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Defining the Oligomerization Domains of Tau Using a Split-Luciferase Strategy

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ABSTRACT

Defining the Oligomerization Domains of Tau Using a Split-Luciferase Strategy

by

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Microtubules (MTs) are highly dynamic components of the cell cytoskeleton that are necessary for many functions, including cell division, cellular locomotion, and intracellular transport. An essential mechanistic feature of MT physiology is dynamic instability, which is characterized by the frequent polymerization and depolymerization of tubulin subunits at MT ends. This dynamicity is critical to MT function and is regulated by MT-associated proteins (MAPs), which interact with tubulin dimers and/or the MTs themselves.

Tau is a prominent neuronal MAP that stabilizes MTs by promoting growth events, stability, and by suppressing shortening events. On the other hand, dysregulation and mutation of tau are associated with pathogenesis in various neurodegenerative diseases, such as Alzheimer's disease (AD), frontotemporal dementia with Parkinsonism-17 (FTDP-17), and progressive supranuclear palsy (PSP). Taken together, it is critical to understand both normal tau physiology as well as how altered tau function leads to disease pathogenesis.

Previous research has suggested that tau is able to dimerize or oligomerize via its N-terminal projection domain as part of its normal function. One currently proposed model, based on *in vitro* data, is that two tau molecules form an "electrostatic

zipper" in which the N-termini of the two molecules associate in an antiparallel fashion, with the C-termini containing the MT-binding region of each tau molecule extending away from one another. If correct, this model could explain many features of tau action. We investigated this hypothesis in mammalian cells using a split-luciferase strategy in order to (i) test the above stated model for tau oligomerization in cells and (ii) identify and map regions of the protein that are capable of tau-tau oligomerization.

We found that constructs containing the N-terminus of tau produce significantly higher luciferase signals indicative of oligomerization compared to constructs containing the C-terminus. More specifically, the construct containing amino acids 1-120 produces the strongest luciferase signal, consistent with our proposed model that the N-terminus of tau is responsible, at least in part, for its oligomerization activity. Interestingly, C-terminal regions of tau are also capable of promoting tau oligomerization. Taken together, our data suggest that both the N- and C- termini of tau are each sufficient to promote tau oligomerization in mammalian cells.

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Chapter 1: Microtubules and Tau

1.1 Microtubules

Microtubules (MTs) are dynamic cytoskeletal polymers that are fundamental to all eukaryotic cells. In proliferating cells, MTs are essential for chromosome segregation and cell division. Further, MTs are especially important in post-mitotic neurons, which have highly elongated axons. Indeed, MTs are critical for establishing axonal morphology and axonal transport (Jordan and Wilson, 2004). MTs are composed of repeating heterodimeric subunits of α- and β- tubulin. These tubulin subunits bind in a longitudinal fashion to form protofilaments, which then assemble laterally into a hollow, cylindrical MT.

An essential feature of MT biochemistry is dynamic instability, which is characterized by periods of MT polymerization and depolymerization of tubulin subunits at MT ends. Dynamic instability consists of five proposed stages: growing, shortening, attenuation (or pause), catastrophe (the transition from growth to shortening), and rescue (the transition from shortening to attenuation or growth). These behaviors depend on the amount of strain in the MT lattice as a result of GTP hydrolysis by β- tubulin. The generally accepted model is that when GTP-tubulin dominates the tubulin subunits at a MT end, this produces a relatively straight structure that readily takes on additional GTP-tubulin subunits, thereby producing a

period of growth. Alternatively, if the end of the MT is dominated by GDP containing tubulin, the MT end takes on a more curved structure that introduces strain into the overall structure, which promotes depolymerization or a shortening event. From an attenuated state, MTs can either "grow" or "shorten". If the rate of GTP hydrolysis at the MT end exceeds the rate of new tubulin subunit addition at the end, then the MT will depolymerize rapidly. MTs undergoing catastrophe can undergo "rescue," where new GTP tubulin subunits are added to begin the assembly process again.

1.2 Tau

Dynamic instability is critical to MT function and is tightly regulated by MT-associated proteins (MAPs) which interact with tubulin dimers and/or the MTs themselves. Tau is an intrinsically disordered neuronal MAP that stabilizes MTs by promoting MT growth and attenuation and suppressing shortening events (Drechsel et al., 1992, Bunker et al., 2004). Normal and pathological tau action is regulated by post-translational modifications (PTMs), most notably phosphorylation and acetylation. For example, pathological tau is hyperphosphorylated in AD brains (Wang et al., 2013) and aberrant acetylation of tau has been shown to impair MT-binding and promote aggregation (Cohen et al., 2011).

Dysregulation and mutation of tau are associated with pathogenesis in a variety of neurodegenerative diseases, such as AD, FTDP-17, PSP, and Pick's Disease. One widely held model for pathological tau action proposes that compromised tau-MT

interactions lead to impaired MT stabilization, which in turn leads to neuronal cell death. At the same time, non-MT bound tau aggregates into neurofibrillary tangles (NFTs), one of the two hallmark pathologies of AD and several related neurodegenerative conditions. However, recent work has demonstrated that insoluble NFTs are not necessary for neurotoxicity, and that soluble, oligomeric tau is sufficiently neurotoxic (Kopeikina et al., 2012). It is also widely held that neurodegeneration spreads in an anatomically progressive manner via the sequential trans-synaptic uptake of pathological tau (Liu et al., 2012). In this model, pathological tau spreads from diseased neurons to healthy ones through stereotyped trans-synaptic connections within the brain. However, the mechanism by which this trans-synaptic pathological tau transfer occurs is poorly understood. Recent work has shown that tau transfer is achieved by direct transmission of exosomes between neurons (Wang et al., 2017) and that uptake might be regulated by the low-density lipoprotein receptor-related protein 1 (LRP1) (Rauch et al., 2020).

