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Tau Prion Strains in Cells, Mice, and Patients

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Tau Prion Strains in Cells, Mice, and Patients
by
David Winland Sanders

A dissertation presented to the
Graduate School of Arts & Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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David Winland Sanders

Washington University in St. Louis

May 2016
Dedicated to Bill and Julia Sanders.
ABSTRACT OF THE DISSERTATION

Tau Prion Strains in Cells, Mice, and Patients

By

David Winland Sanders

Doctor of Philosophy in Biology and Biomedical Sciences

Neurosciences

Washington University in St. Louis, 2016

Professor Marc I. Diamond

Prion-like propagation of tau aggregation may underlie the stereotyped progression of pathology along neuronal networks in neurodegenerative tauopathies such as Alzheimer’s disease. It has become increasingly clear that unique conformations (“strains”) of prion protein (PrP) link aggregate structure to clinical phenotypes in human prion diseases. Intriguingly, vast phenotypic diversity in pathological and clinical presentation is also observed in tauopathies. The mechanisms that account for this phenotypic diversity are completely unknown. During my dissertation work, I put forward and tested the hypothesis that distinct tau amyloid conformations (or “strains”) are responsible for unique tauopathies. First, I found that in experimental models, tau acts as a prion, replicating distinct strains in vitro and in vivo. I observed that tau indefinitely propagates distinct amyloid conformations in a clonal fashion in cell culture. In collaborative work, I found that two tau strains induce distinct pathologies in vivo as determined by successive inoculations into three generations of transgenic mice. Remarkably, tau from these mice re-
created the original strains upon re-introduction to cultured cells. Second, I created a panel of tau prion strains, which have remarkably divergent biochemical and cell biology properties and cause unique patterns of pathology and rates of progression in vivo. Finally, I used my cell system to isolate tau strains from 29 patients with 5 different tauopathies, finding that different diseases are associated with distinct strains. Tau thus demonstrates essential characteristics of a prion. This likely accounts for the phenotypic diversity of tauopathies and could enable more effective diagnosis and therapy.
Chapter 1: Introduction and Perspective
1.1 PREFACE

This chapter contains figures from a previously published manuscript:


*These authors contributed equally to this work.

Author contributions for the citation above: DWS, SKK, and MID made the figures for the paper. DWS, SKK, BBH, and MID wrote the paper.

DWS wrote all text for this introduction, although select paragraphs were structured similarly to portions of the cited review manuscript.
1.2 NEURODEGENERATIVE DISEASES: DISPARATE SYNDROMES WITH STRIKING SIMILARITIES

The twentieth-century featured great breakthroughs in the development of treatments for diseases that prematurely end human lives. Antibiotics and vaccines made many communicable diseases a relic of the past, greatly extending lifespan in the modern world (Andre et al., 2008). However, this incredible human accomplishment has resulted in a larger segment of the population reaching advanced age, bringing with it a new emerging health crisis, that of neurodegenerative diseases (Holtzman et al., 2011; Sloane et al., 2002). The most common neurodegenerative syndrome, Alzheimer’s disease, is estimated to cost the United States close to $200 billion annually in health care costs and is predicted to affect over 100 million people worldwide by 2050 (Holtzman et al., 2011). Due to the fact that there are currently no treatments for this or other fatal neurodegenerative diseases of aging, there is an urgent need to understand the mechanisms that drive these disorders in order to inform targeted therapeutics. Only with these biomedical advancements can we bring much needed dignity to the last years of human life.

Understanding the similarities between these disparate diseases, which are direct consequences of the portions of the nervous system that are affected (Seeley et al., 2009; Zhou et al., 2012), is a critical first step. In addition to age being the primary risk factor, each disease can be caused by rare genetic mutations (Cruts et al., 2012) but far more commonly features sporadic origins of unclear cause. Importantly for understanding the etiology of these diseases, they are progressive, starting in an isolated part of the nervous system, but eventually affecting large swaths of the brain and/or spinal cord prior to death
(Boillée et al., 2006; Hughes et al., 1992; McKhann et al., 1984). This is in contrast to other injuries to the nervous system, such as ischemic stroke, in which damage remains limited to the initial region affected (Astrup et al., 1981; Hossmann, 1994). In addition to their progressive nature, neurodegenerative diseases share a defining pathological characteristic, which may inform mechanisms of pathogenesis: the accumulation of fibrillar deposits of aggregated protein in the affected cells (Chiti and Dobson, 2006; Ross and Poirier, 2004). At a microscopic level, these structures are “amyloids”, fibrils rich in cross-beta-sheet structure that are composed of thousands of repeating subunits or “monomers” of their cognate proteins (Eanes and Glenner, 1968; Knowles et al., 2014). Due to this defining feature of insoluble fibrillar amyloids, neurodegenerative diseases can be grouped based on the protein that aggregates to form their characteristic ultrastructural deposits. Thus, they can be considered as diseases of protein aggregation or “proteopathies” (Walker and LeVine, 2000). Categorical proteopathies, which each comprise multiple syndromes, include the tauopathies (tau; e.g. Alzheimer’s disease) (Lee et al., 2001), the synucleinopathies (α-synuclein; e.g. Parkinson’s disease) (Goedert, 2001), and the TDP43opathies (TDP-43; e.g. amyotrophic lateral sclerosis) (Neumann et al., 2006). A less common infectious group of neurodegenerative diseases are the prionopathies (prion protein; e.g. Creutzfeldt-Jakob disease or CJD) (Prusiner, 1998). A causal role for protein aggregation in disease is supported by the fact that mutations to genes encoding proteins that form the pathogenic amyloid deposits in each major proteopathy, most of which enhance the ability of each protein to form amyloids (Barghorn et al., 2000; Conway et al., 2000; Johnson et al., 2009), cause familial forms of
the disease (Hsiao et al., 1989; Hutton et al., 1998; Polymeropoulos et al., 1997; Sreedharan et al., 2008).

Pathology studies from the past two decades have indicated that each proteopathy’s associated pathological lesion spreads throughout the nervous system in a hierarchical manner, likely occurring in advance of neurodegeneration (Braak and Braak, 1991; 1995; Braak et al., 2003; Brettschneider et al., 2013; McKee et al., 2013; Thal et al., 2002). Thus, spread of protein aggregation to a new region of the brain is a strong predictor of degeneration in that region (Arriagada et al., 1992). For example, in Alzheimer’s disease (AD), accumulation of amyloid beta plaques is hypothesized to trigger the downstream deposition of tau neurofibrillary tangles, which then drives neurodegeneration in specific regions of the brain (Hardy and Selkoe, 2002). Tau pathology is first observed in the locus coeruleus (Braak and Del Tredici, 2012; Braak et al., 2011) before being seen in the hippocampus and entorhinal cortex (Braak and Braak, 1991; 1995). At this stage, memory deficits become apparent, a condition described as mild cognitive impairment (Petersen et al., 2006). However, although AD is classically thought of as a disease of memory loss, the disease further progresses with fibrillar deposits of tau protein eventually being observed in the limbic system and higher regions of the cortex (Braak and Braak, 1991; 1995), which correlates with the more widespread emergence of behavioral impairments, cognitive deficits, autonomic problems, and eventually death. Until recently, however, a mechanistic paradigm to explain this hierarchical spread of pathology and degeneration had been lacking.
1.3 THE PRION CONCEPT AS A MECHANISTIC PARADIGM FOR NEURODEGENERATIVE SPREAD

The prion hypothesis (Prusiner, 1998) has provided such a testable framework to explain these observations of neurodegenerative spread (Aguzzi and Rajendran, 2009; Brundin et al., 2010; Goedert, 2015; Guo and Lee, 2014; Prusiner, 2013; Sanders et al., 2016; Soto, 2012; Walker and Jucker, 2015). Classically, prions (“proteinaceous infectious particles”) have been defined by their infectious (i.e. communicable between individuals) nature (Prusiner, 1982), as these protein-only pathogens were appreciated as causative agents in transmissible spongiform encephalopathies of both sheep (scrapie) (CHANDLER, 1961; PATTISON et al., 1959) and humans (kuru) (Gajdusek, 1977; Gajdusek et al., 1966). In a series of seminal works, Stanley Prusiner and colleagues found that a self-replicating, misfolded, beta-sheet-rich (i.e. amyloid) form (PrP<sub>Sc</sub>) of the physiological prion protein (PrP<sup>C</sup>) was the causative agent in this group of rare, infectious neurodegenerative diseases (Basler et al., 1986; Bolton et al., 1982; Büeler et al., 1993; 1992; Cohen et al., 1994; Pan et al., 1993; Prusiner, 1982; 1998; Prusiner et al., 1983). This finding was a paradigm-shattering affront to scientific dogma, in that an abnormal protein, as opposed to nucleic acids, could act as the material for inheritance of a communicable disease (Prusiner, 1998). However, while this concept remains revolutionary, semantically defining prions as “infectious” has obscured the fact that human prion diseases (CJD and related ailments) today are not generally infectious, in that communication by cannibalism (kuru, observed in the Fore people of Papua New Guinea) (Gajdusek, 1977), infected beef products (variant Creutzfeldt-Jakob disease) (Collinge, 1999), and iatrogenic infection (CJD) (Brown et al., 2000; 2012) has largely been halted by modern
medical and food safety practices. Rather, the vast preponderance (>90%) of human prion
diseases are of sporadic cause, with unknown etiology associated with aging, or feature a
genetic origin, arising from autosomal dominant familial mutations to the PrP gene
(Wadsworth et al., 2003). In both cases, PrP is hypothesized to spontaneously misfold
into a self-propagating amyloid form that spreads throughout the brain to drive
pathogenesis (Prusiner, 1998). On a superficial level, this is eerily similar to what is
observed in more common neurodegenerative diseases such as Alzheimer’s disease
(Prusiner, 1984).

While some maintain that the infectious nature of prions should be their defining
characteristic (Aguzzi and Rajendran, 2009; Brettschneider et al., 2015; Hardy and
Revesz, 2012), others argue that the term prion should be expanded to include other
proteins that form higher-order assemblies that act as heritable elements (Prusiner, 2013;
Sanders et al., 2016; Uptain and Lindquist, 2002; Walker and Jucker, 2015). Due to the
striking similarities between the pathological agents (i.e. toxic amyloids) in classic prion
diseases and other proteopathies as well as physiological assemblies of replication (i.e.
functional amyloids), it may be more useful to define prions as being “proteinaceous
nucleating particles” (Walker and Jucker, 2015) or “protein assembl[ies] that
communicate information stably via template-based amplification of a specific
conformation” (Sanders et al., 2016). We favor this expanded definition as the prion
concept provides a unifying framework by which to understand the mechanisms of
neurodegenerative spread and protein-only based elements of inheritance (Sanders et al.,
2016).
At a biophysical level, proteins that form prions are capable of undergoing a conformational change from a physiological form, which can be natively folded or intrinsically disordered, to a misfolded form rich in beta-sheet structure (Chiti and Dobson, 2006; Knowles et al., 2014). This new alternative conformation is aggregation-prone and rapidly forms thermostable amyloidogenic nuclei or “seeds” that act as templates to corrupt natively folded monomer with similar amino acid sequences (Chiti and Dobson, 2006; Knowles et al., 2014). Monomer can thus be added to the free ends of amyloid fibrils in a process called primary nucleation (Collins et al., 2004a; Jarrett and Lansbury, 1993; Serio et al., 2000), and the process is amplified by fragmentation of fibrils to produce further nuclei with additional free ends for continued growth. Alternatively, individual amyloid fibrils can catalyze the growth of additional fibrils from their sides in a mechanism called secondary nucleation (Cohen et al., 2013; Knowles et al., 2009). At a conceptual level, these proteins act as biophysical switches between a physiological, monomeric form and a self-propagating and heritable fibrillar form (or prion).

At a cellular level, the prion hypothesis posits that fibrillar amyloids are capable of templating their pathological forms between areas of the brain to spread throughout the nervous system (Prusiner, 1998). For extracellular prions such as PrP (Stahl et al., 1987) and amyloid beta (Kang et al., 1987), this could easily be envisioned to occur by bulk flow via the interstitial fluid. Decades of research have indicated that intracerebral inoculation of diverse species with brain homogenates from animals that have succumbed to prion diseases invariably leads to progressive neurodegeneration, PrP prion deposition, and ultimately death (Prusiner, 1998). In fact, more recent work has indicated that
recombinant PrP amyloids are sufficient to cause prion disease in mice (Colby et al., 2009; Legname et al., 2004). Specific co-factors make this process considerably more efficient (Deleault et al., 2007; 2012a; 2012b; Supattapone, 2014; Wang et al., 2010). Initial studies regarding intracerebral transmissibility of AD plaques (amyloid beta prions) in non-transgenic primates were decidedly more mixed (Baker et al., 1993; Goudsmit et al., 1980). Despite the obvious similarities between AD and prion diseases (Prusiner, 1984), studies regarding transmissibility and spread were largely abandoned until the emergence of powerful, new transgenic mouse models of AD amyloid beta pathology (Sturchler-Pierrat et al., 1997). These new models, which greatly accelerated the study of amyloid beta plaque deposition in vivo, allowed for a decade of ground-breaking work by Jucker and Walker (reviewed in (Jucker and Walker, 2013)). These studies demonstrated that intracerebral inoculation of amyloid beta-containing extracts from both AD mice and AD patients greatly accelerated the progressive deposition of plaques throughout the nervous system (Eisele et al., 2009; Fritschi et al., 2014; Langer et al., 2011; Meyer-Luehmann et al., 2006; Rosen et al., 2012; Ye et al., 2015). Follow-up work showed that synthetic amyloid beta fibrils were sufficient for this effect, although induction of pathology was significantly less efficient (Stöhr et al., 2014; 2012). Moreover, amyloid beta plaques have been observed in human patients that developed iatrogenic CJD, suggesting that amyloid beta prions may be transmissible between humans by surgical contamination (Frontzek et al., 2016; Jaunmuktane et al., 2015). Nevertheless, there is no evidence to date to suggest that iatrogenic transmission of amyloid beta (or other non-PrP) prions is sufficient to cause a classic AD-like neurodegenerative phenotype (Irwin et al., 2013).
Many prions, such as tau, α-synuclein, and TDP-43, primarily form intracellular amyloid deposits (Grundke-Iqbal et al., 1986; Neumann et al., 2006; Spillantini et al., 1997). Nevertheless, hierarchical accumulation of associated amyloid deposits is observed in tauopathies (Braak and Braak, 1991; 1995; Braak et al., 2011; 2006), synucleinopathies (Braak et al., 2003), and TDP43opathies (Brettschneider et al., 2013). Indeed, imaging studies suggest that dysfunction in such diseases occurs along specific neuronal networks (Seeley et al., 2009), most parsimoniously explained by the trans-synaptic spread of a pathogenic agent (e.g. a prion) (Raj et al., 2012; Zhou et al., 2012). The idea that a prion could escape a first-order neuron, adhere to a second-order neuron, gain access to the cytoplasm of the second-order neuron, and convert cognate monomer to a prion form was met with rightful skepticism. Nevertheless, trans-synaptic spread and intracellular conversion of PrP prions (Borchelt et al., 1992; Fraser, 1982; Fraser and Dickinson, 1985; Scott et al., 1992; Taraboulos et al., 1990a; 1990b) provided precedent for this concept, although evidence that this occurred outside of the secretory pathway (i.e. in the intracellular space) was lacking. Further, this mechanistic paradigm would potentially explain how active immunization could decrease pathology in transgenic mouse models of tauopathy (Asuni et al., 2007) and synucleinopathy (Masliah et al., 2005) and why α-synuclein amyloids accumulated in the neurons of young dopaminergic grafts implanted into patients with Parkinson’s disease (Kordower et al., 2008; Li et al., 2008).

A flood of studies over the past decade have provided overwhelming evidence that the prion model is indeed correct in cellular and rodent models of intracellular proteopathies (Brundin et al., 2010; Frost and Diamond, 2010; Goedert, 2015; Guo and Lee, 2014; Holmes and Diamond, 2012; Sanders et al., 2016; Walker and Jucker, 2015). Initial
studies on α-synuclein suggested that monomeric and potentially aggregated forms of the protein could be endocytosed and exocytosed by cells in culture (Lee et al., 2005; 2008a; 2008b). Pioneering cell culture studies then demonstrated that tau (Frost et al., 2009a), α-synuclein (Desplats et al., 2009; Luk et al., 2009), and huntingtin (Ren et al., 2009) aggregates can be endocytosed by cells, where they access the intracellular space, and seed cognate monomer to adopt the prion state. Later studies indicated that this was true in a variety of models (dividing cells, primary neurons) of intracellular prion (tau, α-synuclein, SOD1, polyQ, TDP-43) propagation (Grad et al., 2011; 2014; Guo and Lee, 2011; 2013; Münch et al., 2011; Nekooki-Machida et al., 2009; Nonaka et al., 2013; 2010; Pokrishevsky et al., 2016). Meanwhile, a parallel study demonstrated that intracerebral inoculation of brain homogenate containing tau prions resulted in the seeding of intracellular neurofibrillary tau tangles, which spread to distant sites in transgenic mice (Clavaguera et al., 2009). More remarkably, follow-up studies, which intracerebrally inoculated transgenic mice with recombinant tau (Iba et al., 2013; 2015; Stancu et al., 2015) and α-synuclein (Luk et al., 2012a) prions, found that co-factors were not necessary to induce pathology. In particular, α-synuclein prions faithfully replicated known facets of human synucleinopathy, causing rapid and reproducible death in transgenic mice (Luk et al., 2012a; Mougenot et al., 2012) and regional atrophy in wild-type mice (Luk et al., 2012b; Masuda-Suzukake et al., 2013). In all cases, spread of pathology appears to occur along known neuronal networks, as opposed to diffusion by proximity, although it cannot be ruled out that this does not merely reflect trans-synaptic spread of inoculum rather than true propagation of the prion state (Rey et al., 2013). Site-specific expression of tau in diverse models has unequivocally demonstrated that tau can
exit a cell and enter a synaptically connected neuron (de Calignon et al., 2012; Dujardin et al., 2014b; Kim et al., 2010; Liu et al., 2012; Polydoro et al., 2013). Nevertheless, in most cases, there is little evidence for true propagation (i.e. conversion of tau in the recipient neuron to the prion state). Improved models for trans-cellular prion propagation are sorely needed to address lingering concerns regarding the applicability of these many phenomenological studies to mechanisms of neurodegenerative spread in patients (Walsh and Selkoe, 2016).
1.4 MECHANISMS OF TRANS-CELLULAR PRION PROPAGATION

Mechanistic insights into the cellular pathways that regulate trans-cellular propagation of prions have been gleaned from a number of studies over the past several years (Holmes and Diamond, 2012), although in some cases, the data is conflicting. This multi-stepped process requires specific events (Figure 1.1): escape of a prion from a first-order neuron, binding to the surface of a second-order neuron, uptake/endocytosis, escape into the cytosol, “corruption” of endogenous cognate monomer to the prion state, and fragmentation/amplification (Sanders et al., 2016). While much progress has been made regarding how prions enter cells, additional work remains to be performed to clarify the physiologically relevant routes by which they exit neurons. A number of studies have suggested that tau, α-synuclein, and SOD1 prions can enter cells by bulk fluid-phase macropinocytosis (Frost et al., 2009a; Holmes et al., 2013a; Münch et al., 2011). A recent study (Holmes et al., 2013a) suggests that this mode of uptake is dependent on cell-surface receptors called heparan sulfate proteoglycans (HSPGs) (Holmes et al., 2013a), proteins which contain negatively charged sulfate groups (Xu and Esko, 2014) that interact with positively-charged heparin-binding motifs present on many intracellular prions. Furthermore, although all tau species can bind to cell-surface HSPGs, only trimers and larger assemblies are capable of triggering the clustering of HSPGs required to actively stimulate downstream macropinocytosis (Mirbaha et al., 2015). This is consistent with previous data suggesting that PrP trimers are the minimum unit of infectivity in models of prionopathy (Bellinger-Kawahara et al., 1988; Govaerts et al., 2004; Wille et al., 2009), which also insinuates the tantalizing possibility that uptake and
intracellular conversion is required for productive prion infectivity. Not surprisingly, extracellular prions are also able to bind and infect cells using HSPGs (Horonchik et al., 2005; Kanekiyoyo et al., 2011; Sousa and Saraiva, 2001), and a recent study suggests that this pathway may be of critical importance to the development of amyloid beta pathology in transgenic mice and AD patients (Liu et al., 2016). The involvement of this pathway in the metabolism of multiple prions suggests that targeting HSPGs may be an effective therapy for disparate neurodegenerative diseases. Finally, evidence gleaned from cell culture experiments suggests that tunneling nanotubes, filopodia-like extensions that connect the cytoplasm of neighboring cells, could potentially play a role in the spread of diverse prions (Costanzo et al., 2013; Gousset et al., 2009), although additional work is required to confirm the importance of this pathway in vivo.

Once prions enter a cell, they must escape from macropinosomes (or other membrane-bound compartments) to access the intracellular space to convert cognate intracellular monomer into a self-replicating form that further amplifies the prion state. The mechanisms(s) by which prions escape double-membraned vesicles are unclear. It is possible that this occurs by direct penetration (Ren et al., 2009), similar to the hypothesized pathway by which cationic peptides translocate into the cytosol (McNaughton et al., 2009; Thompson et al., 2012). Conversely, macropinosomes may be inherently leaky, as the details regarding their maturation are far from complete (Mercer and Helenius, 2009; 2012). Further work will be required to test whether there are active mechanisms that contribute to delivery of prions into the cytosol or potentially to specific sites where prion replication may occur.
Once translocated into the cytosol, a prion seed must encounter another cognate monomer to amplify its structure. Whether there are active mechanisms that drive this process in the heterogeneous environment of the cytoplasm, where most interactions will be with different proteins, is undetermined. Equally unclear is whether there is cellular machinery that orchestrates further fragmentation and amplification of prion seeds once formed. In *Saccharomyces cerevisiae* (yeast), the Hsp104 chaperone works as a two-tiered hexameric complex, which along with other co-chaperones, “disaggregates” amyloids into additional seeding-competent nuclei (Chernoff et al., 1995; Glover and Lindquist, 1998; Shorter and Lindquist, 2004). However, an Hsp104 homologue does not exist in mammals. Therefore, it was thought that evolution may have selected against this protein in more complex organisms to prevent the formation of toxic oligomers or active propagation of prions (Murray and Kelly, 2012). Recent work suggests that this may not be the case, as *in vitro*, a three chaperone (Hsp40, Hsp70, Hsp110) system can disaggregate amorphous aggregates but not amyloids (Nillegoda et al., 2015; Rampelt et al., 2012; Shorter, 2011). Moreover, a provocative recent paper suggests that at the right stoichiometry, the same system can disassemble pre-formed amyloid fibrils (Gao et al., 2015). It is also conceivable that additional seeds could be generated in lysosomes by proteases, as HTRA1 can re-solubilize tau amyloids (Poepsel et al., 2015). Whether any of these mechanisms occur in living cells to fragment prions and generate additional seeding-competent nuclei remains to be determined.

To continue the process of propagation, an intracellular prion must be able to exit the cell. Numerous studies have indicated that intracellular proteins that can form prions are capable of accessing the extracellular space (Chai et al., 2012; Danzer et al., 2011; de
Alarming, most of these studies have purported to present findings relevant to propagation, but only measure monomer, which is not competent to be actively taken into second-order neurons to seed further aggregation (Mirbaha et al., 2015). Every conceivable mechanism has been put forward including exosomes (Emmanouilidou et al., 2010; Fevrier et al., 2004; Polanco et al., 2016; Rajendran et al., 2006; Saman et al., 2012), ectosomes (Dujardin et al., 2014a), active or unconventional secretion (Chai et al., 2012; Karch et al., 2012; Pooler et al., 2013; Yamada et al., 2011; 2014), and membrane rupture following cell death (Hesse et al., 2001; Palmio et al., 2009). Future studies will need to make use of sensitive bioassays such as real-time quaking induced conversion (Colby et al., 2007; Wilham et al., 2010) or cellular FRET flow cytometry (Furman et al., 2015; Holmes et al., 2014) to delineate which mechanisms are relevant to the spread of seeding-competent prions in individual models.
1.5 STRAINS: DIFFERENT AMYLOID STRUCTURES DRIVE PHENOTYPES IN PRION DISEASES

Long before the widespread acceptance of the “protein-only” prion hypothesis (Griffith, 1967; Prusiner, 1982; 1998), transmissible spongiform encephalopathies (TSEs) were thought to be caused by “slow viruses” (Gajdusek, 1977). In addition to the heretical notion that self-replicating protein entities could cause disease, the existence of TSE disease strains made Prusiner’s hypothesis particularly difficult to accept for many (Bruce and Dickinson, 1987). Pioneering studies by Dickinson, Meikle, and Fraser, demonstrated that different scrapie agents (sheep prions) passaged in inbred mice would result in distinct patterns and degrees of pathology (vacuolation) as well as characteristic incubation periods prior to symptom development (Dickinson and Meikle, 1969; Dickinson et al., 1968; 1969; Fraser and Dickinson, 1973). Later studies suggested that specific prion strains resulted in distinct behavioral phenotypes (Bessen and Marsh, 1992a) and featured infectious agents with variable susceptibilities to thermal deactivation (Dickinson and Taylor, 1978; Kimberlin et al., 1983). Remarkably, if using the same genetic background and inoculate dosage, strain-specific properties can be maintained following passage through multiple generations of mice, even over years of experimental transmission (Bruce and Dickinson, 1987). The simplest explanation for these observations was that despite having as essential PrP protein factor (Basler et al., 1986), the prion agent also contained a self-replicating genetic component and its sequence could infer distinct biological properties (i.e. strain effects) (Bruce and Dickinson, 1987). In other words, prions might merely be very unconventional viruses (Bruce and Dickinson, 1987). Surprisingly, however, decades of inquiry have failed to
find a nucleic acid signature that co-segregates with specific prion strains or even the infectious agent itself (Safar et al., 2005).

