter binding affinities between each site. In the below speculative model, P1, P2, R2, R3, and R’ (ignoring the IR regions) are assumed to be the tightest binding regions. Speculatively, the NTD may alter the affinity of one PRR site and two MTBR sites resulting in a larger number of distinctive microstates in subclass C, but an over all reduction in number of favorable tubulin-binding states. The microstate in which two tubulin dimers are bound in the PRR (indicated by an arrow) in the $E$ subclass is the polymerization initiation favorable state. The MTBR-R’ region increases the number of tubulin subunits bound. As these microstates are in equilibrium with the PRR microstate, they also favor the probability of populating this polymerization-initiation favored microstate.
**Figure 3.11: Model for regulation of tau:tubulin interactions**

**A** The PRR (orange) binds tubulin tightly and stoichiometrically, negatively regulated by the NTD (blue). The MTBR-R' (red) increases the local tubulin concentration through distributed, weak interactions, enhancing the polymerization capacity of tau. The C-terminus is colored black. Increasing both tau and tubulin concentrations favor polymerization. **B** Possible microstates in the first tubulin binding event to regions within the PRR-MTBR-R'. Dark circles indicate favorable binding, and lighter colored regions represent unfavorable binding. Two hypothetical subclasses ‘C1’ and ‘E1’ represent a closed and open formation for the NTD, respectively. These two subclasses, and corresponding the microstates are all in equilibrium with each other. ‘T1’ indicates one tubulin bound. **C** Possible binding microstates upon the binding of a second tubulin. Again, classes ‘C2’ and ‘E2’ represent a closed and open formation for the NTD, respectively. The arrow indicates the microstate in which both tubulin dimers are bound to the PRR region.
Tau’s interactions with microtubules are regulated by phosphorylation (162) and the majority of tau’s 40+ known phosphorylation sites are located in the PRR, including those associated with Alzheimer’s disease (163, 164). Given this, perhaps the relative importance of the PRR in both binding to and polymerizing tubulin should not be so surprising. It has long been known that phosphorylation at serines 199 and 202 and threonine 205 varies along growing axons (165). One very recent study reported a link between phosphorylation of P2 and proper axonal localization of tau (166). However, the relationship between phosphorylation and microtubule binding is not straightforward; to illustrate, while phosphorylation of serine 214 and threonines 212 and 231 in the PRR, as well as serine 262 in the MTBR, all lower the affinity of tau for microtubules, while phosphorylation at other PRR sites has only a minor effect on microtubule binding (167). How phosphorylation at any of these sites impacts binding to soluble tubulin has not yet been tested. Moreover, the MTBR is also modified by lysine acetylation (163). Individual or combinatorial effects of these modifications may alter both the binding affinity and the stoichiometry of tubulin binding. There is at least one example of coordinated modifications to tau in the literature: acetylation at lysines 280 and 281 within the MTBR influences phosphorylation at serines 202 and 205 within the PRR (168). As a whole, post-translational modifications may influence the interaction of the PRR or MTBR with the NTD, suggesting that regulation of binding may be more complex than simply reducing affinity and stoichiometry, but instead an intricate interplay between the NTD, PRR and MTBR domains.
CHAPTER 4: Biochemical and structural elucidation of the core binding sites to soluble tubulin

Part of this chapter is adapted from Fung, H.Y.J., McKibben, K.M., Ramirez J., Gupta, K., and Rhoades E., “Structural characterization of tau in fuzzy tau:tubulin complexes” (2020) 28(3) Structure doi:10.1016/j.str.2020.01.004. As one of the co-authors, I completed requested controls using fluorescence anisotropy to demonstrate the labeling procedure and spectral shifts of the acrylodan data do not interfere with binding (Appendix I.viii). Additionally, I contributed to the data interpretation regarding the spectral shifts of acrylodan along the microtubule surface, and wrote the python code used to analyze the FCS data. Another part of this chapter is adapted from Castle, B., McKibben K.M., Rhoades E., and Odde D., “Tau avoids the GTP cap at growing microtubule plus ends” submitted. As one of the co-authors, I demonstrated tau’s GDP-tubulin preference was unique to the microtubule lattice. I also provided all recombinant protein for the study as well as requested controls. Regarding the unpublished work not incorporated into the aforementioned papers within this chapter, Dr. Joyce Fung collected the binding curve of PRR to tail-less tubulin.

Introduction

As previously discussed, the tau tubulin and microtubule interactions are broadly assumed to be equal, although this has not been clearly demonstrated experimentally. Indeed, the interactions with the MTBR are not equivalent – with R1 and R2 having a higher affinity upon binding to microtubules, and R3 binding tighter to soluble tubulin (62). There are two key differences between soluble tubulin and tubulin incorporated into
the microtubule lattice that may influence these interactions. First, the ordered lattice may occlude tau-binding regions or more simply unfavorably space the tau-binding regions. Second, during GTP-dependent hydrolysis of the GTP-tubulin, there is a coupled chemical (GDP is now bound) and conformational change within the tubulin. The chemical identity of the GDP/GTP cofactor may impact tau binding. Alternatively, the allosteric strain within the heterodimer along the lattice influence tau-tubulin interactions. In this chapter, we present the potential impact of nucleotide and strain on tau binding to the microtubule lattice as well as the preliminary investigations into tau-tubulin versus tau-microtubule interactions.

**Tau prefers GDP-tubulin within the lattice but is insensitive to nucleotide state in solution**

In some cases, MAPs may bind to the growing β(+) end of the microtubule such as end binding protein 1 (EB1) which preferentially interacts with the GTP-cap. Tau is also sensitive to the microtubule nucleotide state, and antagonizes EB1 in a phosphorylation dependent manner (28). Yet, tau not exclusively associated with the end of the microtubule but diffused across the length of the microtubule (47-49). Unfortunately, these prior studies were in the presence of the drug paclitaxel, which has a known influence on microtubule dynamics and tau-microtubule interactions. Therefore, a more complete examination of tau’s potential tubulin nucleotide binding preference and location along the microtubule is needed in the absence of drugs that influence microtubule assembly.
To map tau’s location along microtubules, fluorescently labeled tau$_{2N}$ was incubated with microtubules grown from immobilized stabilized microtubules. Stable microtubules that do not undergo microtubule dynamicity can be formed using a variety of GTP analogs that are hydrolytically incompetent such as guanosine-$5'\cdot[(\alpha,\beta)$-methylene]triphosphate (GMPCPP). Short, stabilized fragments of microtubules are termed microtubule seeds. Microtubule growth occurs when 1 mM GTP is added to solution, and the GTP cap is hydrolyzed to GDP-tubulin; as the GTP cap is comparatively small, the majority of the growing microtubule extension is GDP tubulin. The GMPCPP-seeded region of the growing microtubule was composed of 15% labeled rhodamine-tubulin, while the unstable, GTP-tubulin was only 5% labeled. This allowed the two distinct regions to be easily quantified by brightness using total internal reflection fluorescence microscopy (TIRF) (Figure 4.1A, B). Recombinant tau was labeled with fluorophore AlexaFluor-488 (tau$_{2N}$-A488). Two-color TIRF allowed for the localization of tau along the immobilized microtubules. Clearly, tau$_{2N}$-A488 prefers the GDP-tubulin to the GMPCPP-tubulin seeds. This qualitative preferential binding between GDP-tubulin and GMPCPP-tubulin was quantified by varying the amount of tau and measuring the resulting binding (Figure 4.1C). We found nearly a ~5-fold increase in affinity between GDP-tubulin and GMPCPP-tubulin rather than the nucleotide state itself.

As nucleotide state and tubulin conformation are coupled, we sought to determine whether this preference was linked to the nucleotide itself or the conformational state of tubulin in the GMPCPP or GDP bound state (169). To do this we used FCS to measure the amount of tau$_{2N}$-A488 bound as a function of the increase in diffusion time with
increasing tubulin concentration. We incubated tau_{2N}-A488 with GDP-tubulin and GMPCPP-tubulin under non-polymerizing conditions and measured the diffusion time. Unlike the dramatic difference seen in the microtubule lattice, we see no significant preference between GDP-tubulin and GMPCPP-tubulin in solution. Preliminarily, this suggests that the preference for GDP-tubulin versus GMPCPP-tubulin within the lattice is a result of strain. It should be noted, that the two studies are not directly comparable due to experimental restraints; however, the solution tubulin studies favor observable differences between the two states given stronger tau-tubulin interactions within phosphate buffer than BRB80 buffer used within the TIRF assay.
Figure 4.1: 2N4R tau preferentially binds microtubules composed of GDP-tubulin over GMPCPP-microtubules \textit{in vitro}

(A) Diagram of the TIRF microscopy assay used to measure tau binding to microtubules. Bright (15% rhodamine, 5% biotin labeled) GMPCPP seeds were conjugated to the coverslip using NeutrAvidin. Dim (5% rhodamine label) GTP/GDP extensions were grown from seeds in the presence of Alexa488-tau. (B) Kymographs of individual microtubules grown in the presence of 300 nM tau. Black bars indicate the location of the GMPCPP seeds. Scale bars are 1 µm and 30 s in the x- and y-direction, respectively. (C) Binding of Alexa488-tau to microtubule seeds (black) and extensions (gray) under a range of tau concentrations. Curves indicate best-fit Hill function to all data points. Value of $K_D \pm 95\%$ confidence interval (CI) resulting from the fit is shown. Error bars are $\pm 95\%$ CI. (D) Tau does not exhibit a binding preference for GMPCPP-tubulin or GDP-tubulin in solution. Diffusion time measurements were made using fluorescence FCS. The increase in diffusion time as a function of tubulin concentration reflects binding of Alexa488-tau (20nM) to unlabeled tubulin. Measurements were carried out in phosphate buffer (pH 7.4) at 20°C. Data are presented as mean $\pm 95\%$ CI, n $\geq 3$ independent measurements. Curves indicate best-fit Hill function to all data points. See Appendix I.iii for experimental details.
**Structural features of tubulin- and MT-bound tau are similar but not identical**

Prior work within the lab used an environmentally-sensitive fluorophore acrylodan at specific points throughout tau’s MTBR repeat R3 to measure a periodic pattern consistent with a helix when tau is bound to soluble tubulin heterodimer (160). The higher resolution of the recently published cryo-EM structure suggested instead an extended structure throughout the MTBR when bound to microtubules (1). This prompted a re-examination of the previously proposed structure, as well as suggested there may be structural differences between tau-tubulin interacting and tau-microtubule interactions.