In the adult human brain, six tau isoforms are normally expressed as a result of alternative mRNA splicing of the primary transcript from the *MAPT* gene. These isoforms contain either three or four imperfect MT-binding repeats ("3R" or "4R" tau) and zero, one, or two N-terminal inserts (0N, 1N, 2N tau) (Goedert et al., 1989) (**Figure 1**). The significance of these different isoforms is discussed in more detail below in section 1.3.

1.3 Tau Structure and Function

Because of its intrinsically disordered nature, tau is typically divided into four domains, based on its amino acid composition and known functional capabilities:

1) Microtubule-binding region (MTBR)

2) C-terminal region (CTR and R')

3) Proline-rich region (PRR)

4) N-terminal tail (NTR).

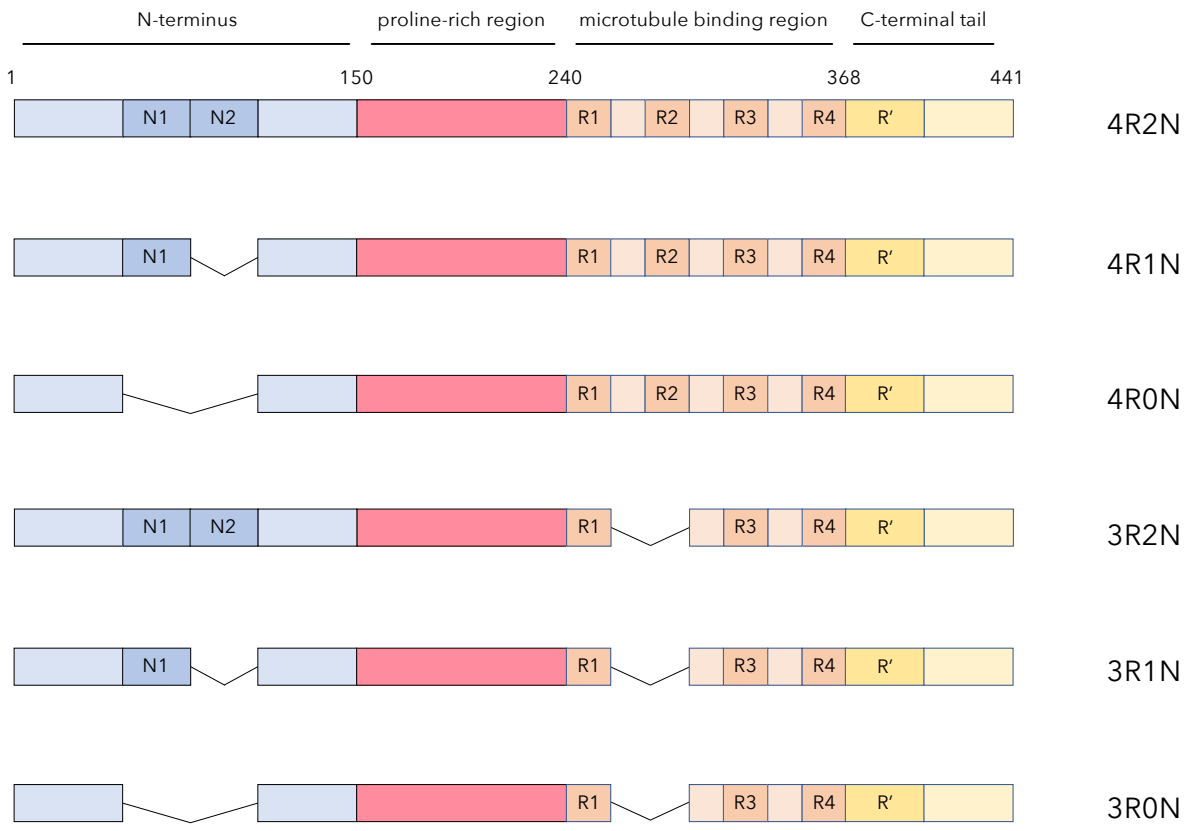

Figure 1 Schematic of Tau Isoforms: The six tau isoforms generated by alternative splicing, with major features labeled, including the N-terminus (blue) which contains zero, one, or two N-terminal inserts (0N, 1N, 2N), the microtubule-binding region (orange) which contains either three or four imperfect MT-binding repeats (3R, 4R), the proline-rich region (red), and the pseudorepeat (R')-containing C-terminal tail (yellow).

These regions are diagrammed above in **Figure 1** and discussed individually below.

1.3.1 Microtubule-binding region (MTBR)

The best understood region of tau is the MTBR (**Figure 1**), which is located in the C-terminal half of the protein and is composed of either three or four 18 amino acid long imperfect repeats separated by 13-14 amino acid long inter-repeats. Alternative splicing of exon 10 of the *MAPT* gene, which encodes the first inter-repeat and the second imperfect repeat, distinguishes 3R tau from 4R tau. Tau mRNA alternative splicing is developmentally regulated. More specifically, 3R and 4R tau are expressed at approximately equal levels in normal adult human brain (Kosik et al., 1989) whereas fetal human brain contains only 3R0N tau. Since mutations that affect tau RNA alternative splicing but do not alter the tau amino acid sequence lead to FTDP-17 and related neurodegenerative tauopathies (Clark et al., 1998), it follows that there must be some functional and/or regulatory differences between 4R and 3R tau. For example, 4R tau has recently been shown to stabilize MT disassembly intermediates more effectively than 3R tau (Best et al., 2019), consistent with observations that 4R tau is more protective against MT shortening events than 3R tau (Bunker et al., 2004).