Rather, a substantial body of work now indicates that the structure of prion protein amyloids mediates the effects of strains (Toyama and Weissman, 2011; Tycko, 2015). An early study suggested that putative PrP amyloids purified from animals that succumbed to unique prion strains featured different susceptibilities to protease (Kascak et al., 1985). However, the idea that different PrP fibril structures drive vast phenotypic differences in prionopathy manifestation was inchoate until the publication of two pioneering studies by Bessen and Marsh, which indicated that PrP isolated from hamsters infected by different transmissible mink encephalopathy strains featured unique biochemical properties (Bessen and Marsh, 1992b; 1994). Moreover, protease-resistant PrP from these strains featured different protected cores, suggesting a structural difference at the level of PrP amyloid replication (Bessen and Marsh, 1994). These strain-specific protease-resistant regions could be imprinted on purified PrP in a cell-free seeding reaction (Bessen et al., 1995). Extensive biochemical characterization of a panel of hamster prion strains solidified the idea that different PrP amyloid conformations drove specific phenotypes (Safar et al., 1998). Together, these observations were consistent with the idea that templated nucleation of specific structures led to fibrils with unique biochemical properties, which propagated throughout the brain at different rates and disproportionately affected specific regions of the nervous system (Collinge and Clarke, 2007). But what were the differences between specific amyloid conformations that lead to such divergent biological outcomes as short vs. long incubation period?
Clues to this question came from a surprising model system: *Saccharomyces cerevisiae* (yeast). This simple single-celled eukaryote features a multitude of proteins that can undergo transitions from a monomeric to a self-propagating and heritable amyloid (i.e. prion) form (Derkatch et al., 1996; Patino et al., 1996; Paushkin et al., 1996; Wickner, 1994). Yeast prions are typically detrimental to the organism (McGlinchey et al., 2011), but in certain environmental situations, confer a growth advantage (Halfmann et al., 2012; Holmes et al., 2013b; True and Lindquist, 2000; True et al., 2004). For example, aggregation of the translation termination factor Sup35 into the prion state can result in read-through errors which can uncover hidden phenotypic traits during times of stress (True et al., 2004). Like mammalian prion protein, yeast prions are capable of forming diverse stably propagating amyloid conformers or strains (King and Diaz-Avalos, 2004; Tanaka et al., 2004). For the Sup35 prion, strain strength is defined by the degree to which the protein loses its ability to terminate translation (Tanaka et al., 2004). In other words, the more the protein aggregates or can seed further aggregation, the stronger the strain. In two seminal studies (Tanaka et al., 2006; Toyama et al., 2007), Weissman’s group determined the physical and structural basis for this phenomenon in several prototypical strains. Surprisingly, they found that Sup35 amyloid conformers with the fastest growth kinetics did not feature the strongest phenotype *in vivo* (Tanaka et al., 2006). Rather, the ability to fragment to produce additional nucleation-competent seeds defined the strength of the strain. This principal was verified by another group using a different yeast prion, Rnq1 (Kalastavadi and True, 2010). In follow-up work using hydrogen-deuterium exchange, the Weissman lab showed that the reason why strong strains fragment more readily is that they feature a smaller amyloid core (Toyama et al.,
Amyloid conformers with small cores are more brittle and break more readily to produce additional seeds that amplify the aggregation process (Toyama et al., 2007) (Figure 1.2A).

These initial observations regarding the biophysical basis for yeast strain diversity appeared to offer great insight into the vast phenotypic diversity with regard to incubation time and disease duration observed in prion diseases. Might strong prion strains (i.e. those that spread rapidly and produce short incubation periods) be caused by more fragile PrP amyloid conformers? This appeared to be the case for prion strains propagated in mice: Prusiner’s group examined the biophysical stability of 30 strain isolates and found that there was a strong correlation (R=0.93) between the stability of the PrP amyloid conformer in denaturant (GdHCl) and the incubation time of the agent when inoculated into transgenic PrP mice (Legname et al., 2006). This principal was upheld by examining the incubation periods of a variety of synthetic PrP amyloid conformations created in vitro (Colby et al., 2009). Additional work examining putative human PrP strains initially seemed to support this idea (Kim et al., 2012a), but recent analysis (Safar et al., 2015) has suggested that this may not be a universal defining principal of strain strength. In at least certain patients, lower conformational stability can be associated with longer disease duration (Safar et al., 2015). Furthermore, the strength of certain yeast strains is dependent on the degree of interaction with chaperone protein clients, not just conformational stability (Stein and True, 2014a; 2014b). Thus, biophysical frangibility alone may be insufficient to fully describe why different prion strains cause different incubation times (Figure 1.2A). Moreover, this principal does not explain why different strains affect different regions of the brain (Bruce et al., 1991; Carroll et al., 2016; Fraser...
and Dickinson, 1973) or unique cell types (Ayers et al., 2011; Carroll et al., 2016; González et al., 2002; 2010) (Figure 1.2B,C).

Like in animals, human prion diseases are phenotypically diverse. They feature infectious (kuru, variant CJD), sporadic (sporadic CJD, sporadic familial insomnia, variably protease-sensitive prionopathy), genetic (familial CJD, fatal familial insomnia, Gerstmann-Straussler-Scheinker syndrome), and iatrogenic (iatrogenic CJD) etiologies (Collins et al., 2004b; Gambetti et al., 2011; Johnson, 2005; Prusiner, 1998). Individual diseases feature striking differences related to age of onset, duration, symptom profiles, and PrP histopathological profiles. A large body of work now indicates that this is due to different stably propagating PrP prion strains, which amplify themselves throughout the nervous system to produce a gestalt phenotype (Collinge and Clarke, 2007; Gambetti et al., 2011) (Figure 1.3A). Fatal familial insomnia and familial CJD, in particular, feature drastically different clinical (insomnia vs. myoclonus/dementia) and pathological (thalamus vs. cortex) presentations despite specific kindreds featuring identical causative point mutations (Medori et al., 1992; Nieto et al., 1991). Identity of a single polymorphism in PrP at amino acid 129 regulates whether such patients develop FFI (M129) or CJD (V129) (Goldfarb et al., 1992). A landmark paper (Telling et al., 1996) determined why this is the case. Prusiner and colleagues inoculated transgenic mice with brain homogenates from patients that died of familial CJD, sporadic CJD, or fatal familial insomnia (Telling et al., 1996). They then analyzed the protease sensitivity of PrP prions isolated from human donors and mouse recipients, finding 21 kD protease-resistant PrP associated with both forms of CJD and 19 kD PrP associated with FFI (Telling et al., 1996). In other words, PrP amyloids in different diseases are biochemically distinct. Two
concurrent studies (Collinge et al., 1996; Parchi et al., 1996) solidified the idea that conformation of PrP is a major contributor to a patient’s clinical phenotype. Gambetti’s group found that sporadic CJD patients featured either 21 kD (type I) or 19 kD (type II) PrP, the first of which was associated with the more common myoclonic form of CJD and the latter of which was associated with the rarer ataxic variant (Parchi et al., 1996).

Further, like with familial prion diseases, codon 129 polymorphism contributed to disease phenotype (Parchi et al., 2011). The second study (Collinge et al., 1996), from Collinge and collaborators, used similar methods to show that a novel prionopathy that disproportionately affected the young (variant CJD) was caused by a distinct PrP strain. Further, they found that this strain was the same as that which caused bovine spongiform encephalopathy (BSE), thus demonstrating that BSE could be transmitted to humans (Collinge et al., 1996). Strikingly, only individuals homozygous at methionine 129 developed variant CJD (Ironside, 2012). This, along with extensive work using transgenic mice expressing PrP from various animal species, has proved that the amino acid sequence composition of PrP is of fundamental importance to the amyloid structures that it can form and what monomers can be corrupted with the pathological conformation (Collinge and Clarke, 2007). In fact, such “seeding barriers” have likely limited the transmissibility of spongiform encephalopathies between species (Collinge and Clarke, 2007). Using common protease digestion protocols, six prion strains have been identified in human patients, each of which is associated with unique clinical and histopathological features (Gambetti et al., 2011). More sophisticated approaches may allow further refinement of the conformational features that contribute to various subtypes of prion disease (Safar et al., 2015).
1.6 PHENOTYPIC DIVERSITY IN NON-INFECTIONOUS NEURODEGENERATIVE DISEASES

Similar to prionopathies, non-infectious human proteopathies are not homogeneous diseases. Rather, each features considerable phenotypic diversity with regard to clinical and pathological presentation (Halliday et al., 2011; Kovacs, 2015; Lee et al., 2001; Van Langenhove et al., 2012). Synucleinopathies include Parkinson’s Disease, multiple system atrophy, and dementia with Lewy bodies, each of which features α-synuclein accumulation in different cell types and distinct regions of the brain (Halliday et al., 2011). TDP-43 aggregation is associated with a spectrum of diseases ranging from frontotemporal lobar dementia to amyotrophic lateral sclerosis (Van Langenhove et al., 2012). The most striking example, however, is the tauopathies, which include over a dozen major neurodegenerative diseases that can be differentiated by symptom profiles, tau isoform accumulation, ultrastructural characteristics of tau fibrils, morphological properties of tau inclusions, cell types affected, and regions of the brain afflicted by degeneration (Kovacs, 2015; Lee et al., 2001). Human tau, a predominantly neuronal microtubule-associated protein that promotes microtubule stabilization and polymerization (Cleveland et al., 1977a; 1977b; Weingarten et al., 1975), is expressed from the MAPT locus, which is alternatively spliced to produce six isoforms that differ with respect to their number of N-terminus inserts (0N, 1N, 2N) and C-terminus tandem repeat sequences (3R or 4R) (Goedert et al., 1989a; 1989b). The fetal brain only features 3R tau, whereas the adult brain features an equal ratio of 3R and 4R tau (Goedert and Jakes, 1990). The evolutionary reasons for the temporal regulation of tau isoform expression possibly relate to 4R tau’s enhanced ability to promote microtubule
polymerization and stabilization relative to 3R tau (Butner and Kirschner, 1991; Goedert and Jakes, 1990). Hyperphosphorylation reduces tau’s affinity for microtubules, leading to a larger free pool of soluble tau in the cytoplasm, thus increasing the likelihood that it spontaneously aggregates to form fibrillar amyloids (Buée et al., 2000; 2010).

Sporadic tauopathies can be subdivided based on the isoforms of tau that accumulate in characteristic phosphorylated tau amyloid lesions in distinct cell types (Kovacs, 2015; Lee et al., 2001). Alzheimer’s disease (AD), the most common tauopathy, features neuronal neurofibrillary tangles composed of 3R and 4R tau (Goedert et al., 1992; Greenberg and Davies, 1990; Greenberg et al., 1992; Lee et al., 1991). Pick’s disease (PiD), a frontotemporal dementia, is defined by the aggregation of 3R tau in globular Pick bodies in neurons of the cortex and hippocampus (Iwatsubo et al., 1994; McKhann et al., 2001; Probst et al., 1996). Conversely, corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP), both of which are movement disorders (Rebeiz et al., 1967; STEELE et al., 1964), feature only 4R tau in neuronal filaments, oligodendrocytic coiled bodies, and astrocytes (Feany and Dickson, 1995; Hauw et al., 1990; Komori, 1999; Komori et al., 1998; Yamada et al., 1992). These two diseases differ with respect to astrocytic pathology: CBD primarily features astrocytic plaques, whereas PSP is differentiated by the presence of tufted astrocytes (Komori et al., 1998).

Regardless, there is thought to be significant overlap between these diseases with regard to clinical and pathological features (Feany et al., 1996). Similar to PSP and CBD, argyrophilic grain disease (AGD) only features insoluble 4R tau (Ferrer et al., 2008; Tolnay et al., 1997), but unlike other tauopathies, it does not feature acetylated tau in its characteristic grain-like inclusions (Grinberg et al., 2013). Other less common sporadic
tauopathies exist, and each features characteristic tau pathological profiles (Kovacs, 2015; Lee et al., 2001). Furthermore, each tauopathy differs with respect to selective neuroanatomical patterns of tau aggregate deposition, which likely contribute to their distinctive clinical phenotypes (Arnold et al., 2013). Even within the same syndrome, there is great variability with respect to progression rate (Armstrong et al., 2000; Huang et al., 2014; Mann et al., 1992; Thalhauser and Komarova, 2012). The mechanistic reasons for these diverse clinical and pathological observations are completely unexplored.

Genetic tauopathies also feature vast phenotypic diversity in presentation (Lee et al., 2001; Reed et al., 2001; van Swieten et al., 1999). Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) is a rare autosomal dominant neurodegenerative disease caused by point mutations to MAPT that enhance tau’s propensity to form amyloids or disrupt the isoform ratio between 3R and 4R tau (Barghorn et al., 2000; Hutton et al., 1998). Seven point mutations have been described that affect at least two kindreds (Cruts et al., 2012). Amongst these, mean age-of-onset can vary between 33.7 (P301S) and 62 (ΔK280), with disease durations between 4.2 (P301S) and 19 (ΔK280) years (Cruts et al., 2012). Furthermore, some mutations are associated with mixed 3R/4R tau pathology whereas others only feature 4R (Reed et al., 2001). Like for sporadic tauopathies, individual tau mutations are associated with distinct histopathological profiles with discrete cell types and regions of the brain being affected as well as characteristic clinical presentations (e.g. memory loss, personality changes, language impairments) (Reed et al., 2001). The mechanistic underpinnings for the spectrum of presentations associated with FTDP-17 remain to be elucidated.
1.7 THE CURRENT PERSPECTIVE: DO STRAINS DRIVE DISTINCT PHENOTYPES IN TAUOPATHIES?

Striking similarities between tauopathies and prionopathies are evident. Most importantly, each progressive proteopathy features neuroanatomical spread of their characteristic fibrillar lesions and vast phenotypic diversity in clinical and pathological presentation. Overwhelming evidence now indicates that self-replicating PrP prion strains drive phenotypic diversity in human prion diseases (Figure 1.3A). This begs the question of whether conformationally distinct tau prion strains contribute to the manifestation of specific tauopathies (Figure 1.3B). For this to be conceivable, a number of criteria must be met:

1.)Tau must form different amyloid conformations in vitro.

2.) These tau amyloid conformations must stably replicate in cells (i.e. they must act as bona fide prion strains).

3.) Tau strains must continue to replicate the same structure when introduced into a new cellular environment.

4.) Specific tau strains must be isolatable and stably propagate through living organisms and across synapses.

5.) Tau must be capable of producing the diversity of strains required to underlie the numerous clinical tauopathies (potentially, over a dozen).

6.) Tau strains must be able to differentially affect specific cell types and brain regions as well as cause characteristic neuronal and glial inclusions. Furthermore, they must spread at different rates. That is, specific strains must be able to produce distinguishable experimental tauopathies in model
organisms.

7.) Tau strains must co-segregate with specific human tauopathies.

When I began my doctoral studies in Marc Diamond’s lab, only the first criterion had been met for tau amyloids. Specifically, we had previously shown that tau could form different amyloid conformations in vitro, which stably replicate certain features in subsequent seeding reactions (Frost et al., 2009b). Other groups had also shown amyloid polymorphism with respect to tau fibrils produced in vitro (Aoyagi et al., 2007; Dinkel et al., 2011; Siddiqua and Margittai, 2010). Similar diversity with regard to fibrillar structures has been observed for amyloid beta (Lu et al., 2013; Paravastu et al., 2008; 2006; Petkova et al., 2005; Tycko, 2015) and α-synuclein (Bouset et al., 2013; Gath et al., 2014; Guo et al., 2013; Peelaerts et al., 2015) induced to fibrillize in a test tube under specific environmental conditions (e.g. high vs. low salt, quiescent vs. shaking incubation). Moreover, inoculation of brain homogenates containing amyloid beta from various sources into transgenic mice induces the formation of extracellular plaques with distinct characteristics (Heilbronner et al., 2013; Meyer-Luehmann et al., 2006), and extracts from different patients seed fibrils with distinct structures (Lu et al., 2013). However, to have clinical significance for diseases that manifest themselves over years or even decades, prion strains must faithfully replicate with tremendous fidelity for significant periods of time, producing predictable pathology upon serial inoculation in vivo. Such strain behavior had yet to be reported for amyloids other than PrP.
1.8 SUMMARY

Neurodegenerative diseases are syndromes of protein aggregation (i.e. proteopathies), resulting from the accumulation of characteristic amyloid deposits in specific cells and regions of the brain. Considerable evidence accrued from in vitro and in vivo studies over the past decade supports the hypothesis that common non-infectious proteopathies progress due to the hierarchical “prion-like” spread of protein aggregates along neuronal networks. However, the reasons why these diseases feature such vast diversity in clinical and pathological presentation have yet to be elucidated. It is now known that different PrP amyloid conformations or prion strains drive phenotypic diversity in the prionopathies (Figure 1.2). This dissertation seeks to determine whether this is also the case for sporadic tauopathies, which each feature disease-specific accumulations of tau fibrils in specific cell types (neurons and/or glia) and regions of the brain. By using cell culture and transgenic mouse models along with patient samples, the work described here unequivocally demonstrates that tau can form a multitude of distinct strains that stably propagate through living systems to drive specific pathological phenotypes. Moreover, it demonstrates that specific strains co-segregate with specific tauopathies, thus suggesting that discrete tau amyloid conformations likely contribute to the etiology of unique tauopathies. This work has fundamental implications for how we differentiate and treat human tauopathies.
1.9 FIGURES

**Figure 1.1. Steps in trans-cellular propagation.** Trans-cellular propagation of prions is likely to involve escape from a first-order cell, binding to a second-order cell, uptake into a second-order cell, seeding of native monomer, and fragmentation and amplification of the seeded aggregates. **(A)** Escape of prions from a first-order cell could occur by direct release into the extracellular space. This may be driven by unconventional secretion, cell death, or membrane penetration. **(B)** Alternatively, prions could escape in exosomes, or **(C)** could directly move to neighboring cells via tunneling nanotubes. **(D)** In the exosomal pathway, cell entry would presumably occur via vesicle fusion. **(E)** More likely, prions bind to heparan sulfate proteoglycans (HSPGs) to trigger macropinocytosis. **(F)** It is theoretically possible that prions gain entry by another form of receptor-mediated endocytosis, although there is not clear evidence for this. **(G)** Prions escape the lumen of vesicles to encounter cognate monomer. **(H)** The seed acts as a template for recruitment of monomer to amplify the prion structure. This likely involves a replication machinery that may also be involved in fibril fragmentation to amplify the number of seeds, which then repeat the cycle of propagation to other cells.
some cases seed (suggested that tau monomer is competent to enter cells and in certain circumstances. For example, two recent investigations proposed that oligomers (monomer or dimer) could act as seeds or cause toxicity through amplification of the prion state. It is unknown whether smaller assemblies, including trimeric units, are required to confirm the importance of this pathway in vivo.

Prions may also require post-translational processing to become competent seeds. In a cell-based assay to detect a putative trans-cytosis step, internalized prions served as templates to convert exogenous seeds co-localize with the aggresome markers, which may have resulted in contamination by tau oligomers. These findings are consistent with a report that tau trimers are required to confirm the importance of this pathway in vivo.

however, these studies relied on less stringent methods for tau detection. Other proteases, such as calpain, asparagine endopeptidase, and cathepsin B, were shown to mediate productive aggregation of the tau repeat domain, a fragment reminiscent of the tau repeat domain. After internalization, prions serve as templates to convert the smallest assemblies that can mediate trans-cytosis. They may also require post-translational processing to become competent seeds. In a cell-based assay to detect a putative trans-cytosis step, internalized prions served as templates to convert exogenous seeds into a cell within the lumen of a vesicle, has not been ruled out. Both tau and -synuclein fragments could act as templates, instead of full-length protein. Genetic or pharmacological inhibition of cathepsin B results in a nonunion of the extracellular space. This may be driven by unconventional secretory processes, although there is not clear evidence for this. The precise cellular sub-compartment where amplification of the prion structure occurs is unknown. There is an involvement in fibril fragmentation to amplify the prion structure. This likely involves a replication machinery that may also be required for productive aggregation of the prion state. Prions are delivered directly to the cytosol via transfection, but the precise role of cathepsin B remains unclear.

Figure 1.1

(A) Escape of prions from a first-order cell could involve escape from a first-order cell, binding to a second-order cell, uptake into a second-order cell, seeding of native monomer, and fragmentation to amplify the prion structure. This likely involves a replication machinery that may also be required for productive aggregation of the prion state. Prions are delivered directly to the cytosol via transfection, but the precise role of cathepsin B remains unclear.

(B) Alternatively, prions could escape in exosomes (B) or directly move to neighboring cells (C).

(C) Tunneling nanotubes (C) may also involve receptor-mediated endocytosis (D). More likely, prions bind to heparan sulfate proteoglycans (HSPGs) to trigger macropinocytosis (E). How-ever, these studies relied on less stringent methods for tau detection. Other proteases, such as calpain, asparagine endopeptidase, and cathepsin B, were shown to mediate productive aggregation of the tau repeat domain, a fragment reminiscent of the tau repeat domain. After internalization, prions serve as templates to convert the smallest assemblies that can mediate trans-cytosis. They may also require post-translational processing to become competent seeds. In a cell-based assay to detect a putative trans-cytosis step, internalized prions served as templates to convert exogenous seeds into a cell within the lumen of a vesicle, has not been ruled out. Both tau and -synuclein fragments could act as templates, instead of full-length protein. Genetic or pharmacological inhibition of cathepsin B results in a nonunion of the extracellular space. This may be driven by unconventional secretory processes, although there is not clear evidence for this. The precise cellular sub-compartment where amplification of the prion structure occurs is unknown. There is an involvement in fibril fragmentation to amplify the prion structure. This likely involves a replication machinery that may also be required for productive aggregation of the prion state. Prions are delivered directly to the cytosol via transfection, but the precise role of cathepsin B remains unclear.

(D) Vesicle escape (G) may also involve receptor-mediated endocytosis (D). More likely, prions bind to heparan sulfate proteoglycans (HSPGs) to trigger macropinocytosis (E). How-ever, these studies relied on less stringent methods for tau detection. Other proteases, such as calpain, asparagine endopeptidase, and cathepsin B, were shown to mediate productive aggregation of the tau repeat domain, a fragment reminiscent of the tau repeat domain. After internalization, prions serve as templates to convert the smallest assemblies that can mediate trans-cytosis. They may also require post-translational processing to become competent seeds. In a cell-based assay to detect a putative trans-cytosis step, internalized prions served as templates to convert exogenous seeds into a cell within the lumen of a vesicle, has not been ruled out. Both tau and -synuclein fragments could act as templates, instead of full-length protein. Genetic or pharmacological inhibition of cathepsin B results in a nonunion of the extracellular space. This may be driven by unconventional secretory processes, although there is not clear evidence for this. The precise cellular sub-compartment where amplification of the prion structure occurs is unknown. There is an involvement in fibril fragmentation to amplify the prion structure. This likely involves a replication machinery that may also be required for productive aggregation of the prion state. Prions are delivered directly to the cytosol via transfection, but the precise role of cathepsin B remains unclear.