Within this study, specific sites within the MTBR repeat R2 were labeled with acrylodan of tau construct PRR-MTBR-R’ which contains all known binding sites to soluble tubulin heterodimers or microtubules to date (Figure 4.2A). Site-specific labeling with the MTBR does not impact binding of tau to tubulin (*Appendix I.viii*). Interestingly, PRR-MTBR-R’ shows biphasic binding with the first plateau before ~2 µM tubulin (Figure 4.2B). As described previously, PRR-MTBR-R’ forms large heterogeneous complexes after ~1.5 µM tubulin (Chapter 3). These complexes are ‘fuzzy’—and consequently highly dynamic and heterogeneous (58). Therefore, the complexes before ~2 µM tubulin represent lower occupancy complexes then those at higher concentrations (Chapter 3, Figure 4.2B). As acrylodan spectral shifts are sensitive to the local chemical environments surrounding the fluorophore, the degree of residue ‘burying’ when tau is bound as well as the average occupancy of any binding site are averaged together (170). To probe the chemical environment of both lower and higher-occupancy complexes, the
acrylodan shifts were measured at both 2 µM and 10 µM tubulin. In all cases, the R2 residues showed a shift in fluorescence emission to lower wavelengths due to a reduction in polarity of acrylodan’s surrounding environment upon binding (Appendix I.xvi). However, the magnitudes of these shifts are both position and tubulin concentration dependent (Figure 4.2D). For both 2 µM and 10 µM tubulin, there is an overall periodic pattern that does not alter dramatically as more tubulin is bound (Figure 4.2D). This suggests that a similar global ensemble exists for the lower and higher occupancy structures, and the additional spectral shift indicates higher occupancy.

The periodic pattern of R2 is very similar to that within R3 – such that the peaks and troughs align well at equivalent residue sites within the repeats. This suggests equivalent tubulin-binding modes between the repeats. Generally, the shift in Δλ max at each residue correlates positively with the surface accessibility (Å²) of the same residue within the cryo-EM structure suggesting these two ensembles are similar (1). Where the periodic pattern between of Δλ max and surface accessibility diverges (residues 292 – 297 in R2 and 323 – 328 for R3) is at the interdimer interface within the cyro-EM tau-microtubule structure, an unstable surface in soluble tau-tubulin structures.
Figure 4.2: Structure of tubulin- and MT-bound MTBR are similar

(A) Schematic of tau (residues 149 – 395) showing proline-rich region (PRR), MT-binding repeats (R1 – R4) and pseudo repeat (R’) domains. Sequence alignment of R1 – R4 is shown below with positions tested in orange. (B) Change in diffusion time ($\Delta \tau_D$) of tau upon tubulin binding. Data points are mean ± SD, n=3. Inset is magnification of $\Delta \tau_D$ for 0 – 3 µM tubulin. See Appendix I.iii for details. (C) Structural model of tau:microtubule (PDB ID:6CVN) where tau is shown as orange cartoon and α(-)/β(+) tubulin as grey/light blue surfaces with their C-terminal residues highlighted in dark grey/turquoise respectively (1). (D) Magnitude of emission shift ($\Delta \lambda_{\text{max}}$) of tau(acrylodan) at different positions in R2 in the presence of 2 or 10 µM tubulin. Data points are mean ± SD, n≥3. See also Figure S2. (E) $\Delta \lambda_{\text{max}}$ in the presence of 2 µM tubulin plotted against surface accessibility in tau:microtubule structure.

PRR and MTBR-R’ share binding sites

As previously mentioned, PRR binding was not seen in the recent cyro-EM structure (Figure 4.2C), but prior NMR studies showed binding of a PRR-like construct (amino acids 166-246) to both stathmin-complexed tubulin and taxol-stabilized
microtubules with 1:2 stoichiometry (65). My work demonstrated PRR binding is tight, and specific, and therefore qualitatively different from MTBR-R’ binding (Chapter 3). One possible interpretation to resolve the differing structural models from cyro-EM and NMR and the qualitative binding differences between the regions is that PRR and MTBR-R’ may bind unique sites on tubulin. To test this, labeled PRR was incubated with unlabeled tubulin prior to addition of unlabeled MTBR-R’. This was also tested with labeled MTBR-R’ incubated with unlabeled tubulin prior to the addition of unlabeled PRR. A decrease in diffusion time indicates a decrease in the number of the labeled tau construct bound to tubulin. Surprisingly, both PRR and MTBR-R’ compete for soluble tubulin with each other. PRR competes completely with MTBR-R’ as indicated by the complete reduction in labeled MTBR-R’ diffusion time to what is expected for MTBR-R’ unbound in solution. However, MTBR-R’ does not completely compete with PRR within the concentration range tested as indicated by the increased diffusion time of labeled PRR from what is expected for unbound PRR. Due to the unknown stoichiometry of MTBR-R’, we cannot determine which region is the stronger competitor or the type of competition. Regardless, our preliminary data suggest PRR and MTBR-R’ either share at least one binding site or are close enough to occlude each other.
Figure 4.3: PRR and MTBR-R’ compete for soluble tubulin

The $\tau_D$ of labeled tau constructs PRR and MTBR-R’ bound to 1 µM soluble tubulin are plotted against increasing competitor concentration (unlabeled PRR or MTBR-R’ respectively). The gray vertical dotted line represents the $K_{D,app}$ of PRR from Figure 3.6 (900 nM). The dotted horizontal lines are the $\tau_D$ for PRR or MTBR-R’ unbound and in the presence of 1 µM tubulin without any unlabeled tau construct. Data are presented as mean ± SD, $n \geq 3$ independent measurements. Measurements were carried out in phosphate buffer pH 7.4 at 20 °C. See Appendix I.iii for details.

Disordered tubulin tails enhance tau binding

It has been suggested that C-terminal most helices H11 and H12 on the tubulin dimers are required for tau binding (68). To the best of our knowledge, the available structural and biochemical data prior to this thesis suggests P1 and R’ do not directly bind the tubulin body (enzymatic chymotryptic digestion of tubulin binding sites (63, 65), and the cryo-EM structure (1)). Additionally, it has been speculated that neutralization of the negative charges on the tubulin tails results in microtubule polymerization (171). This correlation in mutual binding sites between the polymerization-regulating regions of both
tau and tubulin has significant implications, and therefore we sought to test the tail-dependence of PRR binding and R’.

In order to test the role of tubulin tails in tau-tubulin interactions, we digested the tails using substilisin (Appendix I.x). Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) analysis confirmed digestion of both ~ 1 KDa tails from both α(-) and β(+) tubulin of the heterodimer (Fig. A1.4). As demonstrated by FCS, there was nearly a 5-fold weaker affinity than intact tubulin. Furthermore, the binding did not show bi-phasic characteristics. Importantly, treating the tubulin without protease showed similar tau binding to untreated tubulin indicating the handling of tubulin in this manner did not result in weakened binding. We then compared the binding of PRR and MTBR-R’ to intact and tail-less tubulin using FCS. In both cases, the binding of PRR and MTBR-R’ were reduced by upon loss of the tubulin tails. In the case of MTBR-R’, binding was nearly lost. There is a similar dramatic decrease in binding to tail-less tubulin when I tested the K16 (P2-MTBR) construct which lacks P1 and R’. Unfortunately, due to weak binding of the MTBR construct alone to soluble tubulin, I could not test the specificity of tail binding to the MTBR. Collectively the combination of constructs tested suggests none of the proposed binding regions P1, P2, MTBR, or R’ bind uniquely to the tubulin tails.
Figure 4.4 Disordered tails of tubulin enhance tau binding

Binding of tau constructs to intact tubulin, subtilisin digested tubulin, and treated tubulin as measured by an increase in $\tau_D$ as a function of tubulin concentration. Data are presented as mean ± SD, n≥3 independent measurements. Measurements were carried out in phosphate buffer pH 7.4 at 20 °C. Appendix Iii for details of data analysis. (A) PRR-MTBR-R’ binding to intact tubulin (black circle), digested tubulin (light gray circle), and treated tubulin (dark gray diamond). (B) Binding of tau constructs (PRR = square; MTBR-R’ = circle) to intact tubulin (solid color), subtilisin digested tubulin (hollow colored), and treated tubulin (solid gray). (C) Preliminary K16 (P2-MTBR) binding data to tail-less and tailed tubulin (N=1).

The binding dependence on all tested tau constructs to the tubulin tails is curious. It is possible there is no one specific region of tau that the tails bind, and the interaction is largely non-specific electrostatic interactions between the positively charged tau binding domains and the negatively charged tubulin tails. Alternatively, the interaction may be ‘fuzzy’ complex formation in which specific but heterogeneous interactions are forming between the various binding regions on tau throughout the PRR-MTBR-R’ and the IDR tubulin tails.