Recent cryo-EM work has demonstrated that an individual repeat/inter-repeat unit can associate with a single tubulin heterodimer (Kellogg et al., 2018). The multiple imperfect repeats present in each tau molecule allow a single tau to associate with

multiple tubulin heterodimers simultaneously, which likely accounts for the MTBR's ability to promote MT nucleation and assembly (Li and Rhoades, 2017). Traditionally, the inter-repeats were thought to have played a passive role in MT binding, with the imperfect repeats being the primary drivers of the tau-MT interaction. However, the first inter-repeat (present in the alternatively spliced exon 10) has been shown to contain its own MT-binding motif with more than twice the binding affinity than any individual repeat (Goode and Feinstein, 1994). Specifically, the entire MT-binding activity is contained in the ^{265}KVQIINKK^{272} sequence of the first inter-repeat, with Lys^{265} and Lys^{272} being the critical residues for MT-binding (Goode and Feinstein, 1994). The importance of lysine residues in general within the MTBR has recently been highlighted as critical for the pathological internalization of tau by LRP1 (Rauch et al., 2020). The electrostatic interactions between the MTBR-resident lysines and LRP1 are believed to be necessary for the endocytosis and subsequent pathological, trans-synaptic spread of tau through sequential regions of the brain affected by AD and related tauopathies, providing a potential treatment target for these disorders.

Finally, the importance of the MTBR to pathological tau action is highlighted by the fact that a large fraction of the neurodegenerative disease-causing tau mutations that alter the amino acid sequence of tau map to the MTBR, where they appear to interfere with normal tau-tubulin interactions and promote formation of paired helical filaments (PHFs), the principal component of NFTs characteristic of AD (Crowther et al., 1989; Kellogg et al., 2018).

1.3.2 C-terminal Tail (CTT and R')

Regulation of tau-tubulin interactions is not limited to the MTBR. Immediately C-terminal to the MTBR is a highly conserved "pseudo-repeat" region, denoted R', which is in the CTT. The conserved sequence in R' has a higher fraction of charged residues and a higher net positive charge per residue when compared to the repeats. More importantly, these positive charges are clustered within a 5-6 residue span, potentially forming an additional binding motif for tubulin (Li and Rhoades, 2017). Several disease-causing mutations that change the charge distribution within R' display altered MT dynamics (Niewidok et al., 2016), suggesting that R' may play a critical role in tau function, distinct from the MTBR.

1.3.3 Proline-rich Region (PRR)

Immediately N-terminal to the MTBR is a very positively charged, proline-rich region that contains the majority of tau's many phosphorylation sites (**Figure 1**), including many phosphorylation sites associated with AD (Morris et al., 2015). While the functions of the N-terminal half of tau are generally poorly understood, the PRR has been shown to enhance tau's ability to bind to MTs and promote their assembly (Goode et al., 1997). Interestingly, the sequence ^{215}KKVAVVR^{221} within the PRR is similar to the ^{265}KVQIINKK^{272} sequence of the first inter-repeat. Mutagenesis of residues Lys^{215}/Lys^{216} and Arg^{221} to uncharged residues disrupted MT binding,

demonstrating that these charged residues are critical for the effects of the PRR on tau MT-binding activity (Goode et al., 1997).

Recent work has shown that the PRR tightly and stoichiometrically binds soluble tubulin and promotes MT polymerization, describing a novel role for the PRR as an independent tubulin-binding domain with polymerization capability (McKibben and Rhoades, 2019). In a newly proposed model of PRR-MTBR-R' coordination, the PRR serves as the core tubulin binding domain, binding two tubulin heterodimers tightly during initiation of polymerization. The MTBR and R' increases the local concentration of tubulin through distributed weak interactions, enhancing the polymerization ability of tau (McKibben and Rhoades, 2019). It is believed that the differential properties between the two domains ultimately lead to polymerization and stability of a MT. More recently the PRR, but not the MTBR, has been shown to drive tau liquid-liquid phase separation (LLPS) *in vivo* (Zhang et al., 2020). Changing the phosphorylation states of residues in the PRR altered the weak interactions necessary for LLPS; phosphomimetic substitutions abolished light-induced LLPS, suggesting that the phase separation ability of the PRR is modulated by its phosphorylation state.

1.3.4 N-terminal Region (NTR)

The negatively charged NTR of tau is also known as the "projection domain", as it is believed to project away from the MT surface when tau is bound to MTs.

There are two N-terminal amino acid inserts that arise from alternative splicing of exons 2 or 3 (Himmler, 1989), but their functions are poorly understood. It is known that the NTR determines the spacing between bundled MTs (Chen et al., 1992) and can mediate tau's association with the neuronal plasma membrane (Brandt et al., 1995). Although the NTR has very little MT-binding ability on its own, its removal from the full-length protein resulted in an increase in tau's affinity for MTs (Gustke et al., 1994), suggesting that there is interplay between the NTR and the rest of tau.

On the other hand, recent work examining the impact of the NTR on tau-tubulin interactions showed that the presence of the NTR dramatically reduced MT-binding and tubulin polymerization capability (McKibben and Rhoades, 2019). While constructs containing the PRR and/or MTBR+R' alone were capable of binding to MTs and promoting their assembly, constructs including the NTR did not demonstrate appreciable binding to tubulin or significant polymerization (McKibben and Rhoades, 2019). The model that emerges from these data characterizes the NTR as a "gate" that may dynamically shield the weak tubulin binding sites within the rest of tau, negatively regulating the tight, stoichiometric binding of tubulin by the PRR and the weak binding of tubulin by the MTBR+R' (McKibben and Rhoades, 2019). Because tau function is regulated by PTMs, the implications drawn from this model need not be mutually exclusive to those drawn in Gustke et al. (1994).