(E) HSPG-mediated macropinocytosis (E) may also involve receptor-mediated endocytosis (D). More likely, prions bind to heparan sulfate proteoglycans (HSPGs) to trigger macropinocytosis (E). How-ever, these studies relied on less stringent methods for tau detection. Other proteases, such as calpain, asparagine endopeptidase, and cathepsin B, were shown to mediate productive aggregation of the tau repeat domain, a fragment reminiscent of the tau repeat domain. After internalization, prions serve as templates to convert the smallest assemblies that can mediate trans-cytosis. They may also require post-translational processing to become competent seeds. In a cell-based assay to detect a putative trans-cytosis step, internalized prions served as templates to convert exogenous seeds into a cell within the lumen of a vesicle, has not been ruled out. Both tau and -synuclein fragments could act as templates, instead of full-length protein. Genetic or pharmacological inhibition of cathepsin B results in a nonunion of the extracellular space. This may be driven by unconventional secretory processes, although there is not clear evidence for this. The precise cellular sub-compartment where amplification of the prion structure occurs is unknown. There is an involvement in fibril fragmentation to amplify the prion structure. This likely involves a replication machinery that may also be required for productive aggregation of the prion state. Prions are delivered directly to the cytosol via transfection, but the precise role of cathepsin B remains unclear.

(F) It is theoretically possible that prions gain entry to the cytosol via unconventional secretory processes, although there is not clear evidence for this. The precise cellular sub-compartment where amplification of the prion structure occurs is unknown. There is an involvement in fibril fragmentation to amplify the prion structure. This likely involves a replication machinery that may also be required for productive aggregation of the prion state. Prions are delivered directly to the cytosol via transfection, but the precise role of cathepsin B remains unclear.

(G) Vesicle escape (G) may also involve receptor-mediated endocytosis (D). More likely, prions bind to heparan sulfate proteoglycans (HSPGs) to trigger macropinocytosis (E). How-ever, these studies relied on less stringent methods for tau detection. Other proteases, such as calpain, asparagine endopeptidase, and cathepsin B, were shown to mediate productive aggregation of the tau repeat domain, a fragment reminiscent of the tau repeat domain. After internalization, prions serve as templates to convert the smallest assemblies that can mediate trans-cytosis. They may also require post-translational processing to become competent seeds. In a cell-based assay to detect a putative trans-cytosis step, internalized prions served as templates to convert exogenous seeds into a cell within the lumen of a vesicle, has not been ruled out. Both tau and -synuclein fragments could act as templates, instead of full-length protein. Genetic or pharmacological inhibition of cathepsin B results in a nonunion of the extracellular space. This may be driven by unconventional secretory processes, although there is not clear evidence for this. The precise cellular sub-compartment where amplification of the prion structure occurs is unknown. There is an involvement in fibril fragmentation to amplify the prion structure. This likely involves a replication machinery that may also be required for productive aggregation of the prion state. Prions are delivered directly to the cytosol via transfection, but the precise role of cathepsin B remains unclear.

(H) Seeding/fragmentation by replication machinery (H) may also involve receptor-mediated endocytosis (D). More likely, prions bind to heparan sulfate proteoglycans (HSPGs) to trigger macropinocytosis (E). How-ever, these studies relied on less stringent methods for tau detection. Other proteases, such as calpain, asparagine endopeptidase, and cathepsin B, were shown to mediate productive aggregation of the tau repeat domain, a fragment reminiscent of the tau repeat domain. After internalization, prions serve as templates to convert the smallest assemblies that can mediate trans-cytosis. They may also require post-translational processing to become competent seeds. In a cell-based assay to detect a putative trans-cytosis step, internalized prions served as templates to convert exogenous seeds into a cell within the lumen of a vesicle, has not been ruled out. Both tau and -synuclein fragments could act as templates, instead of full-length protein. Genetic or pharmacological inhibition of cathepsin B results in a nonunion of the extracellular space. This may be driven by unconventional secretory processes, although there is not clear evidence for this. The precise cellular sub-compartment where amplification of the prion structure occurs is unknown. There is an involvement in fibril fragmentation to amplify the prion structure. This likely involves a replication machinery that may also be required for productive aggregation of the prion state. Prions are delivered directly to the cytosol via transfection, but the precise role of cathepsin B remains unclear.
Figure 1.2. Prion replication mechanisms and network involvement link strains to biological phenotype. The various steps in replication of a prion provide an explanation for how strains might exhibit different rates of progression, types of neuropathology, and involvement of specific networks. (A) Biophysical and biochemical parameters govern strain replication efficiency. A single protein monomer can adopt multiple potential amyloid configurations, illustrated by squares vs. triangles. Strain-specific fibril stability and the interaction with chaperone/replication machinery may determine the fragmentation rate and production of seeds. This likely determines the rate of spread and disease progression, with strong strains featuring more rapid phenotypes. (B) A strain must propagate pathology by entering a neuron and initiating seeding and subsequent replication of a particular structure. The newly formed aggregates will accumulate in certain regions of the cell, possibly through interaction with specific factors, and may be differentially transported in axons. Finally, strains may have differential rates of release and/or toxicity at the cell membrane/synapse that govern transfer to other cells. Each of these steps may be influenced by strain conformation. (C) Unique strains may target different cell types and neural networks to cause syndromic variation. Aggregate conformers (square vs. triangle) may target one cell type more readily than another. This could be based on parameters discussed above: cell entry, replication, and trans-cellular propagation rates. Distinct neuronal networks might thus be vulnerable to particular strains.
different PrP prion agents contained their own self-replicating genomes and were merely acting as very unconventional viruses. Extensive searching, however, failed to find a nucleic acid that governed strain phenomena (Safar et al., 2005). Two pioneering studies instead suggested that strain variation derives from PrP amyloid conformers with distinct protease-resistant core regions (Bessen and Marsh, 1994; Telling et al., 1996). More extensive characterization of a panel of hamster PrP prion strains (Safar et al., 1998) solidified this hypothesis, and different PrP strains probably cause distinct human prion diseases (Collinge et al., 1996).

The precise mechanisms by which distinct amyloid structures produce different diseases, however, are unclear. The study of yeast prions has provided important clues to the relationship between amyloid structure and biological phenotype. Yeast prions switch between monomeric and aggregated forms, in which the latter is inherited by daughter cells as a stable epigenetic phenotype (Derkatch et al., 1996; Patino et al., 1996; Paushkin et al., 1997). Sup35 is particularly well studied: aggregation blocks its activity as a transcription termination factor and is generally detrimental to the organism (McGlinchey et al., 2011) but may be advantageous in certain environmental situations (Halfmann et al., 2012; Holmes et al., 2013b; True and Lindquist, 2000; True et al., 2004). Like PrP, Sup35 prions form strains with distinct strengths, or degrees of aggregation (Tanaka et al., 2004). A strong yeast prion strain is analogous to a mammalian PrP prion strain with a short incubation period (Figure 2). Two studies determined the physical and structural basis for these phenomena in several Sup35 prion strains. A combination of growth rate and amyloid fibril fragility defined the strength of the strain (Tanaka et al., 2006). Hydrogen-deuterium exchange indicated that strong strains amplify more readily because they contain a smaller amyloid-forming core that fragments more readily to produce additional seeds (Toyama et al., 2007). These studies suggested that bio-physical parameters could account for most of the variance in strain strength. Consistent with these studies, the stability of multiple PrP prion strains in denaturant (GdHCl) strongly correlated with incubation time upon inoculation into transgenic PrP mice (Legname et al., 2006), suggesting that more fragile amyloid conformers produced shorter incubation periods (i.e., were stronger strains). While studies of sporadic Creutzfeldt-Jakob disease (sCJD) and familial CJD (fCJD) have suggested that strain differences contribute to disease outcome, an understanding of the mechanisms by which these differences arise is still emerging. This review will focus on the current state of knowledge regarding the mechanisms by which different strains of the PrP prion agent cause distinct diseases, with an emphasis on recent studies that address these questions.

Figure 1.2

A. Strain and replication. Different strains (left) have distinct fragmentation and replication abilities (middle), leading to variable seed quantities (right). This results in different strain strengths (bottom).

B. Mechanism of differential strain replication. Uptake leads to seeding/replication, transport/localization, and release/toxicity, with different strains having distinct efficiencies at each step.

C. Mechanisms for differential propagation. Strain-specific entry/replication/propagation and strain-specific neuronal or network vulnerability contribute to the observed differences in disease outcome.
Figure 1.3. Do strains account for phenotypic diversity in tauopathies? (A) It is now well established that different fibrillar conformations (i.e. strains) of PrP underlie unique clinical manifestations of the prionopathies. (B) This dissertation examines whether unique tauopathies (e.g. Alzheimer’s disease, corticobasal degeneration, Pick’s disease, progressive supranuclear palsy, argyrophilic grain disease, etc.) are caused by specific tau amyloid conformations (strains), which stably propagate throughout the nervous system to elicit the myriad unique features characteristic of each disease.
Figure 1.3

A

PrP Seed  PrP Monomer  PrP Fibril

Strain A  +  →  Sporadic Creutzfeldt-Jakob Disease

Strain B  +  →  Variant Creutzfeldt-Jakob Disease/BSE

Strain C  +  →  Fatal Familial Insomnia

Templated Seeding  Clinical Phenotype (Strain)

B

Tau Seed  Tau Monomer  Tau Fibril

τ Seed  +  →  Alzheimer’s Disease

τ Monomer  +  →  Corticobasal Degeneration

τ Fibril  +  →  Pick’s Disease

Templated Seeding  Clinical Phenotype (Strain?)
Chapter 2: Distinct Tau Prion Strains Propagate in Cells and Mice and Define Different Tauopathies
2.1 PREFACE

This chapter contains a previously published manuscript:


2.2 ABSTRACT

Prion-like propagation of tau aggregation may underlie the stereotyped progression of neurodegenerative tauopathies. True prions stably maintain unique conformations ("strains") in vivo that link structure to patterns of pathology. We now find that tau meets this criterion. Stably expressed tau repeat domain indefinitely propagates distinct amyloid conformations in a clonal fashion in culture. Reintroduction of tau from these lines into naïve cells re-establishes identical clones. We produced two strains in vitro that induce distinct pathologies in vivo as determined by successive inoculations into three generations of transgenic mice. Immunopurified tau from these mice re-creates the original strains in culture. We used the cell system to isolate tau strains from 29 patients with 5 different tauopathies, finding that different diseases are associated with different sets of strains. Tau thus demonstrates essential characteristics of a prion. This may explain the phenotypic diversity of tauopathies and ultimately could enable more effective diagnosis and therapy.
2.3 INTRODUCTION

Tauopathies are a diverse group of neurodegenerative diseases defined by accumulation of fibrillar deposits of the microtubule-associated protein tau (MAPT) (Lee et al., 2001). Alzheimer’s disease (AD), the most common tauopathy, affects >30 million people worldwide and will afflict >120 million by 2050 (Holtzman et al., 2011). MAPT mutations cause dominantly inherited tauopathies (Hutton et al., 1998), and most increase the propensity of tau to form amyloids (Barghorn et al., 2000), which are paracrystalline protein assemblies rich in beta-sheet structure (Bonar et al., 1969). Most tauopathy cases are sporadic, with variable clinical and pathological presentation (Lee et al., 2001).

The prion hypothesis posits that pathological aggregates of the mammalian prion protein (PrP) cause infectious, sporadic, and familial neurodegenerative diseases (Prusiner, 1998). In contrast, yeast prions are adaptive and confer phenotypic diversity and rapid evolution of new traits in times of stress (True and Lindquist, 2000). Both yeast and mammalian prions form strains, which are encoded by distinct fibrillar structures (Safar et al., 1998; Toyama et al., 2007). Prion strains determine the incubation periods of disease in humans (Kim et al., 2012a) and mice (Legname et al., 2006). In addition, human prion strains are thought to underlie clinical symptoms and pathological presentation (Collinge and Clarke, 2007).

The hypothesis that common neurodegenerative diseases such as AD could be caused by a prion-like mechanism was suggested three decades ago (Prusiner, 1984). Recently, however, experimental work on diverse amyloids has generated new interest (Frost and Diamond, 2010; Guo and Lee, 2014). Human neurodegenerative diseases target unique
neural networks (Braak and Braak, 1995; Seeley et al., 2009), an observation most parsimoniously explained by the network-based spread of a toxic agent (Raj et al., 2012; Zhou et al., 2012). Prior studies suggest that tau aggregates spread among cells via templated conformational change (Frost et al., 2009a; Holmes et al., 2013a). *In vivo* work supports this model (Clavaguera et al., 2009; de Calignon et al., 2012; Iba et al., 2013; Kim et al., 2010; Liu et al., 2012) as has similar work with other intracellular amyloids such as α-synuclein (Desplats et al., 2009; Hansen et al., 2011; Luk et al., 2009; 2012a) and extracellular amyloids including amyloid beta (Eisele et al., 2009; Meyer-Luehmann et al., 2006).

*Bona fide* prions are defined by propagation of distinct conformational strains *in vivo*, and prior studies have hinted at prion-like strain properties of non-PrP human amyloids. For example, amyloid beta protein forms at least two distinct, self-propagating fibrillar conformations *in vitro* (Petkova et al., 2005) and *in vivo* (Lu et al., 2013). Others have demonstrated propagation *in vitro* of distinct tau (Frost et al., 2009b; Siddiqua and Margittai, 2010) and α-synuclein conformers (Bousset et al., 2013; Guo et al., 2013; Sacino et al., 2013). While provocative, these prior studies have not demonstrated that non-infectious proteopathic seeds act as true prions. Specifically, it has not been shown that distinct conformations or “strains” are capable of transmission into a living cell/organism, propagation through multiple generations, extraction, and re-introduction to naïve cells/organisms to replicate the same structural phenotype (Collinge and Clarke, 2007). This is important not for semantic reasons, but because if prion mechanisms underlie human disease, only stably propagating strains can account for stereotyped clinical presentation and network spread. In this study we have found that tau acts as a
prion by these criteria, and, further, that individual human tauopathies are associated with unique strains.
2.4 MATERIALS AND METHODS

Molecular cloning. The restriction endonucleases BamHI (5’) and NotI (3’) were used to remove YFP from the pEYFP-N1 vector (Clontech) and separately insert CBG-Cluc and CBG-Nluc, both derived from the pFRB vector (kind gift from the David Piwnica-Worms lab) (Naik and Piwnica-Worms, 2007). Tau RD (aa 244-372 of the 441 aa full-length tau 4R2N with the following variants: I277P/ΔK280/I308P or 2P, WT, P301L, P301S, P301L/V337M or LM) was PCRed out of the pEYFP vector and placed upstream of each of the split-luciferase constructs using EcoRI (5’) and KpnI (3’), thus generating p-tau RD-Cluc and p-tau RD-Nluc.

For lentiviral constructs, a backbone containing the ubiquitin C promoter IRES-Venus (FCIV FM5) (generously provided by the Jeffrey Milbrandt laboratory) (Araki et al., 2004) was modified to remove IRES-Venus by digestion with BsrGI (5’) and BamHI (3’). DNA was extracted from agarose gel using Qiagen Gel Extraction Kit. Ends were blunted using DNA Terminator End Repair Kit (Lucigen) and the resulting product was purified using Qiagen PCR purification Kit. The blunted product was re-ligated using T4 DNA ligase (New England Biolabs) to create a new plasmid called FM5. The following constructs were then subcloned from the pEYFP vector using Nhel (5’) and AscI (3’): tau RD(ΔK280/I277P/I308P) (2P)-YFP, tau RD(WT)-YFP, tau RD(ΔK280)-YFP, tau RD(P301L/V337M) (LM)-YFP, alpha synuclein(WT)-YFP, huntingtin Exon 1(Q25)-YFP, tau RD-Cluc (various mutants), tau RD-Nluc (various mutants), tau RD P301L/V337M (LM)-HA, full-length tau 4R1N P301S, full-length tau 4R1N P301S-YFP, full-length tau 4R1N WT, full-length tau 4R1N WT-YFP.
To create a plasmid expressing tau RD(LM)-YFP under the TRE (tetracycline response elements) promoter, tTA was subcloned into FM5 using AgeI (5’) and EcoRI (3’) to generate FM5-Ub-tTA. The TRE-Tight promoter was then PCRed using PacI (5’) and NheI/BsrGI/PacI (3’). PacI digestion was then used to subclone the PCR product into FM5-tTA and sequencing confirmed proper orientation, thus generating the FM5-TRE-Ub-tTA plasmid (FM5 Tet Off). Finally, NheI (5’) and BsrGI (3’) were used to subclone tau RD(LM)-YFP into FM5-TRE-Ub-tTA to generate FM5 Tet Off-tau RD(LM)-YFP.

All restriction endonucleases were obtained from New England Biolabs.

**Protein purification and fibrillization.** Amyloid beta (1-42) fibrils were a generous gift from the John Cirrito laboratory: peptide was synthesized by Pepnome and monomer was re-suspended in HFIP, which was subsequently evaporated away under nitrogen gas. The resulting monomer was resuspended at 5 mM in DMSO prior to dilution to 100 µM in 10 mM HCl/150 mM NaCl. Fibrils were prepared by incubating at 37°C for 24 hours. They were then stored at -20°C until use.

Synthetic huntingtin exon1 N17-Q35 peptide was synthesized at the Keck Biotechnology Resource Laboratory of Yale University. Peptide was solubilized in formic acid prior to being dialyzed into phosphate buffer solution. Re-solubilized monomer was fibrillized at room temperature at a concentration of 100 µM for 24 hours. Resulting fibrils were stored at -20°C until use.

α-synuclein fibrils were a generous gift from the Paul Kotzbauer laboratory. Recombinant α-synuclein WT protein was produced in *E. coli* using previously published methods (Giasson et al., 2003; Huang et al., 2005): the purified protein was then dialyzed
overnight in 10 mM Tris-HCl (pH=7.6)/1 mM DTT/50 mM NaCl. Recombinant monomer at a concentration of 2 mg/mL was then incubated with 100 mM NaCl and 20 mM Tris-HCl (pH=8.0) at 37°C for 72 hours with shaking (Eppendorf Thermomixer, 1000 rpm) to induce fibrillization. The fibril mixture was then centrifuged at 15,000xg for 15 minutes to separate fibrils (pellet) from monomer (supernatant). Following manufacturer’s protocol, the concentration of α-synuclein in the supernatant was calculated using a BCA protein assay. The decrease in protein in the supernatant following centrifugation was used to calculate the monomer-equivalent concentration of α-synuclein in the pellet (fibrils). Fibrils were stored at a concentration of 80 µM at -20°C until use.

Recombinant tau RD-HA monomer was prepared as previously described (Frost et al., 2009b; Goedert and Jakes, 1990) by expressing pRK172-Tau RD-HA in Rosetta (DE3)pLacI competent cells (Novagen). Monomer was lyophilized and stored at -80°C as single-use aliquots. Monomer was re-suspended in 25 mM DTT for one hour. The reduced protein was then fibrillized at a final concentration of 8 µM in 2.5 mM DTT/10 mM HEPES (pH=7.4)/100 mM NaCl/8 µM heparin (tau buffer) at 37°C without agitation. Fibrils were stored at a concentration of 8 µM at -20°C until use.

**Cell culture.** HEK293 and HEK293T cell lines were all grown in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (HyClone), 1% penicillin/streptomycin (Gibco), and 1% glutamax (Gibco). Cell lines were maintained and passaged in 10 cm dishes at 37°C, 5% CO₂, in a humidified incubator.
**Lentivirus production.** Lentivirus was prepared as described previously (Araki et al., 2004): HEK293T cells were plated at a concentration of $1 \times 10^6$ cells/well in a 6-well plate. 18 hours later, cells were transiently co-transfected with PSP (1200 ng), VSV-G (400 ng), and FM5 (400 ng) plasmids using 6 µL FuGene HD (Promega). 72 hours later, conditioned media was harvested and centrifuged at 1000xg for 5 minutes to remove dead cells and debris. Supernatant was stored at -80°C until use. For primary neuron transduction, lentivirus was concentrated 10x using lenti-X concentrator (Clontech) with the concentrated pellet being re-suspended in PBS with 25 mM HEPES, pH 7.4.

**Stable HEK293 cell line generation.** HEK293 cells were plated at a concentration of 100,000 cells/well in wells of a 6-well plate. 18 hours later, 1 mL of media was removed and replaced with 1.5 mL conditioned media containing lentivirus. For the split-luciferase aggregation reporter line, cells were co-transduced with 750 µL of tau RD-Cluc and 750 µL tau RD-Nluc lentivirus. These amounts were used for all mutant tau RD split-luciferase pair lines with lentivirus being made in the same batch to ensure roughly equivalent viral titers. For all stable lines, cells were given 5 days to amplify to confluency in the presence of virus. At this point, cells were re-plated in a 10 cm dish and were grown to confluency. For cell lines featuring protein with a fluorescent tag, epifluorescence microscopy was used to confirm that all cells were expressing fluorescent protein. Polyclonal cell lines were then stored in liquid nitrogen until use.

To generate monoclonal lines, polyclonal cell populations were diluted sparsely in 10 cm dishes. Colonies were given 9 days to amplify, at which point cloning cylinders (Bel-Art Products) were used to isolate single colonies. Monoclonal cultures were serially
amplified to confluency using 12-well then 6-well then 10 cm dishes. Resulting monoclonal lines were frozen in liquid nitrogen until use.

**Liposome-mediated transduction of amyloids into HEK293 cells.** A previously used fibril-transduction protocol (Nekooki-Machida et al., 2009) was used with minor modifications: polyclonal HEK293 cells stably expressing either tau RDΔK280/I277P/I308P(2P)-YFP, tau RD(WT)-YFP, tau RD(ΔK280)-YFP, tau RD P301L/V337M(LM)-YFP, α-synuclein(WT)-YFP, huntingtin exon 1(Q25)-YFP, FL tau 4R1N P301S-YFP, or FL tau 4R1N P301S were plated at 250,000 cells/well in 12-well plates. Tau buffer (2.5 mM DTT/10 mM HEPES (pH=7.4)/100 mM NaCl/8 μM heparin) or indicated fibrils were then combined with OptiMEM (Gibco) to a final volume of 100 μL. 96 μL OptiMEM and 4 μL lipofectamine-2000 (Invitrogen) was then added to the OptiMEM mixture to a final volume of 200 μL. After a 20-minute incubation, liposome preparations were added to cells so that fibrils were at the following final concentrations: tau RD (400 nM), α-synuclein (400 nM), amyloid beta (1-42) (1 μM), huntingtin exon 1 N17(Q35) (1 μM), or tau buffer (400 nM tau fibril equivalent). 18 hours later, cells were washed, trypsinized, and re-plated in wells of a 6-well plate. Once confluent, cells were plated on coverslips (thickness = 0.09 to 0.12 mm; Carolina Biologicals) for imaging and quantification (time = Day 6). For transductions involving FL tau, fixed cells were stained with AT8 (see immunocytochemistry methods).

**Quantification of percent cells positive for inclusions.** For inclusion-elimination time course experiments, cells were passaged every two days for 50 days, plating cells for fixation on every other passage. Cells were fixed by incubating with 4% PFA in PBS for 15 minutes. Coverslips were washed twice and stained with DAPI (1:3000 dilution from
1 mg/mL stock) for 5 minutes. Coverslips were mounted on Prolong Gold Antifade Reagent (Invitrogen), sealed with nail polish, and placed at 4°C prior to analysis. To quantify percent cells positive for inclusions, a total of 10 fields, each with 150+ cells, were analyzed per condition. The number of cells per field was determined by counting the number of DAPI-positive nuclei. Then, cells with inclusions were counted and a percentage was calculated. Mean and standard error were subsequently determined and plotted.

**Isolation of monoclonal tau prion strains induced by recombinant tau RD fibrils.** A monoclonal HEK293 cell line stably expressing tau RD(LM)-YFP was generated. Cells were plated at 240,000 cells/well in a 12-well plate. 24 hours later, 400 nM tau RD-HA fibrils were transduced using lipofectamine-2000 (Invitrogen) as previously described. 18 hours later, cells were washed, trypsinized, and re-plated in a 6-well plate. At confluency (Day 3), cells were diluted sparsely in 10 cm dishes so that there were less than 100 cells per dish. Cells were given 9 days to amplify into colonies. At Day 12, epifluorescence microscopy was used to mark colonies featuring cells with inclusions. Cloning cylinders (Bel-Art Products) were used to isolate single clones, which were subsequently serially amplified to confluence in 12-well, then 6-well, and 10 cm dishes. At Day 30, cells were either plated on coverslips for fixation and confocal analysis or were frozen down in single-use pellets at -80°C for later analysis.

**Confocal analysis.** All confocal microscopy was performed using a Zeiss LSM 5 PASCAL system coupled to a Zeiss Axiovert 200M microscope. A pinhole size of 0.8 µm was used for the collection of all images. Images were processed minimally using
ImageJ. When post-hoc processing was performed, the same modifications were made across all images collected for a single experiment.

**Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE).** SDD-AGE was performed as previously described (Kryndushkin et al., 2003) with minor modifications. Cell pellets were thawed on ice, lysed by trituration in PBS containing 0.05% Triton-X and a cOmplete mini protease inhibitor tablet (Roche), and clarified by 5-minute sequential centrifugations at 500xg and 1000xg. Low-SDS 1.5% agarose gels were prepared by dissolving agarose in buffer G (20 mM Tris-Base, 200 mM glycine, in ddH$_2$O) with 0.02% SDS. For each condition, 5 µg of clarified cell lysate was incubated with 0.02% SDS sample buffer for 7 minutes prior to loading. SDD-AGE was ran in Laemmli buffer (Buffer G with 0.1% SDS) at 125 V for 90 minutes or until dye front reached the bottom of the gel. Protein was transferred at 100 V for 90 minutes at 4°C to Immobilin P (Millipore) using a custom-modified SDS-PAGE transfer cassette (Bio-Rad) for accommodation of the thick agarose gel. Membranes were probed for tau RD using rabbit polyclonal anti-tau ab64193 (1:4000, AbCam) overnight, washed four times with TBS-T, counter-probed with goat anti-rabbit HRP (1:4000, Jackson Immunotherapy) for 1.5 hours, and were washed an additional 4 times. Finally, membranes were imaged by exposure to ECL Prime Western Blotting Detection System (Fisher Scientific) for 3 minutes and development using a digital Syngene imager.

**Sedimentation analysis and densitometry.** Clarified cell lysate was prepared as described above (see SDD-AGE). 10% of the lysate was set aside as the total fraction; the rest was centrifuged at 100,000xg for 1 hour. The supernatant was placed aside and the pellet was washed with 1.5 mL PBS prior to ultracentrifugation at 100,000xg for 30
minutes. The supernatant/wash was aspirated and the pellet was re-suspended in RIPA buffer with 4% SDS and 100 mM DTT with the aid of boiling. Bradford assay (Bio-Rad) with BSA standard curve was used to normalize all protein concentrations with additional 0.05% Triton-X in PBS being used for dilution. 1 µg of total protein was loaded per well on a 4-15% SDS-PAGE gel (Bio-Rad). For all samples besides the negative control (Clone 1), supernatant was loaded at a 3:1 ratio relative to pellet and total fractions; for Clone 1, a 1:1 ratio was used. Gels were ran at 120 V for 60 minutes and protein was transferred to Immobilon P (Millipore) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked for an hour with 5% milk, probed with rabbit polyclonal anti-tau ab64193 (1:4000, AbCam) overnight, washed four times with TBS-T, counter-probed with goat anti-rabbit HRP (1:4000, Jackson Immunotherapy) for 1.5 hours, and were washed an additional 4 times. Finally, membranes were imaged by exposure to ECL Prime Western Blotting Detection System (Fisher Scientific) for 3 minutes and development using a digital Syngene imager. Densitometric units were calculated using Syngene GeneTools software with manual band quantification, normalizing against background. Units were then corrected for loading ratios. For calculating densitometric ratios, blots were performed in triplicate and statistical analysis was performed using one-way analysis of variance with Bonferroni’s multiple comparison test.

**Split-luciferase complementation assay.** Polyclonal HEK293 cells stably expressing tau RD(LM)-Cluc and tau RD(LM)-Nluc were plated at 240,000 cells/well in 12-well plates 24-hours prior to cell lysate transduction. Clarified cell lysate was prepared as previously described (see SDD-AGE) and was normalized to final protein concentrations of 2 µg/µL using PBS with 0.05% Triton-X to dilute. 20 µg (10 µL) of each cell lysate was diluted
with 90 µL OptiMEM (Gibco) and incubated with 96 µL OptiMEM and 4 µL lipofectamine-2000 (Invitrogen) for 20 minutes. Liposome preparations were then added to cells (each condition performed in quadruplicate) and 18 hours later, cells were washed, trypsinized, and re-plated in quadruplicate in a 96-well plate. 24 hours later, media was aspirated from wells and replaced with luciferin solution (150 µg/mL D-luciferin potassium salt, Gold Biosciences, in Dulbecco’s phosphate-buffer saline, Gibco). Cells were incubated with luciferin solution for 3 minutes at 37°C prior to reading luminescence with a TecanM1000 fluorescence plate reader. Luminescence reads were first averaged across technical replicates (n=4) and subsequently averaged across biological replicates (n=4 experiments unless otherwise noted) to determine standard errors of the mean. One-way analysis of variance with Bonferroni’s multiple comparison test was used for statistical analysis. For cross-seeding experiments, the same protocol was used with minor exceptions: different stable split-luciferase cell lines were used; recombinant tau RD-HA fibrils were transduced at a concentration of 1 µM; 50 µg of aged transgenic P301S brain (age=12 months) was transduced.

**Protease digestion.** Pronase (Roche) was diluted in PBS to a final concentration of 1 mg/mL and single-use aliquots were stored at -80°C. Clarified cell lysate was prepared as previously described (see SDD-AGE) and protein concentrations were normalized to 1.7 µg/µL, unless otherwise noted. 17 µg (10 µL) of cell lysate was added to 10 µL of pronase at a concentration of 100 µg/mL (diluted in PBS) for a final volume of 20 µL and a final pronase concentration of 50 µg/mL. Cell lysates were digested at 37°C for one hour. Reactions were quenched by addition of 20 µL 2x sample buffer (final SDS concentration of 1%) and boiling for 5 minutes. 15 µL of each sample was loaded onto a
10% Bis-Tris NuPAGE gel (Novex by Life Technologies) and were ran at 150 V for 65 minutes. Protein was transferred to Immobilon P (Millipore) using a semi-dry transfer apparatus (Bio-Rad) and membranes were probed for tau RD as described above.

**X-34 staining of amyloids.** To visualize amyloids, cells were stained with X-34, a derivative of Congo Red (Styren et al., 2000): Cells were fixed with 4% PFA in PBS for 15 minutes, washed twice with PBS, and permeabilized with 0.25% Triton-X/PBS for 30 minutes. They were then incubated with 1 µM X-34 (generous gift from the John Cirrito lab) in X-34 staining buffer (60% PBS/39% ethanol/0.02 M NaOH) for 15 minutes. Stained cells were rinsed briefly with staining buffer 3x followed by two 5-minute washes with PBS. Coverslips were mounted on Prolong Gold Antifade Reagent (Invitrogen), sealed with nail polish, and placed at 4°C prior to analysis.

**Toxicity assays.** To assess relative toxicity of Clone 9 and Clone 10 amyloids, cell growth propensity when lacking or containing tau RD inclusions was examined. Monoclonal HEK293 cells stably expressing tau RD(LM)-YFP were plated at 240,000 cells/well in a 12-well plate. 24 hours later, cells were transduced with 20 µg clarified cell lysate (Clone 9, Clone 10). 18 hours later, transduced cells were re-plated in a 6-well dish and grown to confluency. On Day 3, cells were plated sparsely on coverslips and were given 6 days to amplify into colonies. On Day 9, cells were fixed with 4% PFA and DAPI stained. For each condition, 80 inclusion-positive and 80 inclusion-negative colonies were quantified by size (number of DAPI-stained nuclei per colony). Average colony size was plotted and one-way analysis of variance with Bonferroni’s multiple comparison test was used to assess statistical significance.
Concurrently, at Day 3, Clone 1-, Clone 9- and Clone 10-transduced cells were passaged onto either coverslips or into a new 6-well plate. Every three days, cells were passaged, with cells being fixed and DAPI-stained at Days 4, 17, and 30. Percent cells positive for inclusions at each time point were quantified and plotted as previously described. One-way analysis of variance with Bonferroni’s multiple comparison test was used to assess statistical significance.

For LDH and cell growth toxicity experiments, a polyclonal HEK293 cell line stably expressing tau RD(LM)-HA was generated and transduced with 20 µg clarified cell lysate (Clone 1, Clone 9, Clone 10) or sham treatment in 12-well plates (quadruplicate). 18 hours later, each biological replicate was re-plated at 1:100 dilution in 100 µL total volume in technical quaduplicates in 96-well plates. 72 hours later, confluency was assessed with a Hoechst stain. Media was removed and transferred to new 96-well plates and LDH assay (Roche) was performed according to manufacturer’s protocol. One-way analysis of variance with Bonferroni’s multiple comparison test was used to assess statistical significance.

**Preparation of cells for ultrastructural analysis by transmission electron microscopy.** Cells were pelleted at 15,000 rpm for 5 minutes and fixed in 2.5% glutaraldehyde in 0.1 M mono/disodium phosphate buffer, pH 7.4, for 1 hour at room temperature and then at 4°C on a rotator overnight. After buffer rinsing, the pellets were then post-fixed in 1% (w/v) osmium tetroxide in 0.1 M mono/disodium phosphate buffer, pH 7.4, for 2 hours at room temperature before being rinsed in distilled water and then dehydrated in an ethanol series. After 2x20 min in propylene oxide and infiltrating overnight in 1:1 propylene oxide:TAAB low viscosity resin (TAAB Laboratories Ltd.,
Aldermaston, UK), the pellets were further infiltrated in resin over several days, with several resin changes, before polymerizing at 60°C for 16 hours. The pellets were then sectioned and stained with 0.5% (w/v) aqueous, 0.22 mm-filtered uranyl acetate at room temperature for 1 hour.

**Preparation of cells for immunogold labelling.** Cells were spun at 15,000 rpm and pellets were fixed in fixed in 4% formaldehyde/0.1% glutaraldehyde in 0.1 M mono/disodium phosphate, pH 7.4, for one hour at room temperature and then at 4°C on a rotator overnight. The following procedures were all carried out at 4°C: the fixed pellets were washed in 0.1 M mono/disodium phosphate and dehydrated in an ethanol series, followed by 2:1, then 1:2 100% ethanol/Unicryl resin (British BioCell International, Cardiff, UK) for 30 minutes each. Finally, the pellets were infiltrated in complete Unicryl resin, with several changes over a few days, and light-polymerized as previously described (Thorpe, 1999).

**Immunogold labelling.** Thin sections were cut and collected upon TEM support grids and then blocked for 30 minutes at room temperature in normal goat serum (1:10 dilution in PBS+ (PBS, pH 8.2, modified by the addition of 1% BSA, 500 µL/L Tween-20, 10 mM Na-EDTA, and 0.2 g/L sodium azide)). They were then left in the primary antibody, HJ 9.3 mouse monoclonal (10 mg/ml), overnight at 4°C. The following day, the grids were given three rinses in 1 mL PBS+ for 2 minutes each and then placed into the secondary antibody, 10 nm gold particle-conjugated goat anti-mouse IgG (1:10 dilution in PBS+) for one hour and then rinsed in PBS+ three times for 10 minutes each, followed by four rinses in distilled water for 5 minutes each. Finally, subsequent to drying, the grids were stained in 0.5% (w/v) aqueous, 0.22 mm-filtered uranyl acetate for 1 hour.
**Imaging by transmission electron microscopy.** Grids were examined using a Hitachi-7100 transmission electron microscope operated at 100 kV. Digital images were captured via an axially mounted (2000x2000 pixel) Gatan Ultrascan 1000 charge-coupled device camera (Gatan, Abingdon, UK) and subsequently examined using ImageJ software.

**Immunocytochemistry.** HEK293 cells were fixed in 4% PFA (Vimentin and AT8 stains) or 100% ice-cold methanol (γ-tubulin and PML stains) for 15 minutes followed by permeabilization with 0.25% Triton X-100 for 30 minutes. Coverslips were then blocked in blocking solution (10% fetal goat serum, 25 mg/mL BSA, 0.25% Triton X-100, PBS) for one hour at room temperature. For primary stains, coverslips were exposed to one of the following in blocking buffer overnight at 4°C: mouse monoclonal anti-vimentin V9 (1:50, Santa Cruz), mouse monoclonal anti-phospho-tau AT8 (1:400, ThermoScientific), mouse monoclonal anti-γ-tubulin GTU-88 (1:2500, Sigma Aldrich), or mouse monoclonal anti-PML PG-M3 (1:100, Santa Cruz). Following washes, stained coverslips were counterstained with either Alexa Fluor 488-tagged goat anti-mouse antibody (1:400 in blocking buffer, Life Technologies) or Alexa Fluor 546-tagged goat anti-mouse antibody (1:400 in blocking buffer; Life Technologies) for 1 hour at room temperature. Coverslips were then washed, DAPI-stained, mounted using Prolong Gold Antifade Reagent (Invitrogen), sealed with nail polish, and placed at 4°C prior to analysis by confocal microscopy.

**Primary cortical neuron culture and Triton X-100 extraction.** For primary cortical neuron culture, the cortex of embryonic day 18.5 mouse embryos was isolated and digested with 2 mg/mL papain (Worthington Biochemistry) and 0.1% DNase I (Invitrogen). Neurons in Neurobasal media (Gibco) containing serum-free B-27
(Invitrogen) and GlutaMAX (Invitrogen) were plated in 24-well dishes with coverslips pre-treated with 10 µg/mL poly-D-lysine (Sigma Aldrich). Day 1 post-plating (DIV1), 20 µL of indicated 10x concentrated lentivirus was added per well. On DIV3, 25 µg cell lysate (10 µg/µL sonicated in PBS, clarified and sterilized with 0.22 micron filter) or equivalent amount of PBS, was added to wells. On DIV17, 14 days after inoculation with clarified lysates, neurons were fixed with 4% PFA/4% sucrose/PBS containing or lacking 1% Triton X-100 for 30 minutes, a slight modification from a previously described protocol for extracting soluble cytoplasmic proteins for easy visualization of insoluble inclusions (Volpicelli-Daley et al., 2011). After 30 minute permeabilization with 0.25% Triton X-100 in PBS for 30 minutes, coverslips were placed in blocking solution (10% fetal goat serum, 25 mg/mL BSA, 0.25% Triton X-100, PBS) for one hour at room temperature. Coverslips were then exposed to mouse monoclonal anti-phospho-tau AT8 (1:400, ThermoScientific) overnight at 4°C in blocking solution. Following washes, stained coverslips were counterstained with Alexa Fluor 546-tagged goat anti-mouse secondary (1:400 in blocking buffer; Life Technologies) for 1 hour in blocking solution at room temperature. Coverslips were then washed, DAPI-stained, mounted using Prolong Gold Antifade Reagent (Invitrogen), sealed with nail polish, and placed at 4°C prior to analysis by confocal microscopy.

**Reversing aggregated state using doxycycline in Tet off background.** A monoclonal HEK293 cell line stably expressing tau RD(LM)-YFP under the TRE repressor (Tet Off) was generated and plated at 240,000 cells/well in wells of a 12-well plate. 24 hours later, 20 µg clarified lysate from Clone 10 cells was transduced using lipofectamine-2000 (Invitrogen). 18 hours later, cells were re-plated into a 6-well plate. On Day 3, cells were
plated sparsely in a 10 cm dish and on Day 12, cloning cylinders (Bel-Art Products) were used to select three clones containing inclusions (these clones were picked in order of identification using an epifluorescence microscope). The three clones, annotated Clone 10-1, Clone 10-2, and Clone 10-3, were amplified to confluency in 12-well then 6-well then 10 cm plates and were frozen down in liquid nitrogen. All featured a similar morphology (large juxtanuclear inclusion, no nuclear inclusions) to the original Clone 10. Clone 10-1 was plated in a 6-well plate and media containing 30 ng/mL doxycycline was used to turn off expression for 0, 1, 2, 3, 5, or 7 days. At this point, all cells were re-plated on coverslips, and were given two days to recover in media lacking doxycycline, thus turning tau RD(LM)-YFP expression back on. At this point, cells were fixed with 4% PFA in PBS and DAPI stained, analyzing percent cells positive with confocal microscopy as described above (n=10 fields, each with >150 cells).

Animals. Transgenic mice expressing human 4R1N P301S tau under the murine prion promoter (Yoshiyama et al., 2007) were obtained from The Jackson Laboratory and maintained on a B6C3 background. Mice had food and water ad libitum and were housed with a 12 hour light/dark cycle. All protocols involving animal use were approved by the institutional animal care and use committee at Washington University in St. Louis. For all experiments, conditions were gender-matched (Table S1).

Murine hippocampal injections. P301S and non-transgenic littermates were anesthetized with isoflurane as previously described (Devos and Miller, 2013). Mice were bilaterally injected into the hippocampus (from bregma: −2.5 mm posterior, +/-2 mm lateral, −1.8 mm ventral) with either 2 µL of 5 µg/µL lysate/homogenate or 2 µL of 2.5 µg/µL recombinant tau RD fibrils at an infusion rate of 0.2 µL/minute. 10 µL gas-
tight syringes (Hamilton) and 30 gauge needles with a 60° bevel from the vertical (Hamilton) were used for all injections.

**Tissue collection.** Mice were anesthetized with isoflurane and perfused with chilled 1x PBS with 0.03% heparin. Brains were rapidly removed and for Generations 0-2 transected mid-sagittally. The right hemisphere was micro-dissected into hippocampus, brainstem, cortex, and frontal sections and subsequently snap-frozen and stored at −80°C until further use. The left hemisphere was post-fixed in 4% paraformaldehyde at 4°C for 24 hours and then transferred to 30% sucrose. For Generation 3 mice, whole brains were post-fixed as described above.

**Serial Inoculations.** Hippocampi (right hemisphere) from all Generation 0 mice were micro-dissected and homogenized: while still frozen, samples were sonicated in 1:4 w/v TBS with cOmplete protease inhibitors (Roche) and phosSTOP (Roche) using an Omni-Ruptor 250 probe sonicator at 30% power for 20, 10-second cycles. Crude homogenates were then clarified by centrifugation at 15,000xg for 15 minutes and supernatants were frozen down at -80°C until use. Homogenates were standardized by Bradford assay to final protein concentrations of 5 µg/µL. Hippocampi from the same cohort (e.g. Clone 1) were pooled and 2 µL (i.e. 10 µg) was bilaterally injected into the hippocampi of transgenic P301S mice (Generation 1) as per before (n=3-4 for all inoculation cohorts). Four weeks later, generation 1 mice were sacrificed and the procedure was repeated for generation 2 mice (n=3-4 for all cohorts). For generation 3 mice, 10 µg total protein was unilaterally injected into the right hippocampus so that both ipsilateral and contralateral spread could be examined 5-weeks later (n=2 per cohort).
**Histology and Immunohistochemistry.** 50 µm sections were taken through the entire left hemisphere using a freezing microtome. Slices were first blocked for one hour with 10% goat serum and 3% milk in TBS with 0.25% Triton X-100 (blocking buffer). For DAB stains, brain slices were incubated with either biotinylated AT8 antibody (1:500, ThermoScientific); MC1 antibody (1:500, kind gift from Peter Davies laboratory); or rabbit anti-Iba1 (1:500, Wako Chemicals USA), all overnight in blocking buffer at 4°C. For secondary staining of MC1, slices were incubated with biotinylated goat anti-mouse F(ab’)2 fragment (1:1000 in blocking buffer, Jackson Immunoresearch). For secondary staining of Iba1, slices were subsequently incubated in biotinylated goat anti-rabbit IgG (1:500 in blocking buffer, Sigma) for one hour at room temperature. Using the VECTASTAIN Elite ABC Kit (Vector Labs), all stained slices were then incubated at room temperature for 30 minutes, followed by DAB development using the DAB Peroxidase Substrate Kit with the optional nickel addition (Vector Labs). Histological images and z-stacks were captured using the Olympus Nanozoomer 2.0-HT (Hamamatsu) and analyzed with the NDP viewer software (Hamamatsu). Counts of neurons with AT8-positive tangle-like structures in CA1/CA3 of the hippocampus were performed by a blinded individual. One-way analysis of variance with Bonferroni’s multiple comparison test was used to assess statistical significance.

For immunofluorescence stains, slices were placed in blocking solution for one hour and were then incubated in blocking solution with either AT8 antibody (1:500, ThermoScientific) or rabbit anti-Iba1 (1:500, Wako Chemicals USA) overnight at 4°C. Slices were then incubated with either Alexa Fluor 488-tagged goat anti-mouse antibody (1:2000, Life Technologies) in the case of AT8 or Alexa Fluor 546-tagged goat anti-
rabbit antibody (1:2000, Life Technologies) for one hour at room temperature in blocking solution. Slices were subsequently stained with DAPI as described above and mounted onto coverslips with Fluoromount G (Sigma Aldrich). Slices were imaged by confocal microscopy.

For X-34 staining, sections were incubated in PBS with 0.25% Triton X-100 for 30 minutes, followed by a 20 minute incubation with X-34 (10 µM final concentration) in 40% ethanol, 60% PBS and 1/500 volume of 10 N NaOH. Slices were subsequently rinsed in 40% Ethanol/60% PBS at 3 x 2 minutes followed by rinse for 2 x 5 minutes in PBS. Slices were finally mounted with Fluoromount-G (Sigma Aldrich). Slices were imaged with a Nikon Instruments Eclipse E800 microscope.

**Immunoprecipitation of full-length tau from strain-inoculated mice.** 1:50 HJ8.5 (kind gift from the David Holtzman lab) was added to 120 µL (600 µg) hippocampal homogenates freshly thawed on ice. Homogenate and antibodies were incubated overnight at 4°C with rotation. 18 hours later, 50 µL of protein-G agarose beads (Pierce) were added and samples were again incubated overnight at 4°C with rotation. 24 hours later, samples were centrifuged at 2000xg for 3 minutes at 4°C. Supernatant was discarded and replaced with 500 µL Ag/Ab gentle binding buffer (Pierce). This centrifugation/wash step was repeated three times. After the final aspiration, proteins bound to beads were eluted using 50 µL low pH elution buffer (Pierce) by incubating at room temperature for 5 minutes. Samples were centrifuged at 2000xg for 3 minutes and supernatants were collected. This elution step was repeated to give a total volume of 100 µL. Finally, 10 µL 1 M Tris-Base pH 8.5 was added to the eluate to neutralize the elution
buffer. When seeding of eluate and crude homogenate were compared, volumes were standardized. Eluted samples were stored at -80°C until use.

**Analysis of seeding potential of immunoprecipitated tau from strain-inoculated mice.** Seeding activity of immunoprecipitated full-length tau was examined using split-luciferase complementation and inclusion counts for both generation 0 (un-pooled) and generation 2 (pooled by cohort) mice. IPed material from 60 µg brain homogenate was transduced into split-luciferase cells, following the protocol previously described. Four separate experiments were performed for IPed samples from each brain, each read in quadruplicate 48-hours post-transduction of lysate. Average seeding ratios were calculated for each sample, comparing luminescence versus sham-inoculated cells (lipofectamine-2000 alone). Luminescence values were averaged within mouse cohorts and compared by one-way analysis of variance with Bonferroni’s multiple comparison test was used to assess statistical significance.

For inclusion counts, IPed full-length tau from 60 µg brain homogenate-equivalent was transduced into tau RD(LM)-YFP cells in a 12-well plate. 24 hours later, cells were re-plated onto coverslips. At 96 hours, cells were fixed. Six fields, each with 100+ cells, were analyzed per mouse and averages were calculated for each. These averages were then collapsed within cohorts and compared by one-way analysis of variance with Bonferroni’s multiple comparison test to assess statistical significance.

**Re-introduction of strains passaged through mice into naive tau RD-YFP cells.** IPed FL tau from 60 µg brain homogenate from transgenic tau P301S mice (Generation 0 = un-pooled, Generation 2 = pooled) was transduced into tau RD(LM)-YFP cells in a 12-
well plate. 24 hours later, cells were re-plated into a 6-well plate. At confluency, cells were sparsely plated on coverslips and were given 8 days to amplify into discrete colonies. Cells were then fixed with 4% PFA and DAPI-stained. Colonies with inclusions were imaged by confocal microscopy. Inclusion-positive colonies were scored as either containing or lacking nuclear inclusions. For generation 0 mice, 20+ colonies were scored per mouse and percentage with each phenotype was calculated. For generation 2 mice, 10 coverslips, each with 15+ inclusion-positive colonies, were scored per cohort. Values were averaged within cohorts and compared by student’s t-test.

For isolation of monoclonal lines, this protocol was repeated except inoculated cells were re-plated sparsely into 10 cm dishes following confluency in the first 6-well plate. Monoclonal inclusion-positive lines were isolated as described previously. For Generation 0 mice, one representative colony was blindly picked and amplified per mouse. For Generation 2 mice, 12 colonies were blindly picked and amplified per cohort. Each monoclonal line was examined by inclusion morphology/confocal, pronase digestion, and split-luciferase complementation as described previously.