Tubulin come in a variety of isotypes (discussed in “Tubulin isotypes and the ‘tubulin code’”) which vary significantly in the C-terminal IDR tail. As IDR and IDP interactions are governed primarily by the primary amino acid sequence (discussed in “Intrinsically disordered proteins”), and each tubulin isotype is developmentally
regulated, it suggests the tail region may have a functional origin. The specific isotype, in theory, could influence the formation of the ‘fuzzy’ complex between tau and the IDR tubulin tail due to differing amino acid composition between the tubulin isotypes. As the sampling of PTMs in signal transduction is transient for IDPs, perhaps similarly, tau and tubulin can ‘speak’ to each other through equivalent and matched biophysical mechanisms. In this way, tau isoforms and the tubulin tail isotypes would interact differentially. An underlying recognition mechanism is present, but it is disrupted or enhanced by the specific tau isoform and tubulin isotype present resulting in differing signals between the two proteins and cell-specific behavior. As a preliminary test of this tubulin isotype-dependence of this theory, I designed helical bundles (H9-H12) through the tubulin tails fused to MBP of the two most prevalent tubulin beta isotypes 4A and 2 (TubB4A and TubB2, respectively) within the bovine brain, and measured the binding of PRR. Due to solubility issues, we could not test the binding of PRR to the helical bundles alone for TubB4A, however, PRR does bind to the helical bundle of TubB2 alone. Indeed, PRR alone shows a modest preference for TubB4A over TubB2. As there is little difference in the length or overall predicted compaction of the tails, it is unlikely that a non-specific fishing mechanism applies.
Figure 4.5: Tau binds to helical bundle

(A) Alignment of TubB2 and Tub4A C-terminal tails. The green ‘E’ indicates the polyglutamate PTM site on TubB2. (B) Binding of PRR to MBP fused TubB helical bundles constructs 2 and 4A (MBP-TubB2\textsuperscript{HB} and MBP-TubB4A\textsuperscript{HB} respectively) as measured by an increase in $\tau_{norm}$ as a function of the respective tubulin helical bundle concentrations (MBP-TubB\textsuperscript{HB}). Data are presented as mean ± SD, n≥2 independent measurements. Measurements were carried out in phosphate buffer pH 7.4 at 20 °C. See Appendix I.iii for details of data analysis.

Discussion

Tau-tubulin and tau-microtubule interactions may differ from each other due to tau binding site occlusion within the lattice, potential nucleotide preferences, as well as strain and other spatial constraints along the lattice. Both our current work and prior work (160) suggest that the MTBR repeats upon binding to soluble tubulin and microtubules are similar, and interact with helices H11 and H12 on tubulin. However, it should be
noted neither study resolved R4 or R'; therefore, speculatively either R4 or R' interact with the tubulin tails while R1, R2, and R3 interact predominately with the tubulin body. The cyro-EM paper strongly suggests R4 also interacts with H11 and H12, although the direct evidence of R4 within the full-length protein remains un-modeled due to low resolution of the images (1). Interestingly, PRR still bound weakly, suggesting PRR binds in part along the tubulin body. As resolved by the cryo-EM structure, polar residues within the MTBR region also point outward speculatively toward the β(+)-tubulin tails forming productive interactions (1). All the tested constructs within this brief study bound weaker to tail-less tubulin than intact tubulin, and we cannot determine if there is a specific region within tau that binds to the tubulin tails. Speculatively, it may be that the unresolved regions simply have a preference for the tubulin tails over the tubulin body. Similar to the variable affinities and preference within the MTBR for the lattice (R1 and R2) and soluble tubulin (R3), tau binding to either the tubulin body or the tails is sampled along all its potential binding regions throughout PRR-MTBR-R', and PRR and R' favor tail-binding rather than along the tubulin body. The two tubulin binding hotspots (one on the tails and one on the tubulin body) would then enforce the widely empirically validated 1 tau : 2 tubulin stoichiometry (63, 69).

We have demonstrated lattice-dependent differences between full-length tau’s preferences for GDP over GMPCPP tubulin despite significant structural similarities between the tau-tubulin and tau-microtubule assemblies within the MTBR. Despite the markedly different binding behavior of PRR and MTBR-R', they share similar binding sites and tail-dependent behavior. Strain/nucleotide state, tubulin isotype, PTMs on either
tau or tubulin, and the tau isoform (NTD or MTBR) would then influence the preferential binding towards a more specific tau region and consequently its unique structural and functional characteristics. Therefore, tau-tubulin and tau-microtubule interactions may be emergent properties of the tau global ensemble coupled to the tubulin code (both strain along the tubulin body sensed through the helices H11 and H12 and patterning of isotype and PTMs on the tubulin tails).

CHAPTER 5: Conclusions and Perspectives

My work within this thesis: (1) identifies PRR as an independent binding site to soluble tubulin with polymerization capacity thereby expanding the currently described tau-tubulin binding interactions and adds additional preliminary insight into comparing tau-tubulin and tau-microtubule interactions, (2) demonstrates tau’s conserved NTD/PRR/MTBR conformational ensemble, and finally (3) uses this biochemical information to refine current tau-mediated polymerization models. Collectively, the work within this thesis develops a model that focuses on tau’s interdomain interactions and functions, and expands tau’s functional complexity by further coupling these binding interactions to the tubulin ‘code’.

(1) PRR versus MTBR binding: expanding functional tau binding sites to soluble tubulin

Frequently, it is broadly assumed that tau-tubulin and tau-microtubule interactions are homologous. However, the tubulin lattice is a tube of alternating α(-) and β(+) subunits lined end-to-end and zippered up. This results in a steric-driven system. There
may be potential binding sites accessible only when tubulin is unincorporated. For example, the heterodimeric α(-) end that is buried within the lattice or the side of the tubulin would be oriented toward the lumen once incorporated into the microtubule lattice may be potential tau-binding sites in solution. Furthermore, tau’s interactions with the microtubule are largely driven by electrostatics (55-59). Therefore, if the tubulin dimer is conformationally strained resulting in a deformation of the electrostatic field on its surface, tau would potentially be sensitive to this ‘warped’ tubulin binding region. Consequently, differences in binding site occlusion or even lattice strain may influence soluble tau-tubulin interactions in different ways from the higher-order and constrained tubulin structures. The Rhoades group tests this assumption directly. Prior work within the lab has demonstrated that tau may bind to tubulin tighter than to microtubules (62). Similarly, there are differences in hierarchal binding affinity within the MTBR repeats between microtubules and tubulin (119, 160). To the best of my knowledge, the work presented here represents the first systematic domain-centric search of tau-tubulin interactions beyond the MTBR to soluble unincorporated tubulin without additional stabilizing tubulin binding partners such as stathmin-like proteins. This led to the surprising observation of PRR cooperative binding to soluble tubulin that was comparatively tight and specific in comparison to the isolated MTBR-R' which exhibited weaker binding. Although P2 has long been indirectly associated with tubulin binding and polymerization (63-65, 94), P1 has been missing from most studies, yet it doubles tau’s binding capacity to soluble tubulin heterodimers and binds tubulin independently. Future work on tau-tubulin interactions and tau-mediated microtubule dynamics must
include P1 to fully incorporate all binding sites. Although tau is frequently divided into the ‘projection domain’ (NTD and P1) and the ‘binding domain’ (P2 and MTBR-R’), these broad functional labels are misleading. As tau-tubulin and tau-microtubule interactions are dominated by electrostatic interactions (55-59), terminology and construct designs that emphasize the amino acid content of the tau regions are more nuanced. This would separate tau into three major regions. In the middle, the positively charged ‘core’ containing PRR-MTBR-R’ which is both binding and polymerization competent. Flanking either side of the ‘core’ would be oppositely charged domains C-terminal tail, and the NTD that negatively regulates the formation of tau-tubulin assembly and spaces microtubules.

Although the PRR is capable of binding both lattice-incorporated and unincorporated soluble tubulin, it is beyond the scope of this thesis to fully describe this interaction. As previously mentioned, the recent cryo-EM structure did not resolve the PRR binding on the microtubule lattice. We have presented preliminary evidence that tau binding is heavily regulated though the tubulin tails and exhibits lattice-specific binding preference for GDP-tubulin over GMPCPP-tubulin. However, this tail-dependent binding is not specific to the PRR, and our current data suggests this binding is not isolated to any one region of tau. Furthermore, PRR and MTBR-R’ share binding sites on tubulin; as the MTBR is structurally similar when bound to both unincorporated and incorporated tubulin, this would suggest PRR in part mimics MTBR along the tubulin body. Although the tubulin binding sites may be the same, the binding characteristics of the tau region bound vary (compare PRR to MTBR-R’) and may prove functionally relevant in
combination with either binding region on tubulin. For example, speculatively PRR:tubulin tail interactions may favor microtubule growth in the labile region while MTBR-R:tubulin body interactions may favor microtubule ‘pausing’ or stability. Further structural and biochemical work is needed to resolve PRR binding on the microtubule as well as to soluble tubulin. Speculatively, there are two binding ‘hotspots.’ One binding site is on the tubulin body where tau senses mechanical strain along the lattice or whether the tubulin is incorporated into the lattice. The other binding site on the tails that can be either rapidly modified using PTMs or more statically incorporated by tubulin isotype overall regulating tau binding as well as tau-microtubule interactions with other cellular partners.