Dimerization or higher-order oligomerization of tau has been proposed to be a part of tau's normal mechanism of action (Makrides et al., 2003; Rosenberg et al.,

2008; Feinstein et al., 2016). Indeed, self-association of proteins to form dimers or higher-order oligomers is a very common phenomenon (Marianayagam et al., 2004). Recent work has suggested that the NTR is necessary for tau oligomerization *in vitro* (Rosenberg et al., 2008; Feinstein et al., 2016). The notion of the NTR being responsible for tau oligomerization also raises a possible model for pathological tau action, which is described in more detail below in the Introduction to Chapter 2.

Finally, the NTR might also play a role in LRP1-mediated internalization of tau during the process of trans-synaptic transfer of pathological tau in the progression of AD and related dementias (Rauch et al., 2020). An NTR fragment (amino acids 1-243) was shown to interact with the mLRP2 ligand-binding domain of LRP1. This interaction is believed to be a secondary mediator of tau endocytosis, behind the MTBR's interaction with mLRP4, another ligand-binding domain of LRP1.

1.4 Concluding Remarks

With all the work establishing the NTR as critical for tau function, it is imperative that we understand how this region might regulate normal and pathological tau action. Despite extensive structure-function analyses, the nature of NTR-mediated regulation remains enigmatic. The model that tau functions as a dimer or higher-order oligomer as part of its normal function is attractive, as this model also provides a model for pathological tau action, but tau oligomerization has not been well explored within the cellular context. Though *in vitro* work supports the model that the NTR is

able oligomerize, does the same model hold true *in vivo*? Additionally, if tau does in fact function as an oligomer, precisely which domain(s) that are responsible? Answering these questions are a key next step in elucidating the mechanisms of tau action.

Chapter 2: Defining the Oligomerization Domains of Tau

2.1 Introduction

MTs are dynamic cytoskeletal polymers that are especially important in elongated neurons. Their dynamicity is regulated by tau, which stabilizes MTs by promoting MT growth and attenuation and suppressing shortening events (see Chapter 1; Drechsel et al., 1992; Bunker et al., 2004). Tau also regulates MT-dependent axonal transport (Trinczek et al., 1999). The progressive aggregation of tau into NFTs has long been characterized as a hallmark of several neurodegenerative diseases, but the molecular mechanism(s) by which pathological tau action contributes to neuronal cell death are still poorly understood. The microtubule-binding region (MTBR; see Chapter 1, Figure 1) forms the core of the paired helical filaments (PHFs) that are sufficient for PHF nucleation and assembly into NFTs *in vitro* (Friedhoff et al., 1998; von Bergen et al., 2004). However, although it was presumed for many years that the PHFs and NFTs were the toxic entities, more recent work suggests otherwise (Kopeikina et al., 2012).

Tau hyperphosphorylation and tau fragmentation are also common features of tauopathies (Gamblin et al., 2003), and both have been proposed to be contributors to tau toxicity. For example, an N-terminally derived 17 kDa proteolytic fragment of tau lacking the MTBR is produced in Aβ-induced neurodegeneration (Park and

Ferreira, 2005), has been visualized in brain samples of AD patients and additional tauopathies (Ferreira and Bigio, 2011), and has been shown to be neurotoxic in cultured cells (Park and Ferreira, 2005).

A more recent model that integrates both normal and pathological tau action suggests that dimerization or oligomerization of tau (hereafter referred to as "oligomerization" for the sake of simplicity) is a key part of the normal mechanism of tau action (Makrides et al., 2003, Feinstein et al., 2016). It has been proposed that this oligomerization is mediated by an "electrostatic zipper" that is formed when two NTRs associate with one another in an antiparallel fashion with their C-termini containing the MTBRs extending away from each other. This model was first proposed based upon biophysical analyses using a surface-forces apparatus (SFA), leading to the suggestion that the electrostatic zipper with antiparallel N-termini might be responsible for the uniform spacing between MTs in MT bundles (Rosenberg et al., 2008). More recent work has sought to directly test the hypothesis that the NTR of tau can promote oligomerization. Based on the observations that recombinant full-length tau proteins migrated as multiple bands in native gel electrophoresis (non-denaturing conditions) and N-terminally truncated tau proteins migrated as single bands under the same conditions, it was concluded that full-length tau can oligomerize via the NTR (Feinstein et al., 2016). Additional experiments demonstrated that the 17 kDa N-terminal fragment (amino acids 45-230, containing the PRR and most of the projection domain) formed heptamers and octamers under native conditions.

Surprisingly, the 17 kDa N-terminal fragment was also able to form strong, SDS-resistant dimers even under denaturing conditions (Feinstein et al., 2016). Taken together with the previous SFA analyses, these data provide strong *in vitro* evidence supporting tau oligomerization via its NTR.

The fact that the NTR can homo-oligomerize is particularly interesting in light of the fact that previous work had shown that treatment of cultured primary hippocampal neurons with β-amyloid (Aβ) leads to calpain-1 activation and the generation of a neurotoxic 17 kDa tau fragment (amino acids 45-230), which contains most of the NTR as well as the PRR, but lacks the MTBR (Park and Ferreira, 2005). This same fragment has been shown to be present in neurons in brains from AD patients and several related tauopathies. This, taken together with the oligomerization model, suggests a model for pathological tau action. Since the NTR can oligomerize, it is possible that trans-synaptic transfer of NTR-derived fragments from AD-affected neurons to healthy recipient neurons serves as a means to inactivate the normal tau in the healthy neuron via hetero-oligomerization with the pathological 17 kDa fragment, leading to eventual cell death.

Because oligomerization via the NTR might play a role in mediating both normal and pathological tau action, it is important to know *where* specifically in the NTR this oligomerization occurs. It is also important to note that while tau oligomerization and aggregation has been examined from a biophysical, *in vitro* context, very few studies have assessed multimeric tau behavior in a physiologically

relevant, cellular context. This study employs a split-luciferase strategy in mammalian cells to address both concerns.