**Human patient case selection and neuropathological methods.** Cases were selected from the Neurodegenerative Disease Brain Bank (NDBB) at the University of California, San Francisco (UCSF). The NDBB receives brain and spinal cord materials from patients enrolled in UCSF Memory and Aging Center longitudinal clinical research programs. The fresh brains were cut into ~1 cm coronal slabs, which were alternately fixed in 10% neutral buffered formalin for 72 hours or rapidly frozen, providing tissues preserved with both methods bilaterally for every cut surface. Neuropathological diagnoses were made in accordance with consensus diagnostic criteria (Hyman et al., 2012; Mackenzie et al.,
Using previously described histological and immunohistochemical methods (Kim et al., 2012b). Cases were selected based on neuropathological diagnosis. Blocks (~0.5 g) were dissected from frozen brain slabs, targeting regions for each diagnostic group in which all patients showed no less than a mild-to-moderate tau inclusion burden in the apposed fixed tissue block: middle frontal gyrus (6 AD, 6 PSP, and 6 CBD); inferior temporal cortex (6 AGD) and anterior orbital gyrus (5 PiD). Among the six AGD cases, 2 carried a primary diagnosis of AGD (AGD1 and AGD2) whereas the others carried a primary diagnosis of FTLD-TDP but had incidental AGD (AGD3, AGD4, AGD5, AGD6). The inferior temporal gyrus was chosen for AGD to capture AGD tau while avoiding comorbid AD-related tau as much as possible. See Table S2 for patient characteristics and scoring of morphologies.

**Brain homogenate preparation and clarification.** Human brain samples were shipped from UCSF to Washington University on dry ice. Tau knock-out mouse (Jackson Labs, STOCK Maptlm1(EGFP)Klt/J) brain (male, 12 months of age) was harvested and stored at -80°C. While still frozen, 0.5 gram sections were sonicated in 5 mL TBS with Complete protease inhibitors (Roche) and phosSTOP (Roche) using an Omni-Ruptor 250 probe sonicator at 30% power for 20, 10-second cycles. Crude brain homogenates were then clarified by centrifugation at 15,000xg for 15 minutes and supernatants were frozen down at -80°C until use.

**Immunoprecipitation of tau from human samples and mouse tau knock-out brain.**

1:100 HJ9.3 and 1:100 HJ8.5 (kind gifts from the David Holtzman lab) were added to 1 mL 10% weight/volume brain homogenates freshly thawed on ice. Homogenate and antibodies were incubated overnight at 4°C with rotation. 18 hours later, 50 µL of
protein-G agarose beads (Pierce) were added and samples were again incubated overnight at 4°C with rotation. 24 hours later, samples were centrifuged at 2000xg for 3 minutes at 4°C. Supernatant was discarded and replaced with 500 µL Ag/Ab gentle binding buffer (Pierce). This centrifugation/wash step was repeated three times. After the final aspiration, proteins bound to beads were eluted using 50 µL low pH elution buffer (Pierce) by incubating at room temperature for 5 minutes. Samples were centrifuged at 2000xg for 3 minutes and supernatants were collected. This elution step was repeated to give a total volume of 100 µL. Finally, 10 µL 1 M Tris-Base pH 8.5 was added to the eluate to neutralize the elution buffer. Eluted samples were stored at -80°C until use.

Isolation of brain homogenate-derived tau RD prion strains. Monoclonal Tet Off-tau RD(LM)-YFP cells were plated at 240,000 cells/well in 12-well plates. 24 hours later, 30 µL clarified brain homogenate/70 µL OptiMEM (crude approach) or 100 µL eluted tau (IP approach) was incubated with 96 µL OptiMEM/4 µL lipofectamine-2000 for 20 minutes prior to addition to wells. 18 hours later, transduced cells were re-plated in 6-well plates. On Day 3, cells were plated sparsely in 10 cm plates (<100 cells/plate). Conditions with rounded, dying cells were noted at this time as “toxic.” On Day 12, clones containing inclusions were isolated for each condition. As many positive/mosaic clones as could be identified were picked. Differences in sample size between conditions reflect efficiency of seeding and amplification. Clones were serially passaged to confluency in 12-well, then 6-well, then 10 cm plates. At Day 30, cells were frozen down in liquid nitrogen or plated on cover slips for assessment of morphology by confocal.
2.5 RESULTS

**Homotypic seeding of tau depends on beta-sheet structure.** Amino acid sequence disparities impair cross-seeding between PrP moieties from different species, leading to “seeding barriers” (Collinge and Clarke, 2007). To test the fidelity of hetero- vs. homotypic seeding for tau, we expressed several amyloidogenic proteins and exposed them to a variety of fibrillar seeds. Prolonged expression of full-length (FL) tau can be toxic to dividing cells. Thus for tau, we expressed the aggregation-competent core, termed the repeat domain (tau RD; aa 244-372 of the 441 aa FL tau 4R2N) (Wischik et al., 1988). We generated polyclonal HEK293 cell lines stably expressing tau RD-YFP, α-synuclein-YFP, or huntingtin (htt) exon 1(Q25)-YFP (see Figure 2.2A for construct diagrams). Inclusions did not occur in any line without exposure to exogenous fibrils. However, upon transduction of fibrils (Aβ (1-42), htt exon 1 N17(Q35), α-synuclein, tau RD) using liposomes (Nekooki-Machida et al., 2009), we observed homotypic but not heterotypic seeding for each amyloidogenic protein (Figure 2.1A; Figure 2.2B for quantification), consistent with sequence-specific templating. Prior reports have indicated that in certain cases, α-synuclein aggregates can cross-seed FL tau (Giasson et al., 2003; Guo et al., 2013; Waxman and Giasson, 2011). Cross-seeding has also been reported between other amyloidogenic proteins (Ma and Nussinov, 2012). Thus, we tested this for both YFP-tagged and untagged versions of FL tau 4R1N P301S. We observed only homotypic seeding and no cross-seeding of tau by α-synuclein or any other amyloid (Figure 2.2C,D). This is consistent with sequence-specific templating, although we
cannot rule out the possibility that different amyloid conformers are capable of heterologous seeding, as has previously been reported (Guo et al., 2013).

Amyloids typically feature a cross beta-sheet conformation (Bonar et al., 1969). We exploited two proline substitutions (I277P/I308P) in tau that block its ability to enter into this quaternary form (Bergen et al., 2001) to test whether inclusion formation requires this property. Polyclonal HEK293 cell lines stably expressing tau RD-YFP with no mutations (WT); P301L/V337M (LM: pro-aggregation); ΔK280 (pro-aggregation); or ΔK280/I277P/I308P (2P: anti-aggregation) were transduced with tau RD fibrils. All formed inclusions except tau RD(2P)-YFP, confirming that beta-sheet structure is required for tau RD inclusion formation in our model system (Figure 2.1B).

**Stable inheritance of tau RD aggregates.** Seeded htt exon 1 (Ren et al., 2009), Sup35NM (Krammer et al., 2009), SOD1 (Münch et al., 2011), and α-synuclein (Bousset et al., 2013) form persistent intracellular inclusions in cultured cells. We tested this for tau RD. We transduced tau RD fibrils or buffer into polyclonal tau RD(LM)-YFP (hereafter, referred to as tau RD) cells, chosen for their superior ability to be seeded relative to tau RD(WT)-YFP, and quantified the percentage of cells with inclusions on every other passage. Transduced fibrils induced tau RD inclusions that persisted >50 days post-exposure (Figure 2.1C). We hypothesized that the aggregated state was stably inherited, since inclusion-containing cells formed local clusters (Figure 2.1D). To test this, we sparsely diluted fibril-transduced tau RD cells to isolate individual colonies. These were composed of either 100% inclusion-negative (Figure 2.1E) or 100% inclusion-positive (Figure 2.1F) cells, indicating stable inheritance of the aggregated state.
Tau RD propagates conformationally distinct strains. Only prion protein (PrP) (Birkett et al., 2001) and certain fungal prions (e.g. Sup35 [PSI+]) (Derkatch et al., 1996) unequivocally propagate distinct conformational states, or strains, in cell culture. To test the ability of tau RD to propagate distinct conformers, we diluted fibril-transduced monoclonal tau RD cells and isolated individual clones that stably propagated inclusions (Figure 2.3A). Previous work with the Sup35 protein has indicated that inclusion morphology is a proxy for biochemically distinct yeast prion strains in dividing mammalian cells (Krammer et al., 2009). We thus characterized 20 tau RD clones based on inclusion morphology, numbered in order of isolation. Most (Figure 2.4A) featured small juxtanuclear inclusions with many nuclear speckles, exemplified by Clone 9 (Figure 2.3B). Clone 10 alone propagated a single, large juxtanuclear inclusion (Figure 2.3B). We confirmed that stably propagated tau RD inclusions were amyloids as Clones 9 and 10, but not inclusion-negative Clone 1, bound X-34, a Congo red derivative that stains beta-sheet structures (Figure 2.3C).

To characterize the clones biochemically, we first used semi-denaturing detergent agarose gel electrophoresis (SDD-AGE), a method that differentiates strains based on aggregate size (Kryndushkin et al., 2003). Tau RD species from Clone 10 were larger than those propagated by Clone 9 (Figure 2.3D). Thus, the Clone 10 fibrils may not be as readily fragmented into smaller species (Tanaka et al., 2006). Next, we used sedimentation analysis to differentiate the strains (Tanaka et al., 2006). Clone 1 had entirely soluble tau RD, whereas Clones 9 and 10 had insoluble tau RD (Figure 2.3E). Clone 10 featured more soluble tau RD than Clone 9. To probe for structural differences, we used limited proteolysis as has been used previously for differentiating PrP
conformers (Bessen and Marsh, 1994). Cell lines propagating aggregates (Clones 9, 10) featured pronase-resistant species between 10 and 13 kDa as well as between 20 and 25 kDa in size (Figure 2.3F). Clone 9 produced a smear between 10-13 kDa, while Clone 10 produced a clear doublet. These studies indicated clear differences in biochemical characteristics of the clones, consistent with distinct strain conformations.

Prion strains often have different seeding efficiencies, which can result in variable incubation times in vivo (Legname et al., 2006). Thus we compared the clones, modifying a pre-existing split-luciferase complementation assay (Naik and Piwnica-Worms, 2007) for use as a tau aggregation sensor (Figure 2.2A). Clone 1 contained no seeding activity. However, inclusion-containing lines seeded robustly, especially Clone 9, which seeded more than Clone 10 (Figure 2.3G). Differences in seeding were not an artifact of cell confluency, as determined by normalizing to cell number in seeding experiments (Figure 2.4B).

Next, we compared the toxicities of Clone 9 and 10. Although Clone 9 lysate initially seeded a greater number of cells, these were rapidly eliminated relative to those induced by Clone 10 (Figure 2.3H). Furthermore, cells containing Clone 9-derived inclusions grew more slowly than those derived from Clone 10 (Figure 2.3I). While growth rate of non-transfected HEK293 cells was not affected by inoculation with Clone 9 lysate, growth of tau RD cells was impaired following the same treatment (Figure 2.4C). This was not seen for Clone 10. Finally, an LDH assay suggested that Clone 9 lysate is toxic to tau RD cells relative to a sham treatment (Figure 2.4D).
A previous study reported that tau from human brain can induce aggresome structures in vitro (Santa-Maria et al., 2012). Thus, we examined the subcellular localization of inclusions associated with Clones 9 and 10. Based on anti-vimentin stains (Figure 2.4E), electron microscopy (Figure 2.4F), and anti-γ-tubulin stains (Figure 2.4G), we conclude that juxtanuclear Clone 10 inclusions are canonical aggresomes, unlike the inclusions of Clone 9. Although polyglutamine intranuclear inclusions colocalize with PML bodies (Yasuda et al., 1999), intranuclear Clone 9 inclusions did not (Figure 2.4H). Thus, Clones 9 and 10 propagate conformationally distinct tau prion strains, with different consequences for the cell. To test the fidelity of strain inheritance, we passaged them continuously for 6 months. Inclusion morphologies (Figure 2.3J) and limited proteolysis patterns (Figure 2.3K) associated with Clones 9 and 10 were unaltered. Thus, tau RD prion strains are robust, maintaining their phenotypes indefinitely in cell culture.

**Transfer of strain phenotype to naïve cells.** To rule out an effect of cell background on strain formation, we transduced Clone 9 and 10 lysates into naïve monoclonal tau RD cells, isolating 6 colonies (A-F) for each (Figure 2.5A). We evaluated derivative clones (9C was lost in passage) by inclusion morphology (Figure 2.5B); SDD-AGE (Figure 2.5C); sedimentation analyses (Figure 2.5D, 2.6B); seeding activity (Figure 2.5E); and limited proteolysis (Figure 2.5F). In all cases, derivative clones matched their associated progenitors, indicating that tau RD prion strains are encoded by conformation, independent of cell background. Faithful templating into naïve cells also occurred after passive addition of lysates to media (Figure 2.6A), thus indicating that bypassing physiological uptake is not necessary for templating. With a Tet Off line to control tau
RD expression, we demonstrated that the aggregate-positive phenotype can be cured by stopping expression for 7 days and then restarting it (Figure 2.6C,D).

To rule out an artifact of using artificial truncated tau RD and dividing cell model systems, we examined FL tau inclusion formation in primary cortical neurons. Neurons expressing FL tau P301S-YFP formed detergent-resistant inclusions following treatment with Clone 9 or 10 but not Clone 1 or PBS (Figure 2.5G). Clone 9 seeded very robustly relative to Clone 10 (Figure 2.6E). Clone 9 lysate created inclusions throughout the soma and processes of neurons with untagged and YFP-tagged FL P301S tau, whereas Clone 10 lysate primarily seeded inclusion bodies confined to the soma (Figure 2.5, 2.6F,G).

Corroborating prior studies (Aoyagi et al., 2007; Miyasaka et al., 2001), we observed a seeding barrier between WT tau and P301 mutants (P301L, P301S). Specifically, aggregates from Clones 9 and 10, which feature both the P301L and V337M mutations, never seeded aggregation in neurons expressing FL tau WT-YFP (Figure 2.5H) and FL tau WT (no tag) (data not shown). This seeding barrier was confirmed to be asymmetric by using a panel of split-luciferase tau RD mutant pairs (Figure 2.6H), which demonstrated that WT tau RD can seed all forms of RD (WT, P301L, P301S, P301L/V337M), whereas P301 mutants cannot seed WT.

**Tau strains induce unique pathologies in transgenic tau P301S mice.** Inoculation of recombinant fibrils into transgenic P301S mice (Yoshiyama et al., 2007), which express a form of mutant tau associated with dominantly inherited tauopathy, rapidly induces pathology within weeks (Iba et al., 2013). Thus, we tested whether tau strains formed in cell culture would have similar effects. We inoculated equivalent amounts of lysate from Clones 1, 9, and 10, as well as recombinant tau RD fibrils (RF) bilaterally into the
hippocampi of 3-month old mice (Figure 2.7A). For all experiments, conditions were gender-matched (Figure 2.17). After 3 weeks, RFs induced tangle-like pathology when assessed by AT8 (Figure 2.7B,C), an antibody against FL phospho-tau, as previously reported (Iba et al., 2013). Clone 9 and 10 induced unique pathologies, whereas Clone 1 did not cause any detectable abnormality (Figure 2.7C,D). While Clone 9 induced tangle-like inclusions throughout CA1 and CA3, Clone 10 induced AT8-positive puncta in mossy fiber tracts (Figure 2.7D). Staining with MC1, an antibody against conformationally-altered tau (Jicha et al., 1997) confirmed these differences (Figure 2.8A). X-34, an amyloid dye, primarily recognized Clone 9 pathology (Figure 2.8A), although light staining was observed in CA1 of Clone 10-inoculated mice. Pathological differences could not be explained by differences in the amount of total or insoluble tau RD inoculated (Figure 2.8E,F). Injected WT mice never developed pathology (Figure 2.8B), possibly due to a seeding barrier between inoculated tau RD and WT murine tau (Figure 2.5H, 2.6E,H).

P301S mice inoculated with Clone 10 uniquely accumulated elongated Iba1-positive rod microglia (Figure 2.7E), which aligned end-to-end parallel to CA1 pyramidal axons (Figure 2.8C). Such unique coupling of rod microglia has been observed in a rodent traumatic brain injury model, and may be protective for injured axons (Ziebell et al., 2012). WT mice inoculated with Clone 9 and 10 did not feature this pathology, indicating that endogenous human P301S tau is required for this induced microglial phenotype (Figure 2.8D).

**Tau strains are stably propagated through multiple generations in mice.** Prions can be stably passaged *in vivo* (Bruce et al., 1994). Thus, we performed serial inoculation of
brain homogenates into naïve P301S mice (Figure 2.9A). Brain homogenate from WT or P301S mice inoculated with Clone 1, 9 or 10 (termed generation G0) was inoculated into naïve P301S mice (generation G1). After 28 days, brains were collected for histology and biochemistry, and the process was repeated in a second round of P301S mice (generation G2). Immunohistochemistry demonstrated identical pathology for each generation of mice: Clone 9 serial propagation induced AT8-positive, tangle-like pathology in CA1 and CA3 regions, whereas Clone 10 serial propagation induced AT8-positive puncta in the mossy fiber tracts of the hippocampus (Figure 2.9B,C, 2.10A). Clone 1 induced no pathology in any generation (Figure 2.9B, 2.10B). Homogenate from WT mice inoculated with the original cell tau strains (G0) did not produce pathology upon passage into P301S mice (Figure 2.10C). Therefore, pathology observed in G1 and G2 cannot be due to residual tau RD seeds from the original inoculum, and tau prions propagate unique phenotypes for multiple passages in vivo.

**In vivo tau strains maintain phenotypes upon passage back into cells.** To conclusively test whether tau strains are biochemically stable after passage in vivo, we isolated FL P301S tau from micro-dissected hippocampi of injected mice (G0), using a monoclonal antibody (HJ8.5) that binds an epitope present in FL tau but not tau RD (Yanamandra et al., 2013). We assessed seeding activity in G0 samples by split-luciferase complementation and inclusion counts. Only hippocampi from P301S mice (G0) injected with tau RD aggregates contained seeding activity (Figure 2.11A, 2.12A). This did not correlate with the amount of immunoprecipitated tau (Figure 2.12D). WT mouse hippocampi never seeded, regardless of the inoculum. We next tested whether the strains introduced into G0 mice could be re-isolated in tau RD cells. Scoring of single colonies
based on morphology (containing or lacking nuclear speckles) suggested that strains were unaltered following a single passage through mice (Figure 2.12B,C). To further confirm this, a single representative colony associated with each mouse was blindly selected and amplified. All G0-Clone 9 and G0-Clone 10 samples recapitulated the morphologies of the original Clones 9 and 10 (Figure 2.11B). Limited proteolysis patterns (Figure 2.11C) and seeding propensities were also identical (Figure 2.11D).

Similar experiments were performed following the third passage (G2). IPed tau from pooled G2-Clone 9 and G2-Clone 10 homogenates seeded far more strongly than G2-Clone 1 homogenates (Figure 2.11E). IgG-precipitated material did not seed (Figure 2.11E, 2.12E), and IPed tau seeded as strongly as crude homogenate in a split-luciferase assay (Figure 2.12E). Tau alone thus accounts for the seeding activity reported in these assays.

Next, we introduced IPed material from G2 mice into tau RD reporter cells and scored colonies based on morphology prior to isolation of monoclonal lines. G2-Clone 9 colonies almost exclusively featured nuclear inclusions, whereas virtually all G2-Clone 10 colonies lacked them (Figure 2.12F). The rare inclusion-positive colonies associated with G2-Clone 1 also featured nuclear inclusions, which suggested that some of the G2Clone 10 colonies containing nuclear inclusions could arise from an intrinsic P301S-derived strain. Monoclonal strains (n=12) were blindly selected for each G2 cohort. In all but one case (G2-Clone 10D), inclusion morphologies matched that of the original inoculate (Figure 2.11F, 2.12G). For both Clone 9 and 10 cohorts, 11 of 12 clones matched their parental counterpart based on both limited proteolysis (Figure 2.11G) and seeding activity (Figure 2.11H). Intriguingly, the two outliers (G2-Clone 9G, G2-Clone
10D) had identical proteolysis patterns and seeding ratios, which were unique from those of all other clones. We speculate that these clones result from an intrinsic strain within 4-month P301S mice. We conclude that tau prion strains are stable across numerous passages through cells and animals.

**Spread of tau pathology to distant, synaptically connected regions.** After inoculation with recombinant tau fibrils, pathology can develop in synaptically connected regions (Iba et al., 2013). Our preliminary observations indicated that serial inoculations with Clone 9 induced pathology in the ipsilateral entorhinal cortex (EC) (*data not shown*). To test for spread, we performed a final inoculation (G3) into the left hippocampus of P301S mice. After five weeks, G3-Clone 9 mice had AT8-positive pathology in regions that project to or from the hippocampus (Figure 2.13A) including ipsilateral and contralateral EC, retrosplenial cortex (RSp), and contralateral hippocampus (Figure 2.13B-D, 2.14A) (Andersen, 2007; Neves et al., 2008; van Groen et al., 2003). Ipsilateral EC had robust pathology in layers II/III, whereas contralateral EC pathology occurred in deeper cortical layers, suggesting spread along defined anatomical connections (van Groen et al., 2003). Furthermore, pathology was observed in ipsilateral subiculum and dentate gyrus (Figure 2.14B). In contrast, G3-Clone 1 brain did not show AT8-positivity above baseline (Figure 2.13B,D). Overt spread was not observed in G3-Clone 10 mice (*data not shown*), perhaps due to its decreased seeding ability (Figure 3G, 2.6E). A heat-map summarizes the brain regions with enhanced AT8-positive pathology in G3-Clone 9 mice (Figure 2.13C).

These results agree with previous work suggesting that seeded intracellular amyloids spread along discrete neural networks (de Calignon et al., 2012; Iba et al., 2013; Liu et
We cannot completely exclude the possibility that this was due to trans-synaptic spread of inoculum.

**Intra-patient and inter-disease phenotypic diversity in the tauopathies.** It has been hypothesized that conformationally distinct tau prion strains may be associated with individual tauopathies (Clavaguera et al., 2013b; Frost and Diamond, 2010), and a recent study found that inoculation of transgenic human tau mice with brain homogenates from patients with different tauopathies recapitulates certain pathological features of the diseases (Clavaguera et al., 2013a). To examine whether inclusion morphology is a reasonable indicator of distinct strains, we first used our cell model to examine brain homogenates from three individuals with clinically distinct, pathologically verified tauopathies (all patient samples obtained from the Neurodegenerative Disease Brain Bank at UCSF): Alzheimer’s disease (AD), corticobasal degeneration (CBD), and argyrophilic grain disease (AGD). We transduced immunoprecipitated (IP) or crude (CR) homogenate into the monoclonal Tet Off HEK293 cell line (Figure 2.15A), used for its relatively high tau RD expression and greater seeding efficacy, and characterized resulting colonies morphologically and biochemically (Figure 2.16A-I). Each brain induced a unique inclusion morphology, independent of the transduction method (IP vs. CR) (Figure 2.16A-D). By analyzing three representative clones derived from each brain by sedimentation analysis (Figure 2.16E), seeding (Figure 2.16F,G), and limited proteolysis (Figure 2.16H,I), we concluded that morphology reliably differentiates biochemically distinct strains. Next, we expanded our analysis to include IPed tau from patients with AD (n=6); AGD (n=6); CBD (n=6); Pick’s disease (PiD, n=5); and progressive supranuclear palsy (PSP; n=6) (Figure 2.18). Excepting PiD, a three-repeat tauopathy,
these are predominantly four-repeat (AGD, CBD, PSP) or mixed-repeat (AD) tauopathies that differ in the morphology and distribution of neuronal and glial tau inclusions (Lee et al., 2001). We transduced IPed tau from each sample into the monoclonal Tet Off cell line and isolated clones with inclusions (Figure 2.15A). We identified six morphological phenotypes: 1) No seeding; 2) Toxic (all cells with inclusions died and clones could not be isolated); 3) Mosaic (unstable prion strain); 4) Ordered; 5) Disordered; 6) Speckles (Figure 2.15B). We blindly scored all clones based on tau RD inclusion morphology. This revealed distinct strain compositions across the diseases (Figure 2.15C). AD patient samples revealed remarkable homogeneity, suggesting a predominant strain. Other disorders revealed inter-patient variation. Some patients featured homogeneous strain composition (e.g. certain patients with AGD, PSP), whereas others exhibited considerable heterogeneity. With few exceptions (e.g. AD1-AD4; AGD2; CBD5; PiD3), most patient samples produced two or more strains. The range of phenotypes associated with single patients suggests a diversity of patient-derived tau prion strains. Since the cell-based strain isolation system can likely amplify only a subset of strains, these data suggest that a disease-associated ensemble or “cloud” of conformations exists within individual patients. Nevertheless, certain tauopathies can be differentiated by their strain composition.
2.6 DISCUSSION

Summary. Many papers describing “prion-like” behavior of proteins associated with neurodegenerative diseases have been published in the last several years. In the case of tau, fibrils transmit its aggregated state from the outside to the inside of a cell (Frost et al., 2009a; Holmes et al., 2013a), suggesting that this mechanism could account for the stereotyped progression of tauopathies. This model of disease was subsequently supported in vivo with reports of trans-synaptic spread of pathology (de Calignon et al., 2012; Kim et al., 2010; Liu et al., 2012) and protein-only induction of tau inclusions (Iba et al., 2013). Work with other intracellular amyloids (Desplats et al., 2009; Holmes and Diamond, 2012; Münch et al., 2011; Ren et al., 2009) has suggested that prion-like transmission can explain the progression of many neurodegenerative diseases.