(2) PRR binding and NTD negative regulation: refining polymerization models

Prior work from the lab focused on the functional relevance of ‘fuzzy’ complex formation derived from the number of MTBR-R’ binding sites (58). In this model, the dynamics of tau on the tubulin surface favorably increases the number and size of tau:tubulin complexes. This ‘fuzzy’ complex formation is a dynamic binding property that impacts tau-tubulin interactions in addition to the number of binding sites within tau and their relative affinities to tubulin. In other words, this model added an additional heterogeneous, structural component to the basis of tau-mediated polymerization ‘jaws’ model (64, 94, 95) that focuses on primarily on binding affinity and the number of binding sites. The benefit of this view stems from tau’s potential to transiently sample several binding regions on tubulin through multiple different binding sites within the
PRR-MTBR-R’ (1, 172). I have found the NTD enforces an upper limit on the size of the tau:tubulin complexes and the number of tubulin bound within the these large assemblies. This negative regulation results in a ‘upper limit’ of the extent of tau-tubulin complex formation. Perhaps most interesting is the lack of heterogeneous complex formation when PRR is bound to tubulin, suggesting the initial tubulin binding and microtubule ‘seeding’ step is accelerated by the multiple binding-sites within the MTBR-R’ in a concentration-dependent manner. Therefore, the PRR acts as an initiating clamp flanked by a positive regulator (MTBR-R’) and a negative regulator (NTD). Together, these two findings present a refinement to prior tau-mediated polymerization models (58).

Coupling this polymerization model with the conserved NTD/PRR/MTBR conformational ensemble, the specific combination of PRR and MTBR-R’ binding sites filled may be transiently sampled and therefore influenced by the NTD domain. With this view, although the isoforms may not necessarily have dramatically different binding affinities to either soluble tubulin or microtubules, there may be differences in how these isoforms function in the initial polymerization steps. The interactions between these domains would tip the polymerization capacity of tau to an overall positive or neutral/stabilizing state depending on which domain was dominating the tau structural ensemble. This could be influenced by salt, tau:tubulin concentration and ratio, the NTD or MTBR isoform or PTMs.
To the best of our knowledge, presented within this thesis is the first structural comparison of the NTD isoforms. Surprisingly, the conformational ensemble is conserved. Functionally, the NTD serves to slow polymerization and reduce the number of tau molecules bound. There are subtle differences between all NTD isoforms in the global structural ensembles, the binding heterogeneity at saturation, and a small but significant difference in polymerization of tau\textsubscript{2N}. However, these differences are not as great as one would anticipate given the strict developmental control of expression and the number of added amino acids between isoforms. Perhaps the uniform behavior of these isoforms within an in vitro system is misleading as the developmental regulation of the isoforms implies a dynamic and growing system that is difficult to recapitulate in vitro. Speculatively, the conserved ensemble acts as a general template for tau interactions, with each NTD/MTBR isoform being expressed and decorated with PTMs in both a combinatorial (the precise isoforms and PTM present) and graded (the ratio of isoform and expression level) manner to suit the diverse and rapidly changing demands of a growing cell.

**In summary: complexity in dynamic systems favors biological function**

There are approximately 100 billion neurons with breath-taking specializations varying in shape and size (173). Within differentiated cells, microtubule dynamics along the axon may promote cargo pausing and encourage delivery to presynaptic sites (13). Similarly, microtubule dynamics has recently been suggested to influence memory and
learning with misregulation resulting in several diseases, including Alzheimer’s disease (174). Recent advancements in *in situ* imaging of microtubule dynamics are allowing for the rapidly involving studies of microtubule dynamics *in vivo* (175). One such study demonstrated that suppression of microtubule dynamics during differentiation leads to muscle tissue dysfunction in *Caenorhabditis elegans* thereby suggesting distinct sets of MAPs are needed for tissue biogenesis and tissue function respectively (176). At the same time, it has been suggested tau mRNA expression in neurons can encourage differentiation in resistant or apoptotic sensitive cells (101), and increased tau expression is associated with axonogenesis (177, 178). More specifically, one of the first biomarkers of neuronal polarity is increased tau localization to the neurite that eventually turns into the axon (178).

Salient features of tau are its functional role in neurodegenerative disorders and microtubule dynamic instability, tightly developmentally regulated isoforms, and promiscuous binding and cellular interactions. Tau can polymerize microtubules along both polarities (builds from both the $\alpha$(-) and $\beta$(+) end), phase separate and form microtubule bundles *in vitro* (124), locate itself to the axon specifically through the PRR *in vivo* (166), and potentially sense strain along the microtubule lattice. Two things an injured football player and newborn baby have in common are severe head trauma and differences in tau biology from ‘normal’ adult biomarkers (88, 97, 179). The aforementioned links between (1) microtubule dynamics and neuron-specific communication, (2) tau’s role in regulating microtubule dynamics, and (3) tau’s association with axonogenesis and differentiation as well as loss-of-function disease
mechanisms, lead to speculative theories that tau-tubulin/microtubule interactions play an active role in both neuron differentiation and maintenance. Perhaps tau is involved in maintaining axonal polarity in injured or differentiating cells? Or even more broadly, perhaps tau is partially enforcing a particular regime of microtubule dynamics specific to each cell or cellular compartment to establish or re-establish homeostatic functions? Regulation of tau’s conformational ensemble would favor a subset of tau-mediated cellular interactions over both long (isoform expression) and short (PTMs, environmental sensitivity) time scales with the necessary sensitivity in complex cell signaling without the need for novel regulatory cascades. In this way, tau-tubulin interactions are an omnipresent regulatory mechanism, unlike the specific MAP subsets previously suggested (176). For example, 0N3R may slightly favor TubB4A over TubB2, bind tightly and overall enrich TubB2 within the microtubule lattice during axonogenesis. However, during birth or a sudden injury, established neural networks may need those connections reinforced in a non-specific manner. Continuing with my speculative example, the corresponding PTMs to discourage 0N3R-microtubule binding would be increased as well as PTMs to encourage 1N4R binding specifically along the new axon. 1N4R may have no preference for TubB4A over TubB2 and may increase the concentration of both along the axon. Consequently, upon injury, the previously TubB2-enriched axon becomes a more easily recognizable microtubule mosaic.

In this way, tau would mirror the influx of unincorporated tubulin and its changes both along the lattice and within solution to produce the desired microtubule dynamics for that particular cell given its current homeostatic, diseased/damaged, or growing state.
Tau-tubulin/microtubule interactions would then have greater temporal resolution through modifying PTMs. In this way, a unified and easily modified regulatory template exists across all cells that are swayed simply by favoring certain tau:tubulin ensembles without demanding novel protein factors with more specific regulatory sensitivity. Robust development of the tau-mediated polymerization underpins critical assumptions of this hypothesis, as it is dependent on balancing a variety of equilibriums between the tau-tubulin binding sites and the tau regions themselves. As demonstrated in this thesis, the PRR domain has different and unique behavior to the MTBR-R’ yet each full-length isoform has a conserved ensemble which regulates both regions. How these tau regions interact with each other and the combinatorial interplay between each region and their respective isoform as a function of cellular environment, concentration, and interacting tubulin ‘code’ presents a network problem with tau’s emergent property within a given set of environmental conditions being defined primarily as a node of its collective behavior throughout the cell. In other words, perhaps our diverging experimental observations are simply an isolated subclass within the tau global ensemble with a specific observable function and conformational ‘structure’.
APPENDIX I: Experimental Procedures

i. Tubulin purification and handling

Tubulin was purified from fresh bovine brains as described in (146). Purified tubulin was snap-frozen in BRB80 (80mM PIPES pH 6.8, 1mM MgCl₂, 1mM EGTA). Prior to use, frozen aliquots were rapidly thawed and then clarified at 100,000xg for 6 minutes. BioSpin 6 columns (BioRad) were used to buffer exchange tubulin into the desired assay buffer. The tubulin absorbance at 280 nm was converted to concentration using a molar extinction coefficient of 115,000 M⁻¹ cm⁻¹. Tubulin was used within 2 hours following clarification.

ii. Tau cloning, purification, and labeling

The parent tau plasmid encodes for longest tau isoform, tau₂N. It includes an N-terminal His-tag with a tobacco etch virus (TEV) protease cleavage site for purification (119). For site-specific fluorescent labeling, the native cysteines, C291 and C322, are mutated to serine to allow for the introduction of cysteines at desired locations. Tau₁N, tau₁N* and tau₀N were generated using deletion cloning from the tau₂N plasmid. The nicked DNA fragments were fused using T4 DNA ligase (New England Biolabs) and T4 Polynucleotide kinase (New England Biolabs). The remaining tau fragments were generated using either site-directed mutagenesis to introduce stop codons and cysteines, deletion cloning of the remaining tau amino acids within the parent tau vector or a combination of the two techniques.
For the tau$_{2N_{scr}}$ construct, the Sequence Manipulation Suite at bioinformatics.org was used to generate a series of random sequences based on residues 45-103, corresponding to the N1 and N2 inserts. I chose the shuffled sequence that most closely matched the distribution of hydrophobic and charged residues found in the wild-type sequence without replicating the majority of that sequence. This oligonucleotide was synthesized (Integrated DNA Technologies) with flanking nucleotides in the tau$_{0N}$ (C291S C322S S433C) for splicing by overlap extension (111).