2.2 Materials and Methods

cDNA Plasmids

The full-length tau-luciferase expression vectors were kind gifts from Dr. Suzanne Wegmann (German Center for Neurodegenerative Diseases). Human 4RL tau cDNA was ligated into the HindIII (5') and EcoRV (3') restriction sites in a pAAV-CBA-WPRE vector using Assembly Master Mix (New England Biolabs). A 42 base-pair linker and either half of *Gaussia princeps* luciferase ("luci", amino acids 1-92 or "ferase", amino acids 92-163) were ligated in-frame to the C-terminus of 4RL tau. Deletion constructs of 4RL tau (Δ2-230, Δ256-441, F1, F2, F3, F4) were made using a Q5 Site-Directed Mutagenesis Kit (NEB). The sequence integrity of all plasmids was confirmed by sequencing.

The full-length tau-luci plasmid is 8081 bp long, while the full-length tau-ferase plasmid is 8032 bp long. The various deletion constructs ranged from 6811 to 7220 bp long. Thus, the maximum difference in plasmid size is 17.06%.

HEK 293 Cell Culture and Transfection

HEK 293 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B at 37 °C in a 5% CO_2 atmosphere. For luciferase assays, cells were seeded at a density of 93,750 cells/well into clear, plastic 12-well tissue culture plates coated with poly-L-lysine. This density was calculated to allow cells to reach near-100% confluency on the day of the luciferase assay. Cells were transiently transfected with 0.5 µg total DNA 24 hours after seeding using 2% Lipofectamine 2000 (Invitrogen) and cultured for an additional 72 hours. The conditioned media was then collected and spun down at 13,000 rpm in a microcentrifuge for 2 minutes to pellet any cell debris. To prepare cell lysates, cells were rinsed twice with warm 1x phosphate-buffered saline (PBS) and treated with Luciferase Cell Culture Lysis Reagent (Promega) for 15 minutes at room temperature. The lysate was collected and spun down at 13,000 rpm for 2 minutes in a microcentrifuge to pellet any cell debris.

Luciferase Assay

Luciferase assays were done essentially as described in Wegmann et al. (2016). *Gaussia princeps* luciferase ("gLuc") activity was measured by adding 100 µL of 10 µM coelenterazine (NanoLight) diluted in PBS to 20 µL of conditioned media or cell lysate in solid, white plastic 96-well plates. The emitted photons were counted beginning 1 second after substrate injection for a duration of 10 seconds. Measurements and

substrate injections were performed on a Spark microplate reader (Tecan) and raw values were transformed into photons per second.

Luciferase activity was also measured in lysates, again as described in Wegmann et al. (2016). Consistent with those results, the luciferase signals resulting from media measurement were more robust than those from lysates (see data in results section below). However, the relative relationships between different tested samples were relatively consistent between media and lysate analyses.

Statistical Analysis

All luciferase experiments were performed at least six times, and each experimental condition was read in triplicate by the plate reader. Raw luminescence values from experimental conditions were normalized to an untransfected control and a relative response ratio (RRR) was calculated using the following formula, where the *positive control ratio* was the ratio of FL/FL tau-luciferase signal to the untransfected control signal, and the *negative control ratio* was the ratio of GFP-tubulin signal to the untransfected control signal:

$$\mathrm{RRR} = \frac{(\text{experimental sample ratio}) - (\text{negative control ratio})}{(\text{positive control ratio}) - (\text{negative control ratio})}$$

All RRR values are presented as mean +/- SEM. All statistical analyses were performed using GraphPad Prism 9, using Brown-Forsythe and Welch's ANOVA tests. A descriptive statistics analysis of the data revealed that the standard deviations between conditions were not equal. Thus, Brown-Forsythe and Welch's ANOVAs, which do not assume equal variances, were used. Following the ANOVAs, the Dunnett's T3 post hoc test was applied.

2.3 Results

2.3.1 Full-Length Tau Oligomers are Released by Mammalian Cells

Previous studies have independently shown that i) full-length (FL) tau oligomerizes in cells (Wegmann et al., 2016) and ii) an oligomerization domain of tau can be mapped to the NTR *in vitro* (Feinstein et al., 2016). To further resolve the location of tau sequences capable of oligomerization in cells, we employed a split-luciferase strategy (**Figure 2**). Luciferase is an enzyme that luminesces in the presence of its substrate, coelenterazine. In a split luciferase assay, half of the luciferase protein is expressed as a fusion protein with one of the putative oligomerizing sequences and the other half of luciferase is expressed as a fusion protein with the second putative oligomerizing sequence. If the two putative oligomerizing sequences do indeed oligomerize, the two halves of luciferase will be brought together, and functional luciferase activity will be restored. Neither half of luciferase alone possesses functional luciferase activity. Luciferase signal

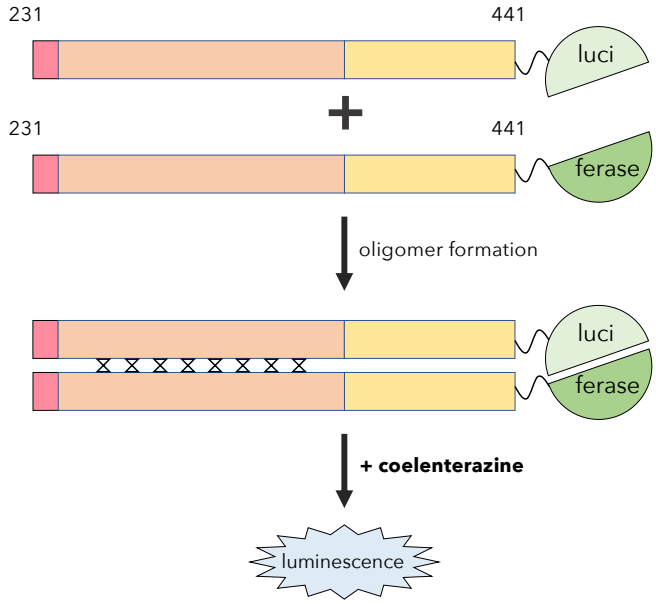

Figure 2 Schematic of Split-Luciferase Assay: Tau was fused to either half of luciferase and expressed in mammalian cells. Oligomerization of tau leads to complementation of both halves of luciferase and restoration of luciferase activity in the presence of coelenterazine.

in the form of photons is measured as a proxy for oligomerization of the sequences of interest. We therefore designed tau-luci and tau-ferase expression constructs that could be expressed alone or in combination (**Figure 3b**).