Whether or not various non-infectious amyloids are “true” prions has become a contentious subject of debate. Some define prions as being capable of inter-organism transmission of pathology and by the ability to survive freely in the environment (Aguzzi and Rajendran, 2009). To date, there exists no evidence that this definition can be applied to proteins other than PrP and serum amyloidosis A protein (Solomon et al., 2007). This restrictive definition, based on early research into prion diseases such as kuru and scrapie, potentially ignores a rich biology that mechanistically unites many common diseases. Importantly, we now know that the vast majority of human prion diseases have non-infectious etiology, and that their great phenotypic heterogeneity can be attributed to strains (Collinge and Clarke, 2007). With respect to prion-like intracellular amyloids in
humans, recent data indicate that homogenates from distinct tauopathies may reproduce certain pathological features of the diseases in transgenic mice, which is consistent with strain behavior (Clavaguera et al., 2013a). Other studies explicitly suggest the existence of α-synuclein strains, based on the production of different α-synuclein conformers in vitro (Bousset et al., 2013; Guo et al., 2013; Sacino et al., 2013). However, to account for phenotypic diversity at a systems level, a prion strain must replicate with remarkable reliability for extended periods of time. A stringent test of this is to ensure that the strain is stable, isolatable, and replicates its phenotype through living systems with high fidelity (Bruce et al., 1994; Prusiner, 1998). Until now, these characteristics have not been linked to a mammalian protein other than PrP. Based on these criteria, our data strongly suggest that tau should be considered a bona fide prion. Fittingly, we also find that different tauopathies are associated with different strains. This has direct implications for understanding the phenotypic diversity of tauopathies.

**Tau as a prion in cell culture and mice.** We began this work by establishing a monoclonal HEK293 cell line that stably expresses the tau repeat domain fused to YFP. In the absence of tau aggregate exposure, these cells propagate only tau RD monomer (“naïve” cells). Induction of aggregation with recombinant fibrils, however, created clonal lines (Clone 9 and Clone 10) that indefinitely propagate unique aggregate structures, or strains, from mother to daughter cells. These strains differ with respect to inclusion morphology, aggregate size, sedimentation profile, seeding capacity, protease digestion patterns, toxicity, and subcellular localization. Importantly, these properties are cell-independent, as we re-created the strains by protein transfer into naïve cells.
Furthermore, the distinct inclusion morphologies we observed may represent specific cellular responses to different aggregate conformations, consistent with their unique patterns of compartmentalization. The cell culture system established here may thus prove useful to detect, propagate and characterize additional tau prion strains, as well as to understand the cellular mechanisms that govern strain replication, subcellular localization, degradation, and toxicity.

*In vivo,* we found that strains 9 and 10 induce unique pathological phenotypes in transgenic P301S mice. Moreover, Clone 10 lysate uniquely results in the formation of rod-shaped microglia, which indicates that distinct tau conformers initiate different physiological responses *in vivo.* More remarkably, we report that the morphological phenotypes breed true through multiple generations of mice, the first time this has been observed for an amyloid other than PrP. We recognize that pathological phenotypes can be prone to bias in detection. Thus, we passaged strains back to naïve tau RD-YFP cells, conclusively demonstrating the robust inheritance of tau conformations. This data also indicates that the repeat domain is sufficient to encode strains that are unaltered by templating of their structure to FL tau. Therefore, the reported cell model is useful for detecting and propagating physiologically relevant tau prion strains. Finally, using unilateral inoculation of Clone 9 lysate, we show that tau aggregation propagates along known anatomical connections, supporting conclusions of previous studies (de Calignon et al., 2012; Iba et al., 2013; Liu et al., 2012). More importantly, however, these cellular and *in vivo* studies indicate for the first time that a mammalian protein amyloid other than PrP templates itself with high fidelity through living systems.
Tau prion strains in human tauopathy brains. Knowing that tau acts as a prion in experimental models, we examined whether this concept could explain phenotypic diversity observed in tauopathies. Brain samples from three patients with distinctive tauopathies induced diverse self-propagating tau prion strains in culture. Our initial work with these novel strains indicated that inclusion morphology is a reliable surrogate for more labor-intensive biochemical characterization. This led us to assess the morphological phenotypes of tau strains derived from numerous patients (n=29) across a spectrum of tauopathies (AD, AGD, CBD, PiD, PSP). Each of the diseases was associated with several cellular inclusion morphologies, although certain diseases (AD, CBD, PiD) are more homogeneous than others (PSP, AGD). It is noteworthy that AD pathology is characteristically more uniform than other tauopathies (Duyckaerts et al., 2009; Feany et al., 1996), and the tau strains isolated from AD brains were by far the most homogeneous. The isolation of multiple conformers from individuals suggests that a tau aggregate ensemble exists within each person, and that standard methodologies (e.g. histopathology, inoculation into mice, protease digestion) will be insufficient for a nuanced understanding of this conformational complexity. Similar to what has been reported for PrP amyloids (Collinge and Clarke, 2007; Li et al., 2010) and virus quasispecies (Eigen, 1996), we speculate that these clouds of tau conformers are prone to selective pressures at the cellular level, which may have implications for therapies that target extracellular tau (Holmes et al., 2013a; Yanamandra et al., 2013).

Although we have now succeeded in categorizing multiple distinct strains, the cell-based isolation method can only detect those that successfully template to tau RD-YFP and propagate without overt cellular toxicity. The inability to re-select Clone 9 derivatives in
the Tet-Off background illustrates this problem. On the other extreme, strains that do not propagate with high fidelity may be lost prior to clonal selection. For example, the strains present in several AGD and PiD samples were not stable in cell culture, making detailed characterization of these strains difficult using our model system. Furthermore, it is likely that seeding barriers between tau from patient brain (consisting of various tau isoforms and post-translationally modified species) and tau RD in cell culture limits the strains we can detect. Our observation of an asymmetric seeding barrier between P301 mutants and WT tau underscores this limitation, as does recent work indicating similar barriers between three-repeat (3R) and four-repeat (4R) tau (Dinkel et al., 2011). Despite some limitations, the model system presented here has many advantages over standard animal inoculations, as it is less resource-intensive and can parse multiple conformations from a single isolate. Finally, knowledge of the existence of multiple strains in vivo may allow us to characterize them on molecular terms and diagnose patients with much greater precision, possibly by determining structures and conformational epitopes specific to individual diseases. This could help facilitate therapeutic strategies tailored toward the underlying protein pathology.

**Expanding the spectrum of prion diseases.** Our data, along with cell culture (Holmes and Diamond, 2012), pathological (Clavaguera et al., 2013b) and imaging (Greicius and Kimmel, 2012) studies are consistent with the model of cell-cell “transmission” of neurodegenerative diseases throughout the nervous system. Our finding of multiple self-propagating conformations in experimental and patient-derived tau preparations suggests that tau should be defined as a prion, because it encodes self-catalyzing conformational information that it propagates indefinitely with high fidelity. Importantly, however, there
is no evidence to suggest that AD or other tauopathies are infectious in the classical sense, as they are not known to be communicable between individuals. The infectious property of PrP$^{Sc}$ might reflect its anomalous biochemical stability or expression profile, whereas a host of other cell biological and biophysical properties, especially the ability to encode self-propagating conformers, will more appropriately unify the growing family of “prion-like” proteins. Indeed, the vast majority (>95%) of human prion diseases appear to be genetic or sporadic, indicating that infectivity should not be a restrictive criterion. We predict that strains associated with distinct clinical phenotypes will also be identified for synucleinopathies and ALS/FTLD spectrum disorders, both of which feature diversity in pathological presentation (Halliday et al., 2011; Van Langenhove et al., 2012). Understanding disparate amyloid neurodegenerative diseases in light of this model should create new possibilities for common diagnostic and therapeutic approaches.
2.7 FIGURES

Figure 2.1. Homotypic seeding produces stably propagated tau RD inclusions. (A) Polyclonal HEK293 lines stably expressing YFP-tagged tau RD, α-synuclein, or htt exon1(Q25) were transduced with buffer, or fibrils of Ab, Htt, α-syn, or tau RD. Cells were DAPI-stained on Day 6. Only homotypic seeding occurred. (B) Polyclonal HEK293 lines stably expressing tau RD-YFP with no mutations (WT), ΔK280 (pro-aggregation), ΔK280/I277P/I308P (2P; anti-aggregation), or P301L/V337M (LM; pro-aggregation) were transduced with either buffer or tau RD fibrils. Upon fibril transduction, all form inclusions, except for 2P. (C) Tau RD(LM)-YFP cells transduced with either buffer or tau RD fibrils were passaged every two days. On every other passage, the percentage of cells with inclusions was quantified (n=10 fields, each with 150+ cells per condition). Inset highlights inclusion-positive cells at later time points. Error bars represent S.E.M. (D) At Day 50 following exposure to fibrils, inclusion-positive cells were visible. (E-F) At Day 3 following exposure to fibrils, tau RD(LM)-YFP cells were diluted sparsely on coverslips and grown for 8 days. Colonies were either 100% inclusion-negative (E) or 100% inclusion-positive (F).
Figure 2.1

RESULTS

Homotypic Seeding of Tau Depends on Beta-Sheet Structure

Amino acid sequence disparities impair cross-seeding between PrP moieties from different species, leading to "seeding barriers" (Collinge and Clarke, 2007). To test the fidelity of hetero- versus homotypic seeding for tau, we expressed several amyloidogenic proteins and exposed them to a variety of fibrillar seeds. Prolonged expression of full-length (FL) tau can be toxic to dividing cells. Thus for tau, we expressed the aggregation-competent core, termed the repeat domain (tau RD; aa 244–372 of the 441 aa FL tau 4R2N) (Wischik et al., 1988). We generated polyclonal HEK293 cell lines stably expressing YFP-tagged tau RD, α-synuclein, or huntingtin (htt) exon1(Q25) were transduced with buffer, or fibrils of Ab, Htt, α-syn, or tau RD. Cells were DAPI-stained on day 6. Only homotypic seeding occurred. See Figure S1A for construct diagrams, Figure S1B for quantification, and Figures S1C and S1D for similar homotypic seeding with full-length (FL) 4R1N tau P301S.

(B) Polyclonal HEK293 lines stably expressing tau RD-YFP with no mutations (WT), ΔK280 (proaggregation), ΔK280/I277P/I308P (2P; anti-aggregation), or P301L/V337M (LM; proaggregation) were transduced with either buffer or tau RD fibrils. Upon fibril transduction, all form inclusions, except for 2P.

(C) Tau RD(LM)-YFP cells transduced with either buffer or tau RD fibrils were passaged every two days. On every other passage, the percentage of cells with inclusions was quantified (n = 10 fields, each with 150+ cells per condition). Inset highlights inclusion-positive cells at later time points. Error bars represent SEM.

(D) At day 50 following exposure to fibrils, inclusion-positive cells were visible.

(E and F) At day 3 following exposure to fibrils, tau RD(LM)-YFP cells were diluted sparsely on coverslips and grown for 8 days. Colonies were either 100% inclusion-negative (E) or 100% inclusion-positive (F).
Figure 2.2. Constructs and lack of cross-seeding. (A) Diagrams illustrating constructs used in this study. (B) Quantification of seeding efficiency of fibril and cell line pairs. Ten fields, each with 150+ cells, were quantified per condition. Percent positive averages are followed by the S.E.M. (C) Polyclonal HEK293 lines stably expressing FL tau 4R1N P301S (with or without YFP tag) were transduced with buffer, Ab, Htt, α-syn, or tau RD fibrils. Cells were stained for phospho-tau (AT8) on Day 6. Only homotypic seeding was observed. (D) Quantification of seeding efficiency of fibrils transduced into FL tau P301S cell lines. Ten fields, each with 150+ cells, were quantified. Percent positive averages are plotted. Error bars represent S.E.M.
Figure 2.2

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<th>Tau RD</th>
<th>Htt N17-Q35</th>
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**A**

Full-Length Tau (4R2N)

- **RD(WT)-YFP**
  - [K P I V]
  - YFP
- **RD(L277P/DK280/I308P)-YFP ("2P")**
  - [F P P V]
  - YFP
- **RD(DK280)-YFP**
  - [I P I V]
  - YFP
- **RD(P301L/V337M)-YFP ("LM")**
  - [I K L M]
  - YFP

**Alpha Synuclein-YFP**

- [IK P I V]
  - YFP

**Htt Exon1 Q25-YFP**

- [IK L I M]
  - YFP

**RD(LM)-Cluc**

- [IK L I M]
  - CBG-Cluc

**RD(LM)-Nluc**

- [IK L I M]
  - CBG-Nluc

**B**

<table>
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**C**

Transduced Fibrils

- **Buffer**
- **Aβ**
- **Htt**
- **αSyn**
- **Tau RD**

**D**

% Cells With AT8-Positive Inclusions

- **FL Tau P301S**
- **FL Tau P301S -YFP**

- **AT8**
Figure 2.3. Generation of stably inherited tau RD prion strains. (A) A monoclonal HEK293 line stably expressing tau RD(LM)-YFP (hereafter referred to as tau RD) was transduced with tau RD fibrils. At Day 3, cells were diluted sparsely in a 10 cm dish. At Day 12, inclusion-positive colonies were identified and picked, amplifying to confluency in separate 10 cm dishes. At Day 30, cells were re-plated for confocal analysis or harvested for subsequent experiments. (B) Confocal analysis of morphologically distinct tau RD prion strains. Clone 1 does not contain inclusions. Clone 9 contains nuclear speckles and a small juxtanuclear inclusion. Clone 10 features one very large juxtanuclear inclusion and no nuclear speckles. (C) Clones 1, 9 and 10 were stained with X-34, an amyloid dye. X-34 staining is only observed in Clone 9 and Clone 10, indicating that the propagated aggregates are amyloids. (D) SDD-AGE demonstrates that Clone 10 features larger aggregates than Clone 9. (E) Sedimentation analysis was performed on Clones 1, 9, and 10. Pellet (P) was isolated from supernatant (S) by ultracentrifugation. For Clones 9 and 10, supernatant was loaded at a 3:1 ratio to pellet and total (T) to allow clear detection; Clone 1, a 1:1 ratio. Clone 1 has all tau RD in the supernatant, whereas Clone 9 has almost all tau RD in the pellet. Clone 10 has mixed solubility. (F) Limited proteolysis (pronase) digests all tau RD in Clone 1, but reveals protease-resistant tau RD peptides between 10 and 13 kDa as well as between 20 and 25 kDa in Clone 9 and 10. Unlike Clone 9, Clone 10 digestion produces a doublet, consistent with a distinct conformation. (G) A split-luciferase assay reports differential seeding efficiency of tau RD prion strains. A polyclonal HEK293 line expressing both tau RD-CLuc and tau RD-Nluc was transduced with lysate from the three clones. Clone 1 does not seed
aggregation. Clone 9 seeds robustly, whereas Clone 10 seeds significantly less. Averages of four separate experiments are shown, each read in quadruplicate 48 hours post-transduction (error bars = S.E.M, * = p<0.05, **** = p<0.0001). (H) Inclusion elimination rates differ between clones. After transduction with lysate from Clone 9 or 10, the percentage of cells containing inclusions was quantified on days 4, 17, and 30 (n=10 fields, each with 150+ cells per condition). Cells with inclusions derived from Clone 9 are eliminated more rapidly from the population. Error bars = S.E.M, **** = p<0.0001. (I) Clone 9-transduced cells grow more slowly. After transduction of stable cells, colonies with inclusions derived from Clone 9 have fewer cells than colonies with inclusions derived from Clone 10. Colonies without inclusions have identical cell numbers (error bars = S.E.M, **** = p<0.0001). (J) Clones 1, 9, and 10 maintain distinctive morphologies after 6 months in culture. (K) Structural characteristics (limited proteolysis digestion patterns) of strains are propagated with high fidelity over six months.
**Figure 2.3**

(A) A monoclonal HEK293 line stably expressing tau RD(LM)-YFP (hereafter referred to as tau RD) was transduced with tau RD fibrils. At day 3, cells were diluted sparsely in a 10 cm dish. At day 12, inclusion-positive colonies were identified and picked, amplifying to confluency in separate 10 cm dishes. At day 30, cells were replated for confocal analysis or harvested for subsequent experiments.

(B) Confocal analysis of morphologically distinct tau RD prion strains. Clone 1 does not contain inclusions. Clone 9 contains nuclear speckles and a small juxtanuclear inclusion. Clone 10 features one very large juxtanuclear inclusion and no nuclear speckles. See Figure S2A for other clones.

(C) Clones 1, 9, and 10 were stained with X-34, an amyloid dye. X-34 staining is only observed in clone 9 and clone 10, indicating that the propagated aggregates are amyloids.

(legend continued on next page)
Figure 2.4. Morphologies of all clones and strain-specific properties of Clones 9 and 10. (A) Confocal images of representative cells from all 20 clones. Nuclear aggregates are present in all clones except Clone 1 (no inclusions) and Clone 10 (one large juxtanuclear inclusion with no nuclear inclusions). (B) Normalization of luminescence based on Hoechst stain reveals that differences in seeding ratios between Clones 9 and 10 are not due to differences in cell confluency (error bars = S.E.M). (C) Lysates were transduced into either non-transfected (NT) cells or those stably expressing tau RD(LM)-HA. Clone 1 and 10 lysate do not affect cell numbers as measured by Hoechst stain at Day 4. However, Clone 9 lysate significantly reduces the number of cells in the tau RD(LM)-HA background, revealing that Clone 9’s toxicity is dependent on expression of tau RD(LM) (error bars = S.E.M, **** = p<0.0001). (D) Tau RD(LM)-HA cells were transduced with lysates from Clones 1, 9, and 10. LDH levels in the media were measured at Day 4 and compared to those of lysed cells (toxic control). Clone 9 is especially toxic relative to sham-treated cells (error bars = S.E.M, ** = p<0.01). (E) Clones 9 and 10 were stained for vimentin, which forms a cage around aggresomes. Vimentin cages are observed around the large juxtanuclear inclusion of Clone 10 cells but not around the inclusion of Clone 9, indicating the Clone 10 aggregates form canonical aggresomes, whereas Clone 9 inclusions do not. (F) Transmission electron microscopic analysis of Clone 10 cells reveals large aggresome structures indenting the nuclear envelope. Clone 9 cells feature small, round, nuclear inclusions. Immuno-EM confirms that both types of inclusions contain tau RD. (G) Co-localization of juxtanuclear Clone 10 but not Clone 9 inclusions with γ-tubulin, a marker of the microtubule-organizing center (MTOC), suggests that only the former are true
aggresomes. Arrowheads point to juxtanuclear tau RD inclusions, whereas arrows point to MTOCs. (H) Staining of nuclear PML bodies, organizing centers for degradation of nuclear aggregates, demonstrates that Clone 9 nuclear inclusions are in a unique compartment.
Figure 2.4
Figure 2.5. Tau RD aggregates transfer strain conformations into naïve cells. (A) Lysates from Clones 9 and 10 were transduced into naïve tau RD-YFP cells and monoclonal inclusion-containing cells were isolated and amplified. Six secondary clones were generated for each condition, but one (Clone 9C) failed to amplify. (B) Morphologies of primary clones are maintained in secondary cell lines. (C) SDD-AGE of lysates from both primary and secondary clones demonstrates similar aggregate sizes in secondary clones relative to the primary ones. A line separates gels run separately. (D) Sedimentation analysis was performed. Secondary clones feature similar sedimentation patterns to the clones from which they were derived. (E) Split-luciferase complementation demonstrates similar seeding efficiencies in secondary lines vs. parental lines. Averages of four separate experiments are shown, each read in quadruplicate 48-hours post-transduction of lysate (error bars = S.E.M, **** = p<0.0001). (F) Limited proteolysis shows that all Clone 10 derivatives feature a doublet whereas Clone 9 derivatives are associated with an unresolvable band between 10 and 13 kDa. Clone 9 derivatives feature a more resistant band between 20 and 25 kDa. (G) Lysates from Clones 9 and 10, but not Clone 1, induce detergent-resistant FL tau P301S-YFP species, which co-localize with AT8 (red) in primary cortical neurons. Clone 9 induces tangle-like structures throughout the soma and neuritic processes. Clone 10 primarily seeds punctate-like structures in the soma. (H) Clone 9 and Clone 10 lysates containing tau RD(P301L/V337M)-YFP, do not seed inclusion formation in neurons expressing WT FL tau.
Figure 2.5

(A) Lysates from clones 9 and 10 were transduced into naive tau RD-YFP cells and monoclonal inclusion-containing cells were isolated and amplified. Secondary clones were generated for each condition, but one (clone 9C) failed to amplify.

(B) Morphologies of primary clones are maintained in secondary cell lines. See also Figure S3A, which demonstrates that this templating of morphology is not dependent on liposome-mediated transduction of lysate.

(legend continued on next page)
Figure 2.6. Templating in absence of liposomes, reversibility of aggregated phenotype, and seeding barriers between tau with different point mutations. (A) Tau RD prion strains template themselves into the inside of the cell without the use of liposomes. 30 µg of clarified Clone 9 and Clone 10 lysate were passively added to separate populations of naïve tau RD-YFP cells. Three monoclonal cell lines were isolated for each. Morphologies of primary clones were recapitulated in these lines (P = passive origin). (B) Three blots were used to construct panels in Figure 2.5D (T=total, S=supernatant, P=pellet). (C) Clone 10 was transduced into a new monoclonal Tet Off-tau RD-YFP HEK293 cell line, and monoclonal derivatives were isolated as described in Figure 2.5A. Tau RD expression was turned off for the indicated period of time (T=days), prior to re-plating in normal media for 48 hours. (D) Quantification of inclusion clearance (n=10 fields, each with 150+ cells, per time point). Repression of tau RD-YFP transcription for 7 days is required to revert all cells to the soluble state (error bars represent S.E.M.). (E) Zoomed-out views of fixed and Triton X-extracted primary neurons expressing either FL tau P301S-YFP or FL tau WT-YFP reveal that tau RD strains seed mutant but not WT tau aggregation (green=YFP, red=AT8). Furthermore, Clone 9 seeds FL tau P301S more robustly than Clone 10, consistent with split-luciferase complementation data (Figure 2.3G). (F) Clone 9 and 10 lysates seed the formation of phosphorylated (AT8-positive, red), detergent-resistant, untagged FL tau P301S species in primary neurons. Similar to FL tau P301S-YFP, Clone 10 inoculation results in detergent-resistant species confined primarily to the soma; Clone 9, throughout the neuron. (G) Clone 9 lysate induces tangle-like insoluble tau inclusions in neuritic processes of both neurons expressing YFP-tagged and untagged versions of FL tau.
P301S. These structures are much less common in Clone 10-inoculated neurons (*data not shown*). (H) Polyclonal HEK293 lines expressing different pairs of split-luciferase-tagged tau RD mutants were generated and transduced with lysate from Clones 1, 9, or 10; 1 µM recombinant tau RD fibrils; or 50 µg brain homogenate from aged tau P301S transgenic mice. Whereas all treatments besides Clone 1 seed tau RD featuring P301L, P301S, or P301L/V337M (“LM”) mutations, only recombinant tau RD WT fibrils seed tau RD WT. This demonstrates that there is an asymmetric seeding barrier between tau containing and lacking P301 mutations. No treatment seeds tau RD 2P, consistent with this mutant’s inability to form beta-sheet structure and amyloids. Averages of four separate experiments are shown, each read in quadruplicate 48-hours post-transduction of lysate (error bars = S.E.M).
Figure 2.6
Figure 2.7. Clone 9 and 10 induce unique tau and microglia pathology in P301S mice. (A) Lysates (10 µg total protein) were injected bilaterally into the hippocampi of 3-month P301S and wild-type (WT) mice. 21 days post-injection, left hemispheres were collected for histology; right hemispheres for homogenization. (B) Recombinant tau RD fibrils (RF) induce tangle-like, AT8-positive tau pathology near the injection site in CA1 (scale bars: hippocampus – 1 mm; inset – 100 µm). (C) Quantification of tangle-like, AT8-positive cell bodies within the hippocampus (CA1 and CA3) of WT and P301S mice. P301S mice injected with Clone 9 lysate have significantly more AT8-positive cell bodies than those injected with Clone 1, Clone 10, or RF (error bars = S.E.M., * = p<0.05, ** = p<0.01). WT mice do not develop pathology after injection. (D) P301S mice were inoculated with Clone 1, Clone 9, or Clone 10 lysate. Representative whole hippocampus images are shown with the corresponding CA3 z-stacks. Arrowheads in Clone 9 CA3 insets highlight an AT8-positive cell body that can be seen throughout both z-stack images. The arrow and arrowhead in Clone 10 CA3 insets each represent a different AT8-positive puncta that is visible in only one z-stack plane (scale bars: hippocampus – 1 mm; CA3 – 100 µm; CA3 inset and AT8 IF – 25 µm; n=3-4 per clone). (E) Iba1 staining of microglia in CA1 of inoculated P301S mice indicates that only Clone 10 induces the formation of rod microglia, which extend highly polarized processes into CA1.
Figure 4. Clone 9 and 10 Induce Unique Tau and Microglia Pathology in P301S Mice

(A) Lysates (10 mg total protein) were injected bilaterally into the hippocampi of 3 month P301S and WT mice. At 21 days postinjection, left hemispheres were collected for histology and right hemispheres for homogenization. See Table S1 for description of mice used in all experiments.