For all constructs (expect tau$_{1N}$) longer than 200 residues, protein expression was induced with 1mM IPTG at OD ~0.6 overnight at 16 °C. For constructs <200 residues, tau protein expression was induced with 1 mM IPTG at OD ~0.8 for 4-5 hours at 37°C. Tau$_{1N}$ constructs were induced with 1 mM IPTG at OD ~0.8 at 25 °C for 4-5 hours. Purification was based on previously reported methods (119). Briefly, cells were lysed by sonication, and the cell debris pelleted by centrifugation. The supernatant was incubated with nickel-nitrilotriacetic acid resin (Ni-NTA) (Qiagen or BioRad) and the recombination protein was bump eluted with 500 mM imidazole. The His-tag was removed by incubation with lab purified tobacco etch virus (TEV) proteinase for either 4 hours at 20 °C (constructs <200 residues) or overnight at 4 °C (constructs >200 residues). Uncleaved protein was removed by a second pass over the Ni-NTA column. Remaining contaminants were removed using size exclusion chromatography on a HiLoad 16/600 Superdex 200 Column (GE LifeSciences) in Buffer C supplemented with 1 mM TCEP. Proteins that did not require fluorescent labeling were buffer exchanged using Amicon concentrators (Millipore) into the final assay buffer of interest, aliquoted and snap frozen.
for storage at -80 °C. Due to the small size and lack of aromatic residues, P1 and P2 were TEV-cleaved as described above but after the fluorescent labeling (below). All other proteins were labeled following elution from the size exclusion column.

All FRET and FCS measurements were carried out in constructs where both native cysteines have been mutated to serine, C291/322S; for FRET, two additional cysteines are introduced at desired locations as indicated in Table 2.1. For FCS, a single cysteine is introduced as indicated in Table 3.2. Site specific labeling of tau for FRET or FCS measurements was carried out as described previously (119). Briefly, tau was incubated with 1 mM DTT for 30 minutes, and then buffer exchanged into labeling buffer (20 mM Tris pH 7.4, 50 mM NaCl, and 6 M guanidine HCl). For FRET labeled constructs, the donor fluorophore, Alexa Fluor 488 maleimide (Invitrogen), was added at sub-stoichiometric ratios (0.3-0.5x), and incubated at room temperature for 15 minutes. A 3-fold molar excess of the acceptor fluorophore, Alexa Fluor 594 maleimide (Invitrogen) was added and the reaction was incubated for another 10 minutes at room temperature, and then moved to 4 °C for overnight incubation. For FCS labeled constructs, Alexa Fluor 488 maleimide was added in 3-fold molar excess and incubated at room temperature for 10 minutes, followed by overnight incubation at 4 °C. Labeling reactions were protected from ambient light and with constant stirring; the dye was added dropwise. For smaller constructs and tau1N, incubation for 1.5 hours at room temperature instead of overnight at 4 °C was used on occasion. The labeled protein was buffer exchanged into 20 mM Tris pH 7.4 and 50 mM NaCl and unreacted dye was removed using HiTrap Desalting Columns (GE Life Sciences). Labeled protein was aliquoted and
snap frozen for storage at -80°C. Cloned constructs were verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.

### iii. FCS instrument and data analysis

All FCS measurements were performed on our home built instrument, as described previously (58). Prior to entering the inverted Olympus 1X-71 Microscope (Olympus), the laser power was adjusted to ~ 5 μW (488 nm diode-pumped solid-state laser, Spectra-Physics) and focused into the sample via a 60x water objective (Olympus). Fluorescence emission from the sample was collected through the objective, separated from excitation light by a Z488RDC long pass dichroic and a 500 nm long pass filter (Chroma). The filtered emission was focused the aperture of a 50 μm diameter optical fiber (OzOptics) coupled to an avalanche photodiode (Perkin-Elmer). A digital correlator (FLEX03LQ-12, Correlator.com) generated the autocorrelation curves.

Measurements were made in 8-chamber Nunc coverslips (Thermo-Fisher) passivated by incubation with (ethylene glycol)poly(L-lysine) (PEG-PLL)(62). The labeled tau (15-25 nM) and tubulin (concentrations vary) were incubated in chambers for 5 minutes prior to measurement. Unless otherwise noted, all FCS experiments were carried out in phosphate buffer pH 7.4 (20 mM phosphate, 20 mM KCl, 1 mM MgCl₂, 0.5 mM EGTA, 1 mM DTT) at 20 °C. Multiple (20-40) 10 second autocorrelation curves were collected per sample and fit to a single-component 3D diffusion equation:

\[
G(\tau) = \frac{1}{N\left(1 + \frac{\tau}{\tau_D}\right)} \sqrt{\frac{1}{1 + \frac{\tau^2}{\tau_D}}} \quad \text{Eq. 2}
\]
where $G(\tau)$ is the autocorrelation function as a function of time ($\tau$), $\tau_D$ is the translational diffusion time of the labeled molecules and $N$ is the average number of fluorescent species. For our instrument, the ratio of the radial to axial dimensions of the focal volume (s) was determined to be 0.2 and consequently fixed for analysis. The recorded intensity trace is divided by N to give counts per molecule (CPM) in kHz.

For some tau constructs, high tubulin concentrations (>1 µM) result in the formation of large, bright species (Chapter 3). These species are not present in the traces of protein in the absence of tubulin (Fig. 3.8). A prior study from our lab demonstrated these species are tau:tubulin specific, electrostatically sensitive and reversible (58). For P2-MTBR, increasing the KCl concentration in our phosphate buffer to 300 mM – previously seen by NMR to disrupt interactions between the PRR and tubulin – results in disassembly of the larger species (Table 3.2) (95). In the case of PRR-MTBR-R', these species persist even at 300 mM KCl suggesting the binding is either tighter or has a more hydrophobic character (Table 3.2).

The individual autocorrelation curves arising from these larger assemblies disproportionally weight the averaged autocorrelation curves used in the analysis described above (Table 3.3 and Fig. A1.1). Working under the premise that removal of these outliers would allow for a more meaningful analysis of the majority tau:tubulin complexes, we developed an algorithm to remove aberrant curves, broadly following the approach we described previously (58). Individual autocorrelation curves were fit with Eq. 2 and assessed the goodness of fit using least-squares $\chi^2 = [G(\tau)_{fit} - G(\tau)_{raw}]^2$ with a
tolerance of $X^2 = 0.0001$ for a consecutive run of 75 ms. In other words, if the fit deviated beyond the $X^2$ for more than 75 ms, the curve was discarded. This process removes 99.5% of curves that cannot be accurately fit using Eq. 2. The frequency of these aberrant curves is ~3% (Fig. A1.1A).

Autocorrelation curves arising from larger assemblies that pass this initial criterion still skew the data towards slower diffusion times (Table 3.3). Descriptive statistics of these diffusion times are reported in Table 3.3 as ‘pre-filtering’. In some cases, such as PRR-MTBR-R' the measured $\tau_D$ could be as large as ~14 ms and up to 4x brighter than unbound tau (Fig. A1.1B,C). Although of potential interest in another context, these species do not represent the majority of the tau:tubulin complexes of interest here. These outliers were removed in an iterative fashion by testing the individual curves using an Anderson-Darling statistical test for either a lognormal or normal distribution. Diffusion times above or below the interquartile range were removed until a stable population was reached and no more curves were removed from the dataset. We did not: (1) enforce a lognormal or normal distribution on the data-set prior to outlier removal; nor (2) continue or use outlier removal if the population is normal or lognormal after testing. This iterative function is demonstrated for tau$_{2N}$ in the absence (Fig. A1.1D) or presence of 10 µM tubulin (Fig. A1.1E). The initial iteration simply tests for normality (seen by the straight line in Fig. A1.1D for a single iteration). This results the removal approximately ~15-25% of curves that passed the initial goodness-of-fit filtering from the data set (Table 3.3).
The fit parameters from the individual filtered curves are presented as scatter box plots throughout Chapter 3, and the descriptive statistics of these values across multiple independent measurements are listed in Table 3.3. There is a general correlation showing that tau:tubulin complexes with larger $\tau_D$s also had larger CPMs, reflecting the presence of multiple tau molecules in these assemblies (Fig. 3.3). In order to allow for straightforward comparison between tau isoforms, we also averaged the filtered curves from each independent measurement and fit the average curve with Eq. 2. These $\tau_D$ values obtained from these fits are reported in Table 3.2 for saturating points, and are graphed in figures with FCS binding curves.
Measurements of tau constructs in the absence and presence of 10 μM tubulin were carried out in phosphate buffer pH 7.4 at 20 ºC. The algorithm written to analyze the data from these experiments is demonstrated here. **(A)** τ_{norm} of MTBR-R’ in the absence or presence of tubulin without any filtering. Inset is a magnification of the y-axis for low τ_{norm}. **(B)** τ_{norm} of tau_{2N}, tau_{1N}, tau_{1N*}, tau_{0N}, tau_{2Nscr} and PRR-MTBR-R’ with 10 μM tubulin after goodness-of-fit filtering, but without iterative filtering with lognormal distribution overlaid. **(C)** The corresponding CPM (kHz) for each point in **(B)** (D) Mean and median τ_{D} (right), the standard deviation of τ_{D} (middle) and number of curves (left) for tau_{2N} over the iteration number (i=1) for tau_{2N} in the absence of tubulin. No curves are selected for elimination in this tau-only sample. **(E)** Mean and median τ_{D} (right), the standard deviation of τ_{D} (middle) and number of curves (left) for tau_{2N} in the presence of 10 μM tubulin over the iteration number demonstrating convergence of the algorithm (at i=9) as described.