As a first experiment, we confirmed that tau-luci FL and tau-ferase FL co-expression generated a robust luciferase signal compared to tau-luci FL and tau-ferase FL expressed individually in HEK 293 cells. Cells that were singly transfected exhibited zero to negligible luminescence compared to the combination (**Figure 4a and 4b**).

2.3.2 Both Terminal Halves of Tau are Sufficient for Oligomerization

To begin mapping the oligomerization domain of tau in cells, we generated constructs with either a large C-terminal deletion (amino acids 256-441, "Δ256-441") or a large N-terminal deletion (amino acids 2-230, "Δ2-230") (**Figure 3b**). Co-expression of tau-luci Δ256-441 and tau-ferase Δ256-441, which contain the N-terminal projection domain and the PRR, showed a 3.32-fold increase in luciferase signal when compared to the FL/FL sample (**Figure 4a**). Interestingly, co-expression of tau-luci Δ2-230 and tau-ferase Δ2-230, which contain a small segment of the PRR, the intact MTBR, and CTT, also led to signal that was comparable to co-expression of FL/FL tau-luciferase (1.02-fold increase).

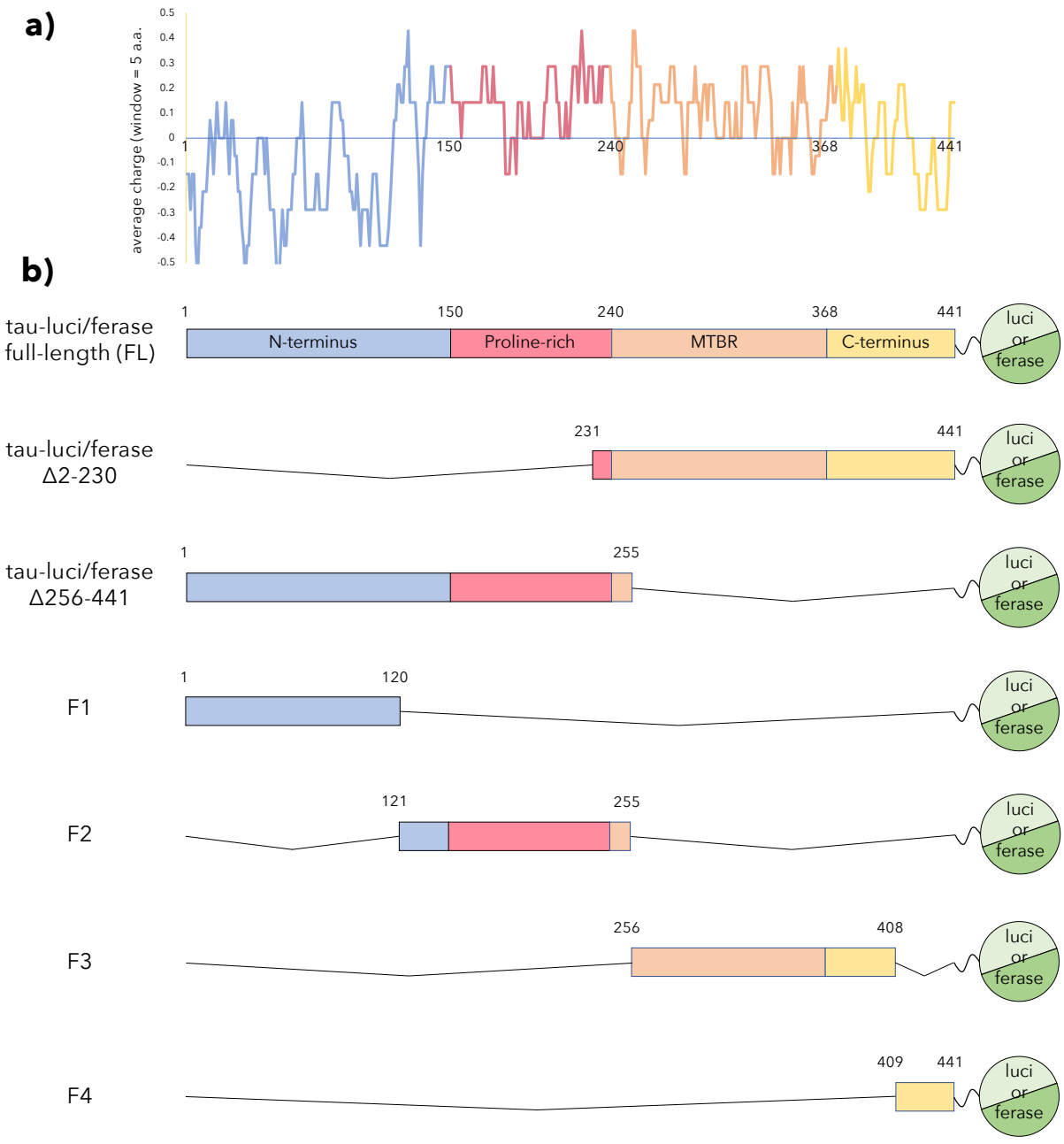

Figure 3 Construct Maps **(a)** Charge distribution profile (using a 5 a.a. window) of tau highlights the charge transitions present within the protein. Major features are color coded accordingly. **(b)** Schematic of regional constructs used in this study. Constructs were fused to either half of luciferase ("luci" or "ferase"). F1 and F2 are subdivisions of the larger tau-luci/ferase Δ256-441 construct, while F3 and F4 are subdivisions of the larger tau-luci/ferase Δ2-230 construct.