(B) Recombinant tau RD fibrils (RF) induce tangle-like, AT8-positive tau pathology near the injection site in CA1 (scale bars represent hippocampus – 1 mm and inset – 100 µm).

(legend continued on next page)
Figure 2.8. Tau RD prion strains induce distinct pathologies in transgenic P301S but not WT mice. (A) Inoculated P301S mice were stained for conformationally-altered tau (MC1) or amyloid (X-34). MC1 staining corroborates morphologies of AT8-positive structures. Strong X-34 positivity is only seen in Clone 9-inoculated mice, although weak signal is seen in the CA1 region of Clone 10-inoculated animals. (B) WT littermates were injected with Clone 1, Clone 9, or Clone 10 lysate. No induced AT8-positive pathology was evident. Representative examples are shown. (C) Rod microglia, which are exclusive to Clone 10-inoculated P301S mice, form columns perpendicular to the cell layer of CA1. (D) Rod microglia are absent in all cohorts of inoculated WT mice, indicating that their formation is dependent on seeding of intracellular FL tau P301S by Clone 10 aggregates. (E) Amounts of insoluble Clone 9 and Clone 10 tau RD do not differ significantly (densitometric quantification, n=4 measurements, error bars = S.E.M.). (F) Total tau RD does not differ between the three cell lines used to inoculate mice (densitometric quantification, n=4 measurements, error bars = S.E.M.).
Figure 2.8
Figure 2.9. Tau strains passage stably through multiple generations of P301S mice.

(A) Lysates (10 µg protein) were injected bilaterally into the hippocampi of 3-month P301S mice (Generation 0/G0). 21 days post-injection, brains were collected for histology and homogenization. Hippocampal homogenate (10 µg) was then bilaterally inoculated into a new round of 3-month P301S mice (Generation 1/G1) followed by a 28-day incubation before the process was repeated for a new cohort of 3-month P301S mice (Generation 2/G2). At G0 and G2, hippocampal homogenates were IPed (anti-tau 8.5; epitope = aa 25-30; outside RD region) and inoculated into the original tau RD-YFP line to test the fidelity of strain inheritance (G0 and G2 clones). For each cohort, n=3-4 animals. (B-C) AT8 staining (DAB = B and immunofluorescence = C) reveals that the morphological phenotypes of phosphorylated tau inclusions breed true through multiple generations of tau P301S mice.
Figure 2.9

Neuron Tau Prion Strains in Cells, Mice, and Patients

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Figure 2.10. Whole hippocampi used to generate images in Figure 2.9 and elimination of seeding following passage through non-transgenic mice. (A) Images of whole hippocampi for representative mice inoculated with indicated lysates/homogenates. Boxes indicate regions that were highlighted in Figure 2.9. (B) AT8 immunofluorescence of CA1 and CA3 regions for serial inoculation of Clone 1. (C) Residual tau RD-YFP seeds do not account for passage of strain phenotypes through multiple generations of mice as inoculated WT mice (Generation 0), which do not form insoluble FL tau, do not seed pathology when passaged into Generation 1 P301S mice.
Figure 2.10
Figure 2.11. Strains transfer faithfully to cell culture after passage through Generation 0 and Generation 2 mice. (A) IPed material was transduced into tau RD-YFP cells prior to passage onto coverslips. At 96 hours, cells were fixed. Only Clone 9-, Clone 10-, and RF-inoculated mice seed inclusions robustly. WT mouse homogenates never seed aggregation. Ten fields, each with 100+ cells, were analyzed per brain, and averages were collapsed within cohorts (error bars = S.E.M., **** = p<0.0001). (B) Inclusion morphologies are maintained following passage through P301S mice (G0). IPed FL tau from individual P301S mice inoculated with Clone 9 or Clone 10 was transduced into tau RD-YFP cells, and a single representative clone per mouse was isolated and amplified. All G0-derived clones continue to propagate the original phenotypes. (C) Limited proteolysis reveals that G0 clones feature similar banding patterns to the original parental lines, with G0-Clone 10 featuring a doublet between 10-13 kDa (vs. smear for G0-Clone 9) and a band between 20 and 25 kDa that is slightly smaller than G0-Clone 9 bands. (D) Split-luciferase complementation demonstrates similar seeding efficiencies in G0 clones relative to original parental lines. Averages of four separate experiments are shown, each read in quadruplicate 48-hours post-transduction of lysate (error bars = S.E.M., **** = p<0.0001). (E) IPed material from pooled G2 mice was transduced into naïve tau RD-YFP cells prior to passage onto coverslips. At 96 hours, cells were fixed. Seeding of inclusion formation is significantly greater for G2-Clone 9 and G2-Clone 10 mice than G2-Clone 1 mice. G2-Clone 1 tau induces inclusions on rare occasions (~1% of cells). Seeding is specific to tau as IgG-precipitated material never seeds. Ten fields, each with 150+ cells, were analyzed per condition (error bars = S.E.M., * = p<0.05, **** = p<0.0001). (F) Inclusion
morphologies are maintained following passage through three generations of mice. IPed full-length tau from pooled G2 homogenates was transduced into tau RD-YFP cells, and 12 clones per cohort were isolated. Representative examples are shown. The two clones boxed in red feature similar limited proteolysis digestion patterns and seeding ratios to each other, which are unique from all 22 other clones. (G) Limited proteolysis reveals that G2 clones feature similar banding patterns to their parental lines, with G2-Clone 10 featuring a doublet between 10-13 kDa (vs. smear for G2-Clone 9) and a band between 20 and 25 kDa that is slightly smaller than G2-Clone 9 digests. Two clones (boxed in red), one for each cohort, are unique in featuring bands at 15 and 25 kDa. (H) Split-luciferase complementation demonstrates similar seeding efficiencies in G2 clones relative to original parental lines. Seeding ratios were averaged across clones, each of which was read in quadruplicate 48-hours post-transduction of lysate (error bars = S.E.M, **** = p<0.0001). Boxed in red are two outlier clones (9G and 10D), which also feature unique inclusion morphologies and limited proteolysis digestion patterns.
Figure 2.11

(A) % Cells with Inclusions

(B) Origin of IPed 4R1S P301S Tau

(C) MW (kDa)

(D) Relative Luminescence

(E) % Cells with Inclusions

(F) Pooled Clone 9

(G) Pooled Clone 10

(H) Unique Strains

IP: 8.5 (Tau) IgG

= Strains with unique proteolysis pattern and seeding efficiency

MW (kDa)

MW (kDa)

Relative Luminescence

MW (kDa)

MW (kDa)

MW (kDa)

MW (kDa)

MW (kDa)

MW (kDa)
Figure 2.12. Strain phenotypes are maintained in cell culture following passage through Generation 0 and Generation 2 mice. (A) Clone 9-inoculated hippocampi (G0) seed robustly relative to other homogenates based on split-luciferase complementation. FL tau was IPed from micro-dissected tau P301S and WT hippocampi inoculated 21 days prior with indicated cell lysate. IPed material from 60 µg total brain homogenate was transduced into split-luciferase cells, following the protocol outlined in Figure 2.3G. Four separate experiments were performed for each sample, each read in quadruplicate 48-hours post-transduction of lysate. Luminescence values were averaged within mouse cohorts (error bars = S.E.M., **** = p<0.0001). (B) Inclusion morphologies are maintained following passage through P301S mice. IPed FL tau from P301S mice inoculated with Clone 9 or Clone 10 was transduced into tau RD-YFP cells in a 12-well plate. 24 hours later, cells were re-plated into a 6-well plate. At confluency, cells were sparsely plated on coverslips and were given 8 days to amplify into discrete colonies. Colonies with inclusions were imaged. Representative examples are shown. Monoclonal strains were also isolated and examined with additional assays (see Figure 2.11B-D). (C) Quantification of inclusion morphologies following passage through P301S mice. Inclusion-positive colonies were scored as either containing or lacking nuclear inclusions. 20+ colonies were scored per mouse, and percentage of each phenotype was calculated. These values were averaged within cohorts (error bars = S.E.M., **** = p<0.0001). (D) Quantification of total tau IPed from G0 mice inoculated with indicated cell lysates (densitometric quantification, n=4 measurements, error bars = S.E.M., * = p<0.05). (E) Split-luciferase complementation reveals that IPed tau from G2 brain seeds similarly to crude homogenate. IPed material from 60 µg brain homogenate or 60 µg crude
homogenate was transduced into split-luciferase cells, following the protocol outlined in Figure 2.3G. Four separate experiments were performed for each sample, each read in quadruplicate 48-hours post-transduction of lysate (error bars = S.E.M.). (F)

Quantification of inclusion morphologies following passage from G2 mice. Inclusion-positive colonies were scored as either containing or lacking nuclear inclusions. Ten coverslips, each with 15+ inclusion-positive colonies per, were scored and averaged (error bars = S.E.M., **** = p<0.0001, Student’s t-test). Representative colonies are shown along with a rare instance of an inclusion-positive G2-Clone 1 colony.

Monoclonal strains were also isolated and examined with additional assays (see Figure 2.11F-H). (G) Confocal images of all 24 F2 colonies (G2-Clone 9 = 12, G2-Clone 10 = 12). All feature the same colony morphology as their parental counterpart, with the exception of 10D, which features nuclear speckles. Red boxes surround images of strains that feature a unique limited proteolysis digestion pattern and seeding ratio.
Figure 2.12
Figure 2.13. Anterograde and retrograde spread of pathology to synaptically connected regions in Generation 3-Clone 9 (G3-Clone 9) mice. (A) Schematic of known projections to and from the hippocampus (DG = dentate gyrus; MEC/LEC = medial and lateral entorhinal cortices; MF = mossy fibers; RSp = retrosplenial cortex; Sub = subiculum). (B) Representative images of AT8 staining in the hippocampi of G3 mice inoculated with 10 µg of G2 brain homogenate. Spread of Clone 9 pathology to the contralateral hippocampus is evident. (C) Summary of pathology present in G3-Clone 9 mice. Gradient represents semi-quantitative analysis of neurofibrillary tangle-like AT8 cell body positivity observed in each region (PPA = posterior parietal association area) both 2.5 and 3.0 mm posterior to bregma. (D) AT8 histopathology observed in brain regions with known projections to/from the hippocampus. Ipsilateral AT8 pathology is observed in the EC and appears in cortical layers II-III, whereas contralateral pathology is observed in deeper layers of the EC. Pathology is also observed in the retrosplenial cortex, especially ipsilateral to the injection site.
**Figure 2.13**

(A) Schematic of known projections to and from the hippocampus (DG, dentate gyrus; MEC/LEC, medial and lateral entorhinal cortices; MF, mossy fibers; RSp, retrosplenial cortex; Sub, subiculum).

(B) Representative images of AT8 staining in the hippocampi of G3 mice inoculated with 10 mg of G2 brain homogenate. Spread of clone 9 pathology to the contralateral hippocampus is evident. See Figure S7A for whole brain slices.

(C) Summary of pathology present in G3-clone 9 mice. Gradient represents semiquantitative analysis of neurofibrillary tangle-like AT8 cell body positivity observed in each region (PPA, posterior parietal association area) both 2.5 and 3.0 mm posterior to bregma.

(D) AT8 histopathology observed in brain regions with known projections to and from the hippocampus. Ipsilateral AT8 pathology is observed in the EC and appears in cortical layers II-III, whereas contralateral pathology is observed in deeper layers of the EC. Pathology is also observed in the retrosplenial cortex, especially ipsilateral to the injection site. See Figure S7B for subiculum and dentate gyrus images.
Figure 2.14. Whole brain slices used in Figure 2.13 and additional sites of spread. (A) Representative images of whole G3 brain slices stained with AT8. Black boxes indicate position of representative images found in Figure 2.13D. (B) Representative images of the subiculum and dentate gyrus (ipsilateral) of G3-Clone 1 and G3-Clone 9 mice.
Figure 2.14
Figure 2.15. Diverse tau prion strains within patients and across diseases. (A)

Schematic illustrating methods used to generate patient-derived tau RD prion strains in a monoclonal Tet Off-tau RD-YFP line. (B) Morphological phenotypes associated with tau RD prion strains induced by patient material: no seeding, toxic, mosaic, ordered, disordered, speckles. Representative examples are shown. (C) IPed tau from 29 patient samples (AD = Alzheimer’s disease, AGD = argyrophilic grain disease, CBD = corticobasal degeneration, PiD = Pick’s disease, PSP = progressive supranuclear palsy) was transduced into tau RD-YFP cells (Tet Off) and as many inclusion-positive clones as could be identified for each patient sample were blindly picked and amplified. Once confluent in 10 cm dishes, morphological phenotypes were scored by a separate blinded experimenter.
Figure 2.15
Figure 2.16. Detailed analysis of strains derived from three human samples. (A) 11 of 12 patient CBD clones isolated using both crude and IP induction (designated with a red *) feature the same phenotype (a wispy, disordered juxtanuclear deposition). CBD-2 is unique in having nuclear speckles. (B) All isolated patient AGD clones lost the aggregated state with time (arrow = inclusion-negative cells). Six representative clones are shown (crude=5, IP=1). An additional 41 clones (crude=30, immunoprecipitation=11) reverted to an inclusion-negative state at an early time point, thus having few cells with inclusions to image at Day 40 (data not shown). All sectoring colonies feature enormous juxtanuclear inclusions with occasional tangle-like aggregates (arrowhead). (C) All 3 Clone 10-derived lines in the Tet Off background feature large, juxtanuclear inclusions. (D) All 12 patient AD clones (8=crude, 4=IP) feature numerous speckles, both cytoplasmic and nuclear. (E) Sedimentation analysis was performed on three representative clones for each patient and average supernatant to total ratios (Sup/Total) were calculated for each using densitometric analysis (n=3 for each clone except Clone 1, n=6). CBD clones contain significantly more material in the pellet than AD clones as reflected by Sup/Total values averaged across clones (lower panel) (error bars represent S.E.M, * = p<0.05). (F) Representative AD clones seed significantly more than CBD clones. Averages of four separate experiments are shown, each read in quadruplicate 48-hours post-transduction of lysate. Seeding values were then averaged across clones (lower panel) (error bars = S.E.M, *** = p<0.001). (G) Lysates derived from all CBD and AD clones were separately transduced into the split-luciferase aggregation-sensor cell line and luminescence was read 48 hours later. Relative luminescence averages for all clones are shown, each measured in quadruplicate (error bars = S.E.M, **** =
p<0.0001, Student’s t-test). (H) Pronase digestion (100 µg/mL) of lysates from representative clones confirms that CBD, AD, and AGD clones are biochemically distinct from each other. AD2 is more sensitive than other clones to pronase, which suggests that its conformation might be slightly different than other AD clones. (I) Limited proteolysis digestion patterns are not affected by amount of insoluble tau in the digested sample. Various amounts of CBD-4 lysate were digested, spiking with non-transfected (NT) cell lysate to maintain a constant level of protein. Regardless of the quantity of CBD-4 lysate, all conditions feature a band around 8 kDa in size.
Figure 2.16
Figure 2.17. Mice used in study.

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Table S1, related to Figure 4, 5, 7 – Characteristics of mice used in all in vivo experiments
Figure 2.18. Characteristics of patient material and morphological analysis of all clones isolated from patient samples.

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**Bold** = most common phenotype associated with tissue sample

*^ = patient carried a primary diagnosis of AGD

*^ = patient carried a primary diagnosis of FTLD-TDP but had incidental AGD
Chapter 3: Tau Prion Strains Dictate Patterns of Cell Pathology, Progression Rate, and Regional Vulnerability *In Vivo*
3.1 PREFACE

This chapter contains a submitted manuscript:


3.2 ABSTRACT

Tauopathies are neurodegenerative disorders that affect distinct brain regions, progress at different rates, and exhibit specific patterns of tau accumulation. The source of this diversity is unknown. We previously characterized two tau strains that stably maintain unique conformations in vitro and in vivo, but did not determine the relationship of each strain to parameters such as regional vulnerability or rate of spread that define a tauopathy. We have now isolated and characterized 18 tau strains in cells based on detailed biochemical and biological criteria. Inoculation of transgenic tau P301S (PS19) mice with these strains causes strain-specific intracellular pathology in distinct cell types and brain regions, and induces different rates of network propagation. In this system, strains alone are sufficient to account for diverse neuropathological presentations, similar to those that define human tauopathies. Further study of these strains can thus establish a structural logic that governs these biological effects.
3.3 INTRODUCTION

Tauopathies are a diverse group of neurodegenerative diseases characterized by clinical heterogeneity, progressive deposition of tau protein aggregates in characteristic brain regions, and distinct cellular pathologies (Lee et al., 2001). The etiology of this clinical and pathological diversity is unknown, but may hold the key to accurate diagnosis, prognosis, and therapy. Tauopathies include Alzheimer’s disease (AD), frontotemporal dementias (FTDs), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and others (Lee et al., 2001). Most tauopathies are sporadic, but dominantly inherited mutations in the MAPT gene, which encodes the tau protein, lead to specific FTD syndromes (Hutton et al., 1998). Disease-associated mutations enhance the ability of tau to form amyloids, which are ordered protein assemblies rich in cross beta sheet structure (Knowles et al., 2014), and support a causal role for tau aggregation in pathogenesis (Barghonn et al., 2000).

In AD, the most common tauopathy (Lee et al., 2001), tau amyloid deposition occurs in an orderly fashion, beginning in the transentorhinal cortex, spreading to synaptically connected regions such as the hippocampus, and eventually moving to more distant regions of the neocortex (Braak and Braak, 1991; 1995). Multiple studies have now documented tau aggregate uptake, “seeding” (i.e. aggregate serving as a template for the conversion of monomer to a pathological, fibrillar form), and transfer of aggregates among cultured cells (Frost et al., 2009a; Guo and Lee, 2011; Holmes et al., 2013a; Nonaka et al., 2010). Experimental evidence suggests that “propagation,” or the movement of tau aggregates between connected neurons with seeding of tau monomer in recipient cells, mediates this progression in vivo (Brettschneider et al., 2015; Sanders et
Importantly, injection of tau aggregates into mice that express human tau protein induces tau pathology that spreads outwards along known brain networks (Ahmed et al., 2014; Clavaguera et al., 2009; Iba et al., 2013; Peeraer et al., 2015; Sanders et al., 2014; Stancu et al., 2015). Transgenic mice that limit the expression of tau to the entorhinal cortex also show spread of tau pathology to distant, connected brain regions (de Calignon et al., 2012; Liu et al., 2012). Together, these studies suggest that propagation of an aggregated state underlies the progression of tau pathology. These observations match the established mechanisms of propagation of pathological prion protein (PrP) (Prusiner, 1998). Controversy exists about whether to refer to proteins other than PrP as “prions” (Aguzzi and Rajendran, 2009; Brettschneider et al., 2015; Prusiner et al., 2015; Sanders et al., 2014; Walker and Jucker, 2015; Woerman et al., 2015). While tau aggregates do not appear to be infectious, we favor the term “prion” principally because it provides a conceptual framework with which to understand pathogenesis, therapy, diagnosis, and heterogeneity of disorders linked to amyloid accumulation.

The pathology of tauopathies occurs in distinct brain regions (Arnold et al., 2013), involves disparate brain networks (Raj et al., 2012; Seeley et al., 2009; Zhou et al., 2012), and features unique tau inclusions in various cell types (Kovacs, 2015). Individuals may develop rapid or slow neurodegeneration even within the same syndrome (Armstrong et al., 2000; Huang et al., 2014; Mann et al., 1992; Thalhauser and Komarova, 2012). The basis of these diverse disease patterns is unknown.

We initially observed that tau adopts multiple, stably propagating conformers in vitro, and speculated that structural variation in amyloids could underlie different tauopathies
(Frost et al., 2009b). We subsequently determined that tau forms discrete prion “strains” that propagate with remarkable fidelity through living systems (Sanders et al., 2014). The concept of prion strains derived from a realization that PrP prions can induce distinct transmissible spongiform encephalopathies with reproducible incubation times and patterns of neuropathology (Collinge and Clarke, 2007). It is now clear that PrP prion strains derive from different PrP amyloid conformations, and produce predictable incubation times and neurodegenerative phenotypes upon serial passage in vivo. Moreover, distinct PrP strains probably account for the myriad features of individual PrP prion diseases (Collinge et al., 1996). The concept of a strain as a stably propagating structure that induces a specific and reproducible phenotype is critical, as it anticipates and enables mechanistic, diagnostic, and therapeutic insights based on knowledge of a defined molecular assembly (Sanders et al., 2016).

In addition to the distinct morphologies of tau fibrils and isoform composition of amyloid deposits in tauopathies (Lee et al., 2001), prior studies have suggested that unique tau amyloid structures might account for some aspects of clinical variation. Injection of homogenate from different tauopathy brains into a mouse model that expresses full-length human tau induced pathology that closely resembles that of the human source cases (Clavaguera et al., 2013a). In a similar study, tau aggregates purified either from AD or CBD brains induced distinct patterns of tau pathology that affected different cell populations in transgenic mice that express 1N4R tau with a P301S mutation (PS19) (Boluda et al., 2015). However, both works relied upon a limited number of patient samples that likely contain a heterogeneous mixture of tau aggregate conformations (Sanders et al., 2014). Thus, the structure and biochemical properties of injected tau
aggregates could not be well defined, making it impossible to directly link amyloid structure to pathology. Further, in the latter study, two different purification paradigms were used for purification of AD versus CBD derived tau, which might also have affected the phenotypes observed (Boluda et al., 2015). While these and other studies support the idea that various tau preparations can induce distinct pathologies following stereotactic injection, the actual cause of diversity in tauopathy syndromes has remained unclear.

Like PrP, tau forms bona fide prion strains that propagate in cells and animals (Sanders et al., 2014). Previously, we generated two stably replicating tau prion strains based on mother-daughter transmission of the aggregated state in HEK293 cells that stably express mutant tau repeat domain (RD) fused to yellow fluorescent protein. We observed maintenance of both tau prion strains through serial hippocampal inoculations in three generations of PS19 mice. Finally, we re-established the original strain phenotypes in culture after transduction of immuno-purified tau aggregates from homogenized mouse brain back into the original cell line that lacked tau aggregates. This work indicated that tau aggregate conformation alone, not brain-specific cofactors, determined the induced pathology in vivo. It also suggested that distinct tau strains might be sufficient to explain different pathological patterns in tauopathies. However, this work did not examine the role of strains with regard to regional vulnerability, rate of spread, or patterns of histopathology that constitute the basis for neuropathological discrimination of tauopathy syndromes.

We have now isolated and extensively characterized 18 putative tau prion strains derived from recombinant, mouse, or human sources that we propagated in tau RD-YFP HEK293 cells. We subsequently performed inoculation experiments in transgenic PS19 tau mice to
test whether tau strains are sufficient to account for the pathological variation that is used to characterize human tauopathies: cellular inclusion morphology, rate of spread through brain networks, glial involvement, and regional vulnerability. This work develops a framework to understand the relationship of tau prion structure to distinct tauopathy syndromes.
3.4 MATERIALS AND METHODS

**Tau purification and fibrillization.** Recombinant preparations of full-length (FL) 2N4R tau protein were prepared as previously described (Goedert and Jakes, 1990). pRK172 FL 2N4R tau was expressed in Rosetta (DE3)pLACI competent cells (Novagen) and tau was purified according to established protocols. After purification, tau was lyophilized using a FreeZone Plus Freeze Dry System (Labconco) and stored at -80°C as single-use aliquots. Prior to fibrillization, monomeric tau was re-suspended in 25 mM DTT for 45 minutes. The protein was then fibrillized at a final concentration of 8 µM in tau buffer (2.5 mM DTT/10 mM HEPES pH=7.4/100 mM NaCl/8 µM heparin, final volume of 200µL) at 37°C for 24 hours prior to addition to cells.