**Figure A1.1: Filtering algorithm**
Tau constructs of different lengths have different diffusion times. To allow for straightforward comparison of the extent of binding between the various constructs, the diffusion times for each construct in the presence of tubulin ($\tau_{D}^{\text{bound}}$) were normalized to that of the construct in the absence of tubulin ($\tau_{D}^{\text{free}}$) as follows:

$$\tau_{\text{norm}} = \frac{\tau_{D}^{\text{bound}}}{\tau_{D}^{\text{free}}}$$  \hspace{1cm} \text{Eq. 3}

iv. FRET instrument and analysis

FRET histograms where the protein signal was readily distinguishable from the ‘zero-peak’ (180), arising from imperfect labeling, were carried out on our lab built instrument as described previously (119). The laser power is adjusted to $\sim$30 $\mu$W (488nm diode-pumped solid-state laser, Spectra-Physics) prior to entering the microscope. Donor and acceptor photons were separated using a HQ585 long pass dichroic and further selected with ET525/50M band pass and HQ600 long pass filters (Chroma). For each path, the emission was focused onto the aperture of a 100 $\mu$m diameter optical fiber (OzOptics) coupled to an avalanche photodiode (Perkin-Elmer). Time traces were collected in 1 ms time bins for 1 hour. As described above, measurements were carried out in PEG-PLL coated Nunc chambers with 20-40 pM labeled tau following 5 minutes incubation with tubulin.

To differentiate photon bursts arising from transit of a labeled molecule from background fluorescence, a photon count threshold of 30 counts/ms was applied. For
each burst, $ET_{eff}$ calculated using a lab-based written software (MATLAB) according to the following equation (181, 182):

$$ET_{eff} = \frac{(I_a - \beta I_d)}{(I_a - \beta I_d) + \gamma(I_d + \beta I_d)}$$  

Eq. 4

where $I_a$ is the intensity of the acceptor photons and $I_d$ is the intensity of the donor photons. Within our system, the bleed through of the donor channel into the acceptor channel ($\beta$) and the difference in the total quantum efficiency of the system and fluorophores ($\gamma$) were determined using Alexa Fluor 488 hydrazine (Invitrogen) and Alexa Fluor 594 hydrazine (Invitrogen) and fixed for analysis. Due to variation in instrument build and detector efficiency over the course of the study, $\beta$ and $\gamma$ were regularly re-determined and checked with DNA standards of 10, 14, and 18 bases labeled with Alexa Fluor 488 and Alexa Fluor 594 (Integrated DNA Technologies). The energy efficiencies were then binned, and the histograms fit using a sum of Gaussians in Origin. One Gaussian described the “zero-peak” (donor-only fluorescence) and the second peak described donor and acceptor labeled protein (main peak fit listed in Table 2.1). In some cases, the distribution was asymmetric (such as tau$^{17-149}$). In these cases, three Gaussians were used to fit the data. The Gaussian that fit the dominant peak is reported in Table 2.1.

At some of the labeling positions, the proteins gave rise to low energy efficiencies with overlap with zero-peak, making it difficult to accurately determine the peak $ET_{eff}$ for the protein sample. To separate donor-only labeled species from the low energy, donor and acceptor labeled species, measurements were repeated on a commercial MicroTime 200 time-resolved confocal microscope (Picoquant) using its
pulsed interleaved excitation FRET (PIE-FRET) mode. The power of the excitation lasers (485 nm and 562 nm) were matched ~30 μW at 40 MHz. The fluorescence emission was focused through a 100 μm pinhole and collected by avalanche photodiode. Fluorescence emission of the donor and acceptor fluorophores were separated using a HQ585 long pass dichroic and further selected with ET525/50M band pass and HQ600 long pass filters. SymphoTime 64 software was used to analyze the photon bursts to yield both the \( ET_{\text{eff}} \) and stoichiometry factors for each burst, using photon threshold, binning and experimentally determined \( \beta \) and \( \gamma \) values as described previously. The binned histograms were fit as described above.

v. Tubulin light scattering assay

Polymerization of soluble tubulin was measured by monitoring the increase in scattered light at 340 nm. The tubulin was clarified as described above and buffer exchanged in phosphate buffer at pH 6.9 (20 mM phosphate, 20 mM KCl, 1 mM MgCl\(_2\), 0.5 mM EGTA, 1 mM DTT) immediately prior to use. For polymerization reactions, 10 μM tubulin was incubated with 5 μM tau (for tau\(_{2N}\), tau\(_{1N}\), tau\(_{1N^*}\), tau\(_{0N}\) and PRR-MTBR-R') or 10 μM tau (for PRR, 2N-PRR, MTBR and MTBR-R') for 2.5 minutes on ice prior to the addition of 1 mM GTP. Immediately after the addition of GTP, the reaction was transferred to a warmed cuvette and the reaction was monitored for 10 minutes at 37 °C in a fluorometer (Fluorolog FL-1039/40, Horiba) with a photon counting module (SPEX DM302, Horiba) with both excitation and emission wavelengths set to 340 nm.
Following polymerization, the samples were quickly returned to 4 °C for 5 minutes; cold depolymerization is evidence that the proteins are not aggregated.

The curves were normalized to account for account for day-to-day variability in the lamp intensity and fit in Origin with:

$$y = \frac{1}{1 + e^{-\frac{t - t_{1/2}}{dt}}}$$  \hspace{1cm} \text{Eq. 5}

where $y$ is the normalized fluorescence intensity, $t$ is time, $t_{1/2}$ is the polymerization half-time and $dt$ is the time constant. The mean and standard deviation of the $t_{1/2}$ values are listed in Table 3.1. The plotted graphs represent the average of the normalized triplicate with standard deviation. Curves with very little polymerization, were normalized to the brightest intensity within the given day.

Polymerization assays were carried out both with wild-type constructs (containing the native cysteines at residues 291 and 322) as well as with constructs that had been designed for FCS measurements but had not been labeled (native cysteines at residues 291 and 322 mutated to serines with an additional cysteine mutation introduced for labeling). For two tau constructs, I directly compared the polymerization kinetics of wild-type and the C291/322S mutants and found that within the resolution of the light scattering assay, the curves are identical (Fig. A1.2). This indicates both that: (1) the removal of the native cysteines does not impact the ability of tau to polymerize tubulin; and (2) the introduction of a non-native cysteine for labeling purposed does not impact the ability of tau to polymerize tubulin. Thus, for the data shown within the thesis in
Figs. 3.5 and 3.6, the triplicate measurements often consist of both wild-type and C291/322S variants. To illustrate, within one triplicate, one curve is obtained with wild-type tau while the other two are with the C291/322S mutant (or vice versa). Similarly, I tested the polymerization competence of tau$_{2N}$ before and after labeling with Alexa Fluor 488. In this study, due to the reduced yield of labeled tau$_{2N}$, the concentrations of tau and tubulin were changed to 2 µM tau and 20 µM tubulin and carried out in BRB80 buffer. Again, there is no significant difference between the two polymerization curves, indicating that within the resolution of our assay, fluorescent labeling does not impact tau-mediated polymerization.
Figure S4. Labeled tau and unlabeled tau polymerize tubulin comparably. Normalized tubulin polymerization is shown as a function of time. Polymerization in the presence of Alexa488-tau is shown in green while that in the presence of unlabeled tau is shown in gray. Polymerization of soluble tubulin was measured by monitoring the increase in scattered light at 340 nm. Tubulin was clarified as described in the Materials and Methods and then buffer exchanged in BRB80 buffer. For polymerization reactions, 20 $\mu$M tubulin was incubated with 2 $\mu$M tau for 2.5 minutes on ice prior to the addition of 1 mM GTP. The reaction was then transferred to a warm cuvette at 37 °C. The arrow indicates cold depolymerization at 4 °C.

Figure A1.2: Neither FCS mutations nor dye impact tubulin polymerization

Tubulin polymerization as measured by scattered light at 340 nm as a function of time. Data are presented as mean ± SD following normalization, n=3 independent measurements. Arrows indicate depolymerization at 4 °C. (A) The polymerization kinetics for wild-type PRR-MTBR-R’ and PRR-MTBR-R’T149C-C291/322S, the variant created for site-specific labeling for FCS, are indistinguishable indicating that under the conditions of our assay, removal of tau’s native cysteines and introduction of a new cysteine does not impact tubulin polymerization. Likewise, the polymerization kinetics of wild-type PRR and the variant created for site-specific labeling for FCS, PRRT149C, are comparable. For these assays, 10 µM PRR or 5 µM PRR-MTBR-R’ were incubated with 10 µM tubulin in phosphate buffer pH 6.9 with 1 mM GTP at 37 °C. (B) The polymerization kinetics for 2N4R T149C-C291/322S (unlabeled tau) and 2N4R T149C-C291/322S labeled with AlexaFlexa-488 (Alexa-488 tau) are indistinguishable under the conditions of our assay indicating dye does not impact tubulin polymerization. For these assays, 2 µM 2N4R was incubated with 20 µM tubulin in BRB80 with 1 mM GTP at 37 °C.

The polymerizations were carried out in phosphate buffer at pH 6.9 while the FCS experiments were carried out at pH 7.4. This chosen primarily due to PRR solubility issues at pH 7.4 in phosphate buffer at high concentrations as above ~ 7 µM PRR begins to aggregate without glycerol present as indicated by increased absorbance signal at 340 nm. It has been suggested that tau binding is pH sensitive through histidine-mediated binding located throughout its MTBR region (75). To test whether the polymerization assay condition at pH 6.9 unfavorably biases polymerization of the MTBR-R’ region, I measured the binding of fluorescently labeled PRR-MTBR-R’ to unlabeled tubulin using
FCS. There is no significant difference in PRR-MTBR-R' binding to soluble tubulin between the two pH conditions indicating the polymerization conditions do not screen MTBR-R' polymerization.

![Graph showing pH effects on binding](image)

**Figure A1.3: pH does not affect PRR-MTBR-R' binding**

The $\tau_D$ of PRR-MTBR-R’ is plotted against increasing tubulin concentration at two different phosphate buffer pHs. Measurements were carried out in phosphate buffer at 20 °C. See *Appendix I:iii* for details of data analysis.