Taken together, the data indicate that while constructs containing the N-terminal half of tau are most effective at forming oligomers, constructs containing only the C-terminal half of tau are also capable of forming oligomers in mammalian cells.

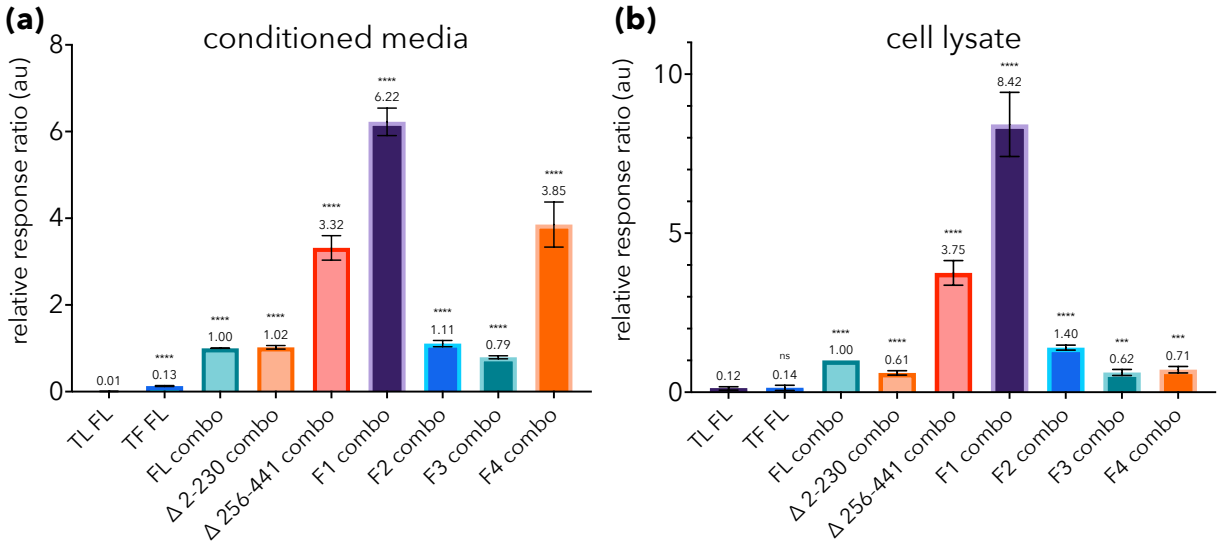

Figure 4 Luciferase Data of Conditioned Media and Cell Lysate Shows All Regions of Tau are Capable of Oligomer Formation **(a)** luciferase assay of conditioned media from transiently transfected HEK 293 cells. **(b)** luciferase assay of cell lysate from transiently transfected HEK 293 cells. All data were normalized as a relative response ratio as detailed in the Materials and Methods section. All data are shown as Mean ± SEM, $n = 6$ for all experimental conditions. Other singly transfected controls not shown. **** $p < 0.0001$; *** $p < 0.0002$; ns, not significant, $p \geq 0.05$. All significance values are versus the TL FL single control.

2.3.3 Resolving the Oligomerization Domains of Tau

To more specifically map the regions of tau capable of oligomerization, additional deletion constructs lacking more and more of the N- and C-terminal fragments were generated (**Figure 3b**). The N-terminal half of tau was subdivided into Fragment 1 ("F1", amino acids 1-120) and Fragment 2 ("F2", amino acids 121-255), while the C-terminal half was subdivided into Fragment 3 ("F3", amino acids 256-408) and Fragment 4 ("F4", amino acids 409-441). Since the oligomerization interactions have been hypothesized to be electrostatic in nature, these subdivisions were created at points where there were charge transitions within the protein (**Figure 3a**). Co-expressing F1-luci with F1-ferase exhibited a marked 6.22-fold increase in luciferase signal when compared to the FL/FL combination (**Figure 4a**). Interestingly, while F2-luciferase and F3-luciferase exhibited signals similar to the FL/FL combination (1.11-fold and 0.79-fold greater, respectively), the signal for F4-luciferase was 3.85-fold greater than the FL/FL combination. Taken together, the subdivision data suggest that the F1 and F4 regions of tau oligomerize more effectively than F2 and F3, though F2 and F3 are still equally as capable as the FL/FL tau combination at forming oligomers.

2.4 Discussion

Previous work has hypothesized that tau oligomerization is mediated by charge distributions within the N-terminal region of the protein (Feinstein et al. 2016). One model suggests that the remarkable charge distribution within the N-terminal half (the negatively charged N-terminal projection domain and the positively charged PRR (**Figure 3a** and **Table 1**) promotes assembly of an anti-parallel electrostatic zipper between two or more tau molecules (Feinstein et al., 2016). Another model suggests that the positively charged, C-terminal flanking region of the MTBR and the negatively charged CTT can also mediate oligomerization via electrostatic interactions (Donhauser et al., 2017). However, both models draw conclusions based on *in vitro* observations. More recent work has shown that FL tau oligomerizes in cells (Wegmann et al., 2016), but does not resolve which regions are responsible for this behavior. Our present work supports a model in which multiple regions of tau can mediate oligomerization, with the strongest activity residing in the N- and C-terminal regions.