### vi. Electron microscopy imaging

*EM images were taken by Dr. Changsong Yang of the Svitkina group.*

Polymerization reactions using PRR, 2N-MTBR-R’, and PRR-MTBR-R’ were carried out and monitored as described above. After 10 minutes of polymerization at 37 °C, the sample was transferred to Cu-coated 200 mesh electron microscopy grids (Electron Microscopy Sciences, FCF200-Cu) and incubated with 20 µM taxol on the grid.
for 1.5 minutes, as generally described in published protocols (183). The grids were washed with BRB80 buffer to remove interfering phosphate salts and stained with 2% uranyl acetate. Images were taken on a JEOL JEM-1011 transmission electron microscope.

**vii. Microtubule pelleting assay**

Taxol stabilized microtubules were made by incubating ~65 μM clarified tubulin with 4 μM taxol for 15 minutes at 37 °C. The taxol concentration was increased to 40 μM and incubated for another 15 minutes. The polymerized microtubules were pelleted by ultracentrifugation at 353,000xg for 20 minutes at 25 °C. The resulting pellet was resuspended in phosphate buffer adjusted to pH 6.9. The concentration of the harvested microtubules was determined by absorbance at 280 nm (ε=115,000 M⁻¹cm⁻¹) after cold and chemical denaturation in 8 M urea at 4 °C. The taxol-stabilized microtubules were diluted to 10 μM in phosphate buffer pH 6.9, and incubated with 1 μM Alexa Flour 488 labeled tau for 10 minutes at room temperature. The microtubules and associated tau were pelleted by centrifugation at 353,000xg for 20 minutes. The pellet was resuspended in an equal volume as the collected supernatant (70 μL) and cold denatured. Tau in the absence of microtubules was subjected to the same assay as a control. Quantification of microtubule binding was by SDS-PAGE; band intensity of Alexa Fluor 488 fluorescence was analyzed using ImageJ. The gels were imaged using using Typhoon FLA 7000. Both Alexa Fluor 488 fluorescence and Coomassie staining were recorded for the same gel.
viii. Fluorescence anisotropy

Fluorescence emission of tau\textsubscript{acrylodan} and tau\textsubscript{488} labelled at position T149 were measured on a JASCO-8300 fluorometer with temperature control accessory ETC-815. For tau\textsubscript{acrylodan} was excited at 390 nm and scanned from 400 to 600 nm. For tau\textsubscript{488} was excited at 470 nm and scanned from 480 to 650 nm. Both excitation and emission slits were set to 2.5 nm. Clarified tubulin was buffer exchanged into phosphate buffer (20 mM potassium phosphate pH 7.4, 20 mM KCl, 1 mM MgCl\textsubscript{2}, 0.5 mM EGTA, and 1 mM DTT) and incubated at varying concentrations with 300 nM tau for 5 min prior to measurement. Each spectra was integrated to overcome spectral shifts in acrylodan. The anisotropy signal (r) was calculated according to Eq. 6:

\[
I = \frac{I_{VV} - I_{VH}^2}{I_{VV} + 2I_{VH}^2}; \quad G = \frac{I_{HV}}{I_{HH}}
\]

Eq. 6

Where \(I_{VV}, I_{VH}, I_{HH},\) and \(I_{HH}\) are the integrated intensities for each respective excitation and emission polarizer position.

The normalized change in anisotropy (\(r\text{\scriptsize{norm}}\)) is calculated by subtracting the measured anisotropy signal subtracted from the unbound tau anisotropy signal and dividing by the difference between the bound signal at 2 \(\mu\)M tubulin and the unbound signal according to Eq. 7:

\[
\frac{r\text{\scriptsize{norm}}} = \frac{r - r\text{\scriptsize{unbound}}}{r\text{\scriptsize{bound}} - r\text{\scriptsize{unbound}}}
\]

Eq. 7
A1.4 Labeling positions or common dye sizes do not affect binding

Anisotropy ($r$, right and $r_{\text{norm}}$, left) of tau labeled with acrylodan at I277 and K281 or with Alexa Fluor 488 at T149 titrated with tubulin. Data points are mean ± SD, n=2 – 3. Error bars in normalized plot on the left are not error-propagated. The $r$ of Alex Fluor 488 and acrylodan differ, however, the binding curves are the same ($r_{\text{norm}}$) are comparable. As the spectral shifts of acrylodan differ between I277 and K281, the spectral shift is not a reflection of weakened binding due to point mutation and labeling, but rather the local environment when bound.

ix. Tubulin tail digestion

Tailless tubulin was generated by modification of a published protocol (184). Briefly, tubulin was quickly thawed and buffer exchanged into 1/10X MES buffer (0.1 M MES pH 6.9, 1 mM MgCl$_2$ and 1 mM EGTA) using Bio-spin 6 columns and diluted to 50 µM. GTP was added to 1 mM and the mixture was incubated at room temperature for 5 mins. Subtilisin was then added to 16.8 µg/mL (1:300 w/w) and the reaction was incubated for 45 mins at room temperature. PMSF (0.1 mM) was added to quench the reaction and the mixture was incubated on ice for 10 mins before centrifugation at 110,000xg for 20 mins. Supernatant containing tailless tubulin was buffer exchanged back into MES buffer for storage at -80°C. Complete cleavage of the tails was confirmed by MALDI-TOF mass spectrometry. All subtilisin-treated tubulin had a loss of ~1.8-2
kDa when compared to intact tubulin, consistent with the loss of tails from both α(-) and β(+) subunits. Prior to use, aliquots of tailless tubulin were clarified and buffer exchanged as described previously.

![Figure A1.5: Digestion of tubulin tails](image)

MALDI-TOF spectra of intact (upper) and tailless (lower) tubulin. The difference in mass between intact and tailless tubulin is <2 kDa, consistent with the removal the disordered tails from both tubulin monomers (184).

**x. MBP-TubB\(^{HB}\) Purification**

The TubB\(^{HB}\) genes for TubB4A (amino acids 310-444) and TubB2 (amino acids 310-445) were codon optimized for bacterial expression and synthesized by Genescript, and then cloned into the pET His6 maltose binding protein (MBP) TEV ligation independent cloning (LIC) vector (plasmid #29656) using traditional LIC techniques. Alignment of the TubB\(^{HB}\) component for MBP-TubB4A and MBP-TubB2 in Figure A1.4.
pET His6 MBP TEV cloning vector (1M) was a gift from Scott Gradia (Addgene plasmid # 29656; http://n2t.net/addgene:29656; RRID:Addgene_29656)

Figure A1.6: Alignment of TubB helical bundles

Alignment of amino acid sequences of bovine TubB4A and TubB2 with secondary structures highlighted and numbered according to PDB 1JFF (185). Blue highlighted regions indicate β-sheets and red regions indicate α-helices.

The MBP-TubB<sub>HB</sub> constructs were induced with 500 µM IPTG at OD ~0.6 at 25 °C overnight in LB media. The collected pellet was resuspended in Ni-Buffer A supplemented with 2 mg/ml lysozyme and 1 EDTA-free Complete inhibitor cocktail tablet and stored at -80 °C. Cells were lysed by sonication, and the cell debris pelleted by centrifugation. The supernatant was incubated with nickel-nitrilotriacetic acid resin (Ni-NTA) (Qiagen or BioRad) and the recombination protein was bump eluted with 500 mM imidazole. Remaining contaminants were removed using size exclusion chromatography.
on a HiLoad 16/600 Superdex 200 Column (GE LifeSciences) in Buffer C (25 mM Tris pH 8.0, 100 mM NaCl) supplemented with fresh 1 mM TCEP.

**xi. TubB^{HB} Purification**

The TubB2 plasmid encodes for an N-terminal His-tag with a TEV protease cleavage site for purification. TubB2 were induced with 1 mM IPTG at OD ~0.8 at 37 °C for 3.5 hours in unsupplemented terrific broth media. The collected pellet was resuspended in BRB80 supplemented with 10 mM imidazole and 50 mM NaCl, 2 mg/ml lysozyme, and 1 EDTA-free cOmplete inhibitor cocktail tablet and stored at -80 °C. Cells were lysed by sonication, and the cell debris pelleted by centrifugation. The supernatant was incubated with Ni-NTA resin (Qiagen or BioRad) and the recombination protein was bump eluted with 500 mM imidazole. The His-tag was removed by incubation with lab purified TEV proteinase for 2.5 hours at 20 °C. Uncleaved protein was removed by a second pass over the Ni-NTA column. Remaining contaminants were removed using size exclusion chromatography on a HiLoad 16/600 Superdex 200 Column (GE LifeSciences) in Buffer C supplemented with fresh 1 mM TCEP.

**xii. CD Spectroscopy**

Circular dichroism (CD) confirmed helical structure of the tubulin bundles. Spectra were taken in 20mM phosphate buffer pH 7.4. The measured CD signal (θ_{obs}(λ)) was converted to molar ellipticity ([θ]) using Eq. 8:

$$[\theta] = \frac{\theta_{obs}(\lambda)}{n_{ph} \times 10 \times l \times c}$$

Eq. 8
where \( n_{pb} \) is the number of peptide bonds (number of amino acids – 1), \( l \) is the pathlength and \( c \) is the concentration.

![Graph](image)

**Figure A1.7: Purified TubB\textsuperscript{HB} constructs are predominately helical**

All tested constructs form predominately helical bundles as indicated by characteristic negative molar ellipticity ([\( \Theta \)] deg·cm\(^2\)·dmol\(^{-1}\)) at ~ 195 nm and ~ 225 nm as measured by CD.

xiii. **Preparation and functionalization of imaging chambers**

*Slide preparations were performed by Dr. Brian Castle of the Odde group.*

Imaging chambers for TIRF microscopy were assembled and functionalized as described in (186), with few modifications. First, an acid cleaned coverslip was rendered hydrophobic by brief incubation in Rain-X® Original Glass Water Repellent (ITW Global Brands, Houston TX) at room temperature (187). Coverslips were then allowed to dry completely before remaining residue was wiped away using lens paper. Hydrophobic coverslips were then mounted to acid cleaned glass slides using double sided tape, forming three separate imaging channels.
Imaging chambers were functionalized by flowing in solutions of 0.1 mg/mL NeutrAvidin (ThermoFischer Scientific, Waltham, MA) in PBS, followed by 5% Pluronic® F-127 (Sigma-Aldrich, St. Louis, MO) in PBS, and then doubly stabilized GMPCPP microtubule seeds (5% or 15% rhodamine-labeled, 5% biotin-labeled) in BRB80 (80 mM PIPES/KOH pH 6.9, 1 mM EGTA, 1 mM MgCl$_2$). For each separate solution, chambers were allowed to incubate at room temperature for 10 mins and then washed with 8-10x chamber volume of BRB80. After the last wash, 2x chamber volumes of the imaging solution was flowed through the imaging chamber before moving to the microscope for imaging. Imaging solution consisted of indicated concentration of porcine brain tubulin (5% or 15% rhodamine-labeled, Cytoskeleton Inc., Denver CO) and Alexa488-labeled tau in BRB80 supplemented with 1 mM GTP, 40 mM D-glucose, 8 µg/mL catalase, 20 µg/mL glucose-oxidase, and 0.1 mg/mL casein. To prevent sample drying during imaging, individual imaging chambers were sealed using CoverGrip sealant (Biotium, Fremont, CA).

xiv. TIRF microscopy

TIRF microscopy experiments were performed by Dr. Brian Castle of the Odde group.

Unless otherwise noted, all proteins were handled and stored as described previously (186). Rhodamine-labeled microtubules growing from double-stabilized GMPCPP microtubule seeds were imaged by TIRF microscopy using a 100x, 1.49NA Apo TIRF objective on a Nikon TiE inverted stand equipped with the Perfect Focus, H-TIRF module and LU-N3 laser launch (Nikon Instruments Inc., Melville, NY) under control of NIS-Elements software (v4.xx, Nikon Instruments). Images were collected on a Zyla 4.2 PLUS sCMOS camera (Andor, Belfast, UK) with a high speed emission filter.
wheel (HS-632; Finger Lakes Instrumentation, Lima, NY) placed between the camera and stand for color separation. Additional 1.5x tube lens in the microscope stand resulted in a total magnification of 150x (42 nm/pixel). For tau binding and microtubule dynamics imaging, 488 nm and 561 nm TIRF lasers were reflected up through the rear aperture of the objective using a triple band pass filter set (TRF69901; Chroma Technology Corp., Bellows Falls, VT). Unless otherwise noted, all images were collected using 200 ms exposure at 20% laser power. For FRAP experiments, a 488 nm 100 mW Argon-ion laser (Spectra-Physics, Santa Clara, CA) shuttered by a Uniblitz VS35 shutter (Vincent Associates, Rochester, NY) was focused on the imaging plane as previously described using a separate light path from that used for TIRF imaging (188). Bleach event timing was set to a 3 s delay and 100 ms exposure using a VMM-TI shutter driver/timer (Vincent Associates). Simultaneous TIRF imaging was accomplished by replacing the triple band pass filter above with an 80/20 beam splitter. To compensate for the beam splitter, the 488 nm TIRF laser was increased to 100% power such that 20% laser power used for imaging was maintained. Temperature was maintained at 37°C using an objective heater (OkoLab S.R.L., Pozzuoli, Italy) and airstream incubator (Nevtek, Burnsville, VA).

xv. Microtubule tip tracking and fluorescence profile analysis

_Microtubule fluorescence analysis was performed by Dr. Brian Castle of the Odde group._

The dynamic microtubule end was tracked using our previously described semi-automated algorithm, TipTracker (version 3.1), without modification (189, 190). Briefly, fluorescence profiles along the determined microtubule axis (x’’-axis) are fit with a Gaussian survival function (Eq. 9):
where $\mu_{PF}$ is taken to be the position of the microtubule tip, $I_{MT}$ and $I_{BG}$ are the fluorescence intensity on the microtubule and the background, respectively, and $\sigma_{PF+PSF}$ is the spread of the fluorescence due to the combination of the point spread function and the taper or spread of protofilament lengths at the microtubule tip. For averaging purposes, fluorescence profiles along the microtubule axis from all channels (tubulin, tau, and EB1) were aligned to $\mu_{PF}$ determined from the tubulin channel (either rhodamine-tubulin or mCherry-α-tubulin). Fluorescence offset values were the difference between $\mu_{PF}$ resulting from tracking the tubulin and tau channels (offset = $\mu_{PF, tub} - \mu_{PF, tau}$). In vitro fluorescence profiles were normalized to the maximum value while in vivo profiles were normalized to an average of the first 10 values.

xvi. Acrylodan labeling

Tau construct purification and acrylodan labeling was carried out by Dr. Ho Yee Joyce Fung in the adapted figures within this thesis. Jennifer Rameriez and myself purified additional constructs for other figures within the published paper (142). Jennifer Rameriez labeled those constructs.

For labeling, the proteins were concentrated to $\sim$300 $\mu$M ($\varepsilon_{\lambda=280nm} = 4470$) and treated with 1 mM DTT for 30 mins at room temperature to reduce the cysteines. The proteins were then buffer exchanged into Buffer E (20 mM Tris pH 7.4, 50 mM NaCl) with 6 M guanidine HCl using HiTrap desalting columns. For Alexa Fluor 488 labeling, the C5 maleimide dye in a DMSO stock was added in 2 – 4 times molar excess and incubated at room temperature for 30 mins followed by overnight incubation at 4°C.
acrylodan labeling, DMSO was added to the protein sample to 10% final concentration prior to the addition of dye to 4 times molar excess and incubated for 4 hrs at room temperature. In both cases unconjugated dye and guanidine HCl was removed from the protein samples by two rounds of buffer exchange into Buffer E using concentrators and a final desalting column step. Labeled proteins were aliquoted, snap frozen and stored at -80°C until use. Complete labeling of tau proteins were confirmed by MALDI-TOF mass spectrometry using a Bruker Ultraflex III instrument. Sinapic acid dissolved in 50:50 acetonitrile:water (v/v) and 0.1% TFA was used as a matrix.

xvii. Acrylodan fluorescence
Acrylodan measurements were performed by Dr. Ho Yee Joyce Fung.

Fluorescence emission of tau<sub>acrylodan</sub> was measured using a Horiba Fluorolog-3 fluorometer in a Quartz cuvette. Excitation was set at 390 nm and emission was scanned from 400 to 600 nm. Both excitation and emission slit widths were set at 2 nm. The cuvette holder was held at 20°C. 300 nM tau<sub>acrylodan</sub> was mixed with varying tubulin concentrations in Buffer F and incubated in an eppendorf tube for 5 mins prior to measurement. Either 3 or 4 independent measurements were made for each tau construct using different tubulin aliquots across multiple days.

xviii. Analysis of acrylodan emission peak shifts
Acrylodan analysis was performed by Dr. Ho Yee Joyce Fung.

Each emission trace was background subtracted with buffer or tubulin only measurements. Maximum emission wavelength was determined using Origin peak search algorithm using 1<sup>st</sup> derivative method with smoothing. For each labeling position, measurements were made of tau in the absence and presence of tubulin and the mean
emission maximum of tau in the absence of tubulin was used to calculate the shift in the emission peak, $\Delta \lambda_{\text{max}}$. The emission maximum for tau labeled with acrylodan in the absence of tubulin was relatively insensitive to position, with peaks ~522 nm (Figure A1.7). The emission peaks shifts are reported as means and standard deviations. The error is propagated in the calculations for changes in peak shifts. In Figure 3B and C, normalization was performed using the measured peak shift at 10 µM tubulin and the standard deviation was error-propagated. Data were plotted using Origin and the accompanying structure figure (Figure 4.2) was generated using Pymol. Surface accessibility of residues in Figure 4.2 was calculated using GetArea (191).

**Figure A1.8: Raw acrylodan data**

(A) Emission spectra of tau acrylodan in all positions tested in this study. Intensities are normalized to peak maximum for comparison. Peak emission was not affected by location of acrylodan conjugation. (B) Representative plots of emission blue-shifting ($\Delta \lambda_{\text{max}}$) of tau acrylodan (at K281) in the presence of 2 µM and 10 µM tubulin.
APPENDIX II: FCS Python code

The original code developed to analyze FCS data was written in MATLAB and required curves to be discarded ‘by eye’ prior to weighting and fitting the data. The individual curves were analyzed by a separate program that imposed a Gaussian over the data and removed any curves outside of this distribution. Again, the goodness of fit of each curve was not assessed but was handled at the user’s discretion. In the case of the later program, it was developed to analyze K16 data – which suggests only one tau is bound at 10 µM tubulin – and therefore discarded up to 70% of the PRR-MTBR and PRR-MTBR-R’ data sets. To correct for the redundant problem of biased datasets towards smaller species and user dependent curve filtering, I wrote a new program using Python which analyzes all the desired information without user input. The parameters and additional description are listed in Appendix 1.iii. I would like to thank Joshua Straquadine for helpful advice and assistance in the development of this program.


162. L. Buée, T. Bussière, V. Buée-Scherrer, A. Delacourte, P. R. Hof, Tau protein isoforms, phosphorylation and role in neurodegenerative disorders11These authors contributed equally to this work. *Brain Res Rev* **33**, 95-130 (2000).


I did it!