Construct	No. of Residues	Net Charge	Mean Net Charge
full-length	441	2.9	0.01
Δ 2-230	212	12.5	0.06
Δ 256-441	255	-6.8	-0.03
F1	120	-23.9	-0.20
F2	135	16.9	0.13
F3	153	12.5	0.08
F4	33	-3.2	-0.10
17 kDa	186	-3.0	-0.02

Table 1 Charge Profile of Tau Constructs: The net charge was calculated at pH 7.4 using ProtCalc. The mean net charge is the net charge divided by the number of amino acids in each construct.

While tau Δ256-441, which contains the N-terminal projection domain and the PRR, exhibited strong luciferase activity, F1, which lacks the PRR and contains most of the negatively charged N-terminal projection domain with few positively charged residues, exhibited the highest luciferase activity out of all the constructs. This would appear to eliminate the simplest version of the electrostatic zipper model presented in Rosenberg et al. (2008), since that model required both the negatively and positively charged sequences in the projection domain and the PRR. In contrast, F2, which contains part of the projection domain and the entire PRR, exhibited luciferase activity comparable to FL/FL tau constructs. Taken together, it is possible that F2 has some sort of an inhibitory effect on F1-mediated oligomerization in tau Δ256-441 while nonetheless retaining some oligomerization capability. Though it has been suggested that the tubulin-binding ability of the PRR is negatively regulated by the projection domain (McKibben and Rhoades, 2019), this work further highlights the intricate interplay between the projection domain and PRR.

Our work also supports the hypothesis that N-terminally derived neurotoxic oligomers are formed and released by cells. Tau Δ256-441, which produced the third strongest luciferase signal, contains the neurotoxic 17 kDa tau$^{45\text{-}230}$ fragment that has been found in the cerebrospinal fluid (CSF) of AD patients (Ferreira and Bigio, 2011) and other tauopathies as well as in the spinal cord of amyotrophic lateral sclerosis patients (Vintilescu et al., 2016). Considering that F1 (amino acids 1-120) produced the highest luciferase signal, it is possible that the oligomerization ability of the

neurotoxic 17 kDa fragment is localized to amino acids 45-120. However, we must also consider the fact that F2 (amino acids 121-255) produced luciferase signal comparable to FL/FL tau. The fact that all three constructs containing either the entire neurotoxic fragment (Δ256-441) or parts of it (F1 and F2) exhibit luciferase activity suggests that it can form stable oligomers and is also released from cells. Further truncations will need to be made to further resolve the details of tau oligomerization mediated by this region of the protein.

The question of how tau is released from cells remains unclear, since it lacks a conventional signal sequence. Trans-synaptic transfer of aberrant, misfolded tau from diseased to healthy neurons is a hallmark of AD (Braak and Braak, 1991; Liu et al., 2012). Several mechanisms for tau propagation have been proposed, one of which suggests that exosomes mediate neuron to neuron transmission of tau (Wang et al., 2017). Tau oligomers have been identified in AD-CSF derived exosomes, though there are discrepancies between the sizes of the oligomeric species (Saman et al., 2012; Wang et al., 2017). Other work has suggested that stable FL tau oligomers released from healthy cells are largely soluble and not associated with secreted membranes, and that these oligomers are readily internalized by cells (Wegmann et al., 2016). While the mechanism by which our tau constructs are released into the culture medium is still unknown, it would be practical to investigate whether the different constructs used in this study can differentially mediate exosomal secretion.

Since previous work using this tau-luciferase system suggested that stable tau oligomers are formed intracellularly and then released (Wegmann et al., 2016), we expected to observe significant luciferase signal in the cell lysate as well. Interestingly, raw luciferase activity from cell lysates was considerably less than that observed in culture medium, although the trends were still generally consistent between the constructs (**Figure 4a and 4b**). The same phenomenon was observed when Wegmann et al. (2016) co-expressed FL- luci and FL- ferase and measured luciferase activity at 48 hours; lysate signals were approximately three-fold lower than media signals. One possibility for these observations may be that the detergent in the lysis buffer might interfere with the interactions required for oligomerization, though the lysis buffer itself is claimed to be "luciferase-safe".

Given the observation that several of our tau-luciferase constructs were able to oligomerize, the question arises of how much of an interaction is sufficient for oligomerization *in vivo*? If oligomerization is electrostatically mediated, it must require both positively and negatively charged regions. When designing the regional tau fragments (F1-4), cutoffs for each region were made at points where there were charge transitions within the protein. The objective was to create four regional tau fragments that did not have steep charge transitions (such as the one seen in $tau^{106\text{-}144}$). The fact that oligomerization was still observed in all the fragments can be interpreted in several ways. Perhaps the minor charge transitions still present within the regional fragments are sufficient to mediate electrostatic oligomerization. Another possibility is that

individual point charges along the protein can mediate oligomerization in a promiscuous, nonspecific manner. One final possibility is that the nature of tau oligomerization is not electrostatic at all, though there are multiple lines of *in vitro* evidence that suggest otherwise (Rosenberg et al., 2008; Feinstein et al., 2016). Further investigations combining fragments of tau with different net charges (F1+F2, F3+F4, ..., see **Table 1**) as well as site-directed mutagenesis of individual amino acids will be necessary for determining whether oligomerization is indeed electrostatically mediated.

Many mechanistic questions regarding normal and pathological tau action remain. If tau functions as a dimer or oligomer as part of its normal mechanism of action, how does it regulate MT bundling or dynamics? If tau fragmentation is a hallmark of neurodegeneration, how might these fragments exert their neurotoxic effects on healthy neurons? How might post-translational modifications of tau affect oligomerization? At the core of this work, we have been able to establish that multiple regions of tau, whether positively or negatively charged, have the ability to oligomerize in mammalian cells. Though more work assessing the functional implications of tau oligomerization will need to be done, our investigation provides strong evidence supporting the hypothesis that tau can form oligomers in cells and that this behavior is not exclusive to the N-terminal half of tau.

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