**Cell culture.** All HEK293T and HEK293 cell lines were grown in complete media: Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Gibco). Cells were cultured and passaged at 37°C, 5% CO₂, in a humidified incubator. Dulbecco’s phosphate buffered saline (Life Technologies) was used for washing the cells prior to trypsinization with 0.05% Trypsin-EDTA (Life Technologies).

**Lentivirus production.** Lentivirus was prepared as previously described (Araki et al., 2004; Sanders et al., 2014). HEK293T cells were plated at 1x10⁶ cells/well in 6-well plates. After 24 hours, cells were transfected with a three-component plasmid system for virus production: VSV-G (400 ng), PSP (1200 ng), FM5 (400 ng). DNA was mixed with 7.5µL TransIT-293 (Mirus) and 250µL OptiMEM for 15 minutes prior to addition to cell media. After 48 hours, media was harvested and spun at 500g for 5 minutes to remove
debris. For generation of HEK293 cell lines, lentivirus-containing supernatant was stored at -80°C prior to addition to cells. For primary neurons, lentiviral supernatant was concentrated 50x using lenti-X concentrator (Clontech) according to manufacturer’s protocol. The final concentrated virus was re-suspended in PBS with 25 mM HEPES (pH = 7.4).

**Stable HEK293 cell line generation.** Monoclonal HEK293 cell lines were generated that stably overexpress fusion proteins containing the tau repeat domain (amino acids 244-372 of the 2N4R isoform of tau) with various mutations (P301L, P301S, V337M) previously shown to cause familial tauopathy (FTDP-17) (Hutton et al., 1998). Two monoclonal HEK293 cell lines were previously described: Clone 1/DS1, which expresses tau RD P301L/V337M-YFP (Sanders et al., 2014) and the tau RD P301S FRET biosensor (ATCC CRL-3275), which expresses tau RD P301S-CFP and tau RD P301S-YFP (Holmes et al., 2014). To generate additional polyclonal lines, HEK293 cells were plated at 50,000 cells/well in a 12-well plate. After 18 hours, 300 µL conditioned media containing lentivirus was added to the wells (RD LM FRET polyclonal: 150 µL tau RD P301L/V337M-CFP and 150 µL tau RD P301L/V337M-YFP; RD P301S split-luciferase polyclonal: 150 µL tau RD P301S-Cluc and 150 µL tau RD P301S-Nluc). For both polyclonal lines, cells were given 3 days to grow in the presence of virus. Cells were then re-plated in a 10 cm dish, grown to confluency, and stored in liquid nitrogen until use.

To generate monoclonal lines, the two polyclonal populations (RD LM FRET and RD P301S split-luciferase) were diluted sparsely in 10 cm dishes, so that there were fewer than 30 cells per dish. Cells were given seven days to grow into visible colonies. At this point, cloning cylinders (Bel-Art Products) were used to isolate single colonies, which
were passaged to new wells. Monoclonal lines were serially amplified to confluency using 24-well then 6-well then 10 cm dishes. Upon reaching confluency in 10 cm dishes, monoclonal lines were frozen in liquid nitrogen until use. LM10 was the tenth monoclonal line selected from the RD LM FRET polyclonal, and was chosen due to its ability to differentiate toxic and non-toxic strains (described below). The RD P301S-split luciferase biosensor was selected due to its high signal to noise ratio in differentiating lysates with and without aggregates.

**Lysate production for generation of strain library.** Confluent cells from 6-well plates were harvested, pelleted, and stored at -80°C for the following cell lines (Sanders et al., 2014): Clone 1-derived (Clone 1, Clone 9, Clone 10), OFF1-derived (AGD2/mosaic, CBD3/disordered, CBD5/disordered, CBD3/speckles, AD1/speckles, PiD2/ordered). Cell pellets were thawed on ice and subsequently lysed in PBS with 0.05% Triton X-100 and a Complete mini protease inhibitor tablet (Roche) by triturating 10x and incubating at 4°C for 10 minutes. Sequential 5-minute centrifugations were then performed at 500xg and 1000xg to clarify the lysate. A Bradford assay (Bio-Rad) was performed on the supernatants, and protein concentrations were normalized to 5 µg/µL with addition of lysis buffer. Lysates were stored at -80°C prior to addition to cells.

To produce brain homogenates, the following samples were used: transgenic P301S mouse brain (age = 9 months), an Alzheimer’s disease patient brain (patient AD1) (Sanders et al., 2014), and a late-stage chronic traumatic encephalopathy patient brain that was a generous gift from Dr. Ann McKee. For each, 0.4 gram sections were sonicated in 5 mL TBS with Complete protease inhibitors (Roche) using an Omni-Ruptor 250 probe sonicator at 30% power for 3-second pulses x 30 cycles. Crude homogenates
were then clarified by centrifugation at 15,000xg for 15 minutes. The supernatants were set aside, a Bradford was performed, and protein concentrations were normalized to 4 µg/µL with addition of TBS buffer. Homogenates were stored at -80°C prior to addition to cells.

**Generation of a library of monoclonal strains.** To generate a library of isogenic tau strains, several monoclonal tau RD P301L/V337M-YFP cell lines were examined for their ability to stably propagate a diversity of strains. Clone 1/DS1 (Sanders et al., 2014) was selected for its capacity to amplify both highly toxic strains and strains prone to sectoring, which can be attributed to its intermediate level of tau RD expression. Clone 1 cells were plated at 240,000 cells/well in 12-well plates. After 24 hours, cells were treated with 400 nM tau fibrils or 20 µg lysate/homogenate (see lysate production for generation of strain library), and prepared as transduction complexes (4 µL lipofectamine-2000 (Life Technologies), protein source, and OptiMEM to 200 µL volume). After 18 hours, cells were washed, trypsinized, and re-plated in a 6-well plate. At confluency (day 3), cells were diluted sparsely in 3 x 10 cm dishes (per condition) so that there were less than 30 cells per dish. Cells were given 9 days to amplify into visible colonies. At day 12, epifluorescence microscopy was used to find colonies featuring cells with inclusions and these were marked. Cloning cylinders (Bel-Art Products) were used to isolate up to six colonies per condition, and each individual colony was separately amplified to confluency in 12-well, then 6-well, and 10 cm dishes. At approximately day 30, cells were either plated on coverslips for fixation and confocal microscopy or were frozen down in single-use pellets at -80°C for analysis by seeding and protease digestion. A total of 90 monoclonal lines were examined. Based on preliminary analysis by
inclusion morphology, seeding, and protease digestion, 21 were selected for rigorous analysis by both the aforementioned assays as well as sedimentation analysis and toxicity assays. Finally, 18 lines were suspected to be distinct strains and were designated DS2 to DS19 (DS = David/Sarah).

**Fixation of cells for confocal microscopy.** Cells were grown for 48 hours on coverslips in 24-well plates. Media was removed and replaced with 4% PFA for 15 minutes. PFA was removed and replaced with PBS. Cells were washed once with additional PBS and DAPI-stained for 10 minutes in 0.1% Triton-X. Stain was removed and replaced with PBS. Coverslips were mounted using Prolong Gold Antifade reagent (Life Technologies), sealed with nail polish, and placed at 4°C prior to confocal analysis.

**Confocal analysis of strains based on inclusion morphology.** For confocal microscopy, a Zeiss Axiovert 200M microscope was coupled to a Zeiss LSM 5 PASCAL system. For the collection of all images, a pinhole size of 0.8 µm was used. Representative images of each monoclonal cell line were taken and were scored based on inclusion morphology: diffuse (no aggregates), mosaic (loss of aggregated state with division), ordered (large, dense juxtanuclear inclusion with no nuclear aggregates), speckles (small juxtanuclear inclusion with numerous nuclear speckles), threads (long, fibril-like inclusions in cytoplasm with rare nuclear speckles), disordered (diffuse aggregates that wrapped around nucleus with no nuclear speckles).

**Limited proteolysis.** Lyophilized pronase (Roche) was re-suspended in PBS to a final concentration of 1 mg/mL and aliquots were snap-frozen and placed at -80°C. Cell pellets were thawed on ice and lysed in PBS with 0.05% Triton X-100 and a cOmplete mini
protease inhibitor tablet (Roche) by triturating 10x and incubating at 4°C for 10 minutes. Sequential 5-minute centrifugations were then performed at 500xg and 1000xg to clarify the lysate. A Bradford assay (Bio-Rad) with BSA standard curve was performed and protein concentrations were normalized to 4 µg/µL with addition of lysis buffer. 60 µg (15 µL) of cell lysate was added to 15 µL of pronase (diluted in PBS) at a concentration of 60 µg/mL for a final volume of 30 µL and a final pronase concentration of 30 µg/mL. Cell lysates were digested at 37°C for one hour. Reactions were quenched by addition of 30 µL 2x sample buffer (4 µL BME, 11 µL 10% SDS, 15 µL 4x Laemmli buffer) followed by five minutes of boiling. 13 µL of each sample was loaded onto a NuPAGE 10% Bis-Tris gel (Life Technologies). Gels were run at 150 V for 70 minutes. Protein was transferred to Immobilon P (Millipore) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked for 1 hour in 4% milk. Membranes were probed for tau using mouse monoclonal anti-tau 2B11 (0.7 µg/µL, Clontech) for 18 hours, washed four times with TBS-T, counter-probed with goat anti-mouse HRP (1:4000, GE Healthcare) for 1.5 hours, and were washed four additional times with TBS-T. Finally, membranes were exposed to ECL Prime Western Blotting Detection System (Fisher Scientific) for 2 minutes and were developed using a digital Syngene imager.

**Sedimentation analysis and densitometry of strain library.** Cell lysates were prepared and clarified as described above (see Limited proteolysis) in biological quadruplicates for each strain. A Bradford assay (Bio-Rad) with BSA standard curve was performed to determine the protein concentrations for the 76 samples, normalizing each to 1.6 µg/µL. 1 mL of each was centrifuged at 186,000g for 60 minutes, with the remainder being set aside as the total fraction. After centrifugation, the supernatant fraction was placed aside.
and the pellet was washed with 1 mL lysis buffer. The ultracentrifugation step was repeated for 30 minutes, and the wash fraction was aspirated. Final pellets were re-suspended in 1 mL of 4% SDS/1% BME (in PBS) with the aid of five minutes of boiling. For each sample, 1 µg total (or equivalent volume of supernatant or pellet) was run on a NuPAGE 10% Bis-Tris gel (Life Technologies) at 150 V for 35 minutes. Protein was transferred to Immobilon P (Millipore) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked for 1 hour in 4% milk and then probed overnight with rabbit polyclonal anti-tau ab64193 (1:5000, AbCam) and rabbit polyclonal anti-cofilin (1:28,000, Sigma). Following four washes with TBS-T, blots were counter-probed with goat anti-rabbit HRP (1:4000, Jackson Immunotherapy) for 90 minutes at room temperature. Finally, blots were washed an additional four times and membranes were imaged with a digital Syngene imager following exposure to ECL Prime Western Blotting Detection System (Fisher Scientific) solution for two minutes. Biological replicates were imaged separately, with four blots being developed at a time. Densitometric units were calculated using Syngene GeneTools software with manual band quantification. The brightness of each tau band (total, supernatant, pellet) was normalized relative to the signal calculated for the condition’s associated total cofilin. Ratios were then averaged across biological quadruplicates.

**Split-luciferase complementation assay.** A monoclonal HEK293 cell line expressing tau RD P301S-Cluc and tau RD P301S-Nluc was generated (see Stable HEK293 cell line generation) and plated at 24,000 cells/well in 96-well plates. After 18 hours, when the cells were at 50% confluency, cells were transduced with cell lysate: indicated amounts of clarified cell lysate (see Limited Proteolysis) were added in transfection complexes
with 0.4 µL lipofectamine-2000 (Life Technologies) to each well. 60 hours after addition of lysate, media was aspirated and replaced with luciferin solution (150 µg/mL D-luciferin potassium salt, Gold Biosciences, in Dulbecco’s phosphate-buffer saline, Gibco). Cells were incubated in luciferin solution for two minutes at 37°C prior to reading luminescence with a Tecan M1000 fluorescence plate reader. Each condition was performed in biological quadruplicate with each replicate being performed on a separate plate to control for differences between plates. Seeding ratio was calculated relative to sham control for an individual plate. Seeding ratios were then averaged across quadruplicates and standard errors of the mean were calculated and plotted. Inflection point was defined as the amount of lysate required to achieve a 50% increase in luminescence relative to sham treatment. EC$_{50}$ and peak seeding were calculated using a non-linear regression with one-phase decay fit.

**Toxicity assay.** A monoclonal cell line expressing tau RD P301L/V337M-CFP and tau RD P301L/V337M-YFP was generated and given the name LM10 (see Stable HEK293 cell line generation). LM10 cells were plated at 240,000 cells/well of 12-well plates. After 18 hours, wells were transduced with 20 µg clarified lysate (see Limited Proteolysis) in transfection complexes with 4 µL lipofectamine-2000. Each condition was performed in biological triplicate. After 24 hours, media was aspirated and cells were trypsinized and re-plated into 6-well plates. After 48 hours (i.e. 72 hours post-treatment), media was removed, cells were trypsinized, and pelleted, followed by re-suspension in flow cytometry buffer (HBSS with 1% FBS and 1 mM EDTA). FRET-positive (aggregate-containing) and FRET-negative (aggregate-lacking) cells were gated according to published protocols (Furman et al., 2015; Holmes et al., 2014). Using a
FACS Aria II SORP cell sorter (BD Biosciences), 7000 FRET-positive cells were collected in 1.4 mL media for each condition (DS2 to DS19). For the negative control (DS1), 7000 FRET-negative cells were sorted. 200 µL (i.e. 1000 cells) were plated in sextuplicate for each condition in 96-well plates. Cells were given 7 days to amplify. Cells were then harvested with 0.05% trypsin, pelleted, and fixed in 2% paraformaldehyde (Electron Microscopy Services) for 10 minutes. Cells were re-pelleted and re-suspended in flow cytometry buffer. A MACSQuant VYB (Miltenyi) was used to perform FRET flow cytometry according to previously published protocols (Holmes et al., 2014), and total FRET-positive and FRET-negative cells were counted for each condition. Totals were averaged across technical sextuplicates and then averaged across biological triplicates. Error bars represent the standard error of the mean for biological triplicates.

**Statistical analysis of in vitro correlations.** For the correlation of toxicity, seeding, and sedimentation metrics, normality of the data was assessed using both D’Agostino/Pearson and Shapiro-Wilk tests. If both tests were passed, data sets were considered normal and a Pearson correlation was performed. Else, the data sets were not considered normal and a more conservative Spearman correlation was performed. Correlations were considered significant if p was less than or equal to 0.05.

**Primary neuron culture and staining.** Cortical and hippocampal neurons were isolated from E18.5 CD1 mice and cultured according to previous literature (Yano et al., 2014). Briefly, neurons were incubated with 0.5% trypsin in HBSS with glucose (1:6 dilution) for 20 minutes at 37°C. Subsequently, neurons were triturated and passaged through a 70 µm filter, followed by plating 100,000 cells per well on poly-D lysine- or
polyethyleneimine (PEI)-coated plates for cortical or hippocampal cultures respectively. Neurons were treated at DIV3 with 2 µL of 50x-concentrated 1N4R Tau P301S-YFP lentivirus (see Lentivirus production) per well of a 96-well plate. Neurons were subsequently treated at DIV6 with 10 µg of cell lysate or PBS. Neurons were examined for intracellular aggregates each day with an epifluorescence microscope (Nikon Eclipse TI), and scored using a 0-5 scale (No seeding – Peak seeding). Neurons were fixed at 5, 8 or 11 days post lysate addition with a modified triton-X extraction to remove soluble tau protein (Volpicelli-Daley et al., 2011). Briefly, cells were incubated with 4% PFA/4% sucrose in PBS with 1% Triton-X 100 for 30 minutes. Cells were subsequently washed and stained with MC1 antibody (1:500; Peter Davies) in 10% NGS in PBS overnight at 4°C. Cells were washed and treated with anti-mouse IgG 546 (1:400, Life Technologies), and stained with DAPI. Cells were imaged with the In Cell Analyzer 6000 at 40x resolution with the assistance of the UTSW HTS Core facility.

**Primary neuron seeding assay.** Hippocampal neurons were plated at 75,000 cells per well on PEI-coated plates. At DIV3, cells were treated with 1 µL of 50x concentrated TauRD P301S-CFP and 1 µL of TauRD P301S-YFP lentivirus. At DIV6, cells were treated with 10 µg of cell lysate or an equivalent volume of PBS. Cells were subsequently incubated for four days. A MACSQuant VYB (Miltenyi) flow cytometer was used to assess FRET-positive neurons according to previously published literature (Furman et al., 2015; Holmes et al., 2014).

**Cell lysate production for animal inoculation experiments.** DS1-19 cell lines were grown in 3 x 10 cm dishes until 90% confluency. Cells were trypsinized, re-suspended in media and centrifuged at 500xg. Cell pellets were washed with 1x PBS and stored at -
80°C until use. Pellets were thawed on ice and re-suspended in 1x PBS with cOmplete protease inhibitors (Roche) using an Omni-Ruptor 250 probe sonicator at 30% power for 30, 3-second cycles. The probe sonicator was washed with 100% ethanol and ddH₂O between cell lines. Strains were subsequently centrifuged at 1000xg, normalized to 7 µg/µl by Bradford assay (Bio-Rad) and stored as aliquots at -80°C until use.

**Sedimentation analysis and densitometry of injected lysates.** Sonicated and clarified cell lysate was thawed on ice, and diluted to 2 µg/µL in 1x PBS with cOmplete protease inhibitors (Roche). Samples were subdivided into a total protein and ultracentrifuge (UC) aliquots. The UC aliquots were centrifuged at 186,000xg for 90 minutes. Supernatant was removed and stored with the total lysate aliquot at -80°C until use. Pellets were washed with 1x PBS and centrifuged at 186,000xg for an additional 30 minutes. Pellets were re-suspended to their original volume. SDS-PAGE was performed on total, soluble or insoluble fractions of each cell line (1:2:1 ratio) using 5-20% gradient acrylamide gels. Gels were transferred as described above. Membranes were cut at the 20 kilo-Dalton ladder mark and incubated at 4°C overnight with a rabbit polyclonal anti-tau antibody (1:4000, Abcam ab64193) or a rabbit polyclonal anti-cofilin antibody (1:4000; Sigma). Anti-rabbit ECL HRP conjugated secondary antibody (1:4000, GE Lifesciences) was added for one hour, and blots were developed using ECL Prime Western Blotting Detection System (Fisher Scientific). Washes were performed as described above. Densitometry was performed by measuring the mean grey value (mgv) of bands with ImageJ, and normalizing to cofilin mgv. Samples were averaged across biological triplicate. A one-way ANOVA with Bonferroni’s multiple comparisons correction was
performed by comparing DS1-1 to each sample. A one-way t-test was performed to directly compare DS10 and DS4.

**Animal tissue collection.** P301S or WT mice were anesthetized with isoflurane and perfused with chilled PBS + 0.03% heparin. Brains were post-fixed in 4% paraformaldehyde overnight at 4°C and then placed in 30% sucrose in PBS until further use.

**Histology.** Brains were sectioned at 50 µm using a freezing microtome. Slices were first blocked for one hour with 5% milk in TBS with 0.25% Triton X-100 (blocking buffer). For DAB stains, brain slices were incubated with biotinylated AT8 antibody (1:500, Thermo Scientific) overnight in blocking buffer at 4°C. Slices were subsequently incubated with the VECTASTAIN Elite ABC Kit (Vector Labs) in TBS prepared according to the manufacturer’s protocol for 30 minutes, followed by DAB development using the DAB Peroxidase Substrate Kit with the optional nickel addition (Vector Labs). Slices were imaged using the Olympus Nanozoomer 2.0-HT (Hamamatsu). For astrocyte staining, slices were permeabлизed in 0.25% Triton X-100 in TBS, followed by blocking with 3% milk and 10% normal goat serum (NGS) for 30 minutes, followed by incubation with AT8 (1:500, Thermo Scientific) and GFAP (1:500, Dako) overnight at 4°C. Slices were incubated with 1:500 anti-mouse 488 and anti-rabbit 546 antibodies (Life Technologies), followed by imaging using a Zeiss LSM780 inverted confocal microscope.

**Quantification of Tau pathology.** Images of AT8 DAB stained slices were collected as above. A blinded analysis of the level of tau pathology was performed using a semi-
quantitative 0-3 scale (no pathology, mild, moderate, and severe, respectively) as previously reported (Lace et al., 2009). Briefly, images of each brain slice were randomized and blinded to the scorer. Individual brain regions in each slice were assessed for AT8 positive neuronal inclusions. Scores were recorded unless a slice was damaged such that the region of interest was not present in the slice. The level of pathology was averaged among biological replicates for each region within a condition. For the regional vulnerability experiments (Figure 3.9), pathology was normalized by subtracting the average DS1 pathology in a region, and heat maps were generated with discrete bins for different pathology levels as described within the figure. For the time course experiments (Figure 3.12), averages were plotted as a heat map with a gradient from 0 to 3 as reported in the figure. Heat maps were generated using MATLAB.

**P301S FRET Flow Seeding assay.** Bilateral hippocampus, sensory cortex, or thalamic 1 mm punches were isolated from mice injected with DS1, 4, 6, 7, 9, 10 or diluted DS6 and DS9, 8-weeks post inoculation. Samples were placed into 1xTBS at a dilution of 1mm³/mL (v/v) and sonicated with a water bath sonicator (Qsonica Q700MPX with Chiller and tubing set) at 4°C at 50% power for 30 minutes. Seeding assays were performed as previously described (Furman et al., 2015; Holmes et al., 2014). Briefly, 2.5 μL of each sample was added in triplicate to wells of a 96-well plate onto the tau RD P301S FRET biosensor cell line. Cells were incubated for 48 hours and then trypsinated, fixed with 2% PFA in 1x PBS, and incubated in flow cytometry buffer. A FACS LSRFortessa SORP was used to perform FRET flow cytometry as previously described (Furman et al., 2015; Holmes et al., 2014). Integrated FRET density (IFD = percent cells positive for FRET multiplied by the median fluorescent intensity of FRET-positive cells)
was averaged for every condition and normalized to lipofectamine-2000 (sham) IFD. A one-way ANOVA with Bonferroni corrections was performed for each region by comparing DS1 ipsilateral IFD to all other conditions within that region.
3.5 RESULTS

**Generation of a library of tau strains.** In previous work, we created a monoclonal HEK293 cell line (initially termed Clone 1, now DS1) that stably expresses the repeat domain (amino acids 244-372) of 2N4R tau with two disease-associated mutations (P301L and V337M) (Sanders et al., 2014). Importantly, these cells never produce aggregates unless stimulated by tau seeds in the culture medium. Exposure to exogenous seeds converts intracellular tau to an aggregated form, which propagates as a stable phenotype from mother to daughter cells. We extensively characterized two biochemically distinct tau strains *in vitro* and in the PS19 mouse tauopathy model (Clone 9/DS9 and Clone 10/DS10) and further found that distinct inclusion morphologies associate with unique tauopathy syndromes (Sanders et al., 2014). However, the range and diversity of amyloid structures that tau could adopt in this cellular context was not known.

We thus treated DS1 cells with tau aggregates from diverse recombinant, mouse, and human brain samples (Figure 3.1A). We isolated and amplified 90 monoclonal cell lines that stably propagated tau aggregates and froze them for later analyses. Following preliminary analyses by several assays (inclusion morphology, limited proteolysis, seeding by tau split-luciferase complementation as described previously (Sanders et al., 2014)) we rigorously analyzed 21 cell lines. This indicated that we had isolated 18 putatively distinct strains (DS2-DS19; see Figure 3.2A for origin of each strain). These strains featured several striking differences in their subcellular distribution of aggregated tau (Figure 3.1B): a single, large juxtanuclear inclusion (Ordered: DS3, 10, 14, 19), prominent nuclear inclusions (Speckles: DS4, 5, 7, 8, 9, 12, 16, 17, 18), aggregated tau
that failed to organize into ordered inclusions (Disordered: DS11, 13), fibril-like ribbons of aggregated tau throughout the cytoplasm (Threads: DS6, 15), and one strain that sectored with time, reverting from the aggregated state to the soluble diffuse state (Mosaic: DS2). Importantly, with the exception of the mosaic strain DS2, every daughter cell featured the same inclusion morphology even after months of passage, suggesting that each monoclonal cell line stably propagated a single strain.

To examine whether the tau inclusions were composed of structurally distinct tau amyloids, we used limited proteolysis, an assay previously shown to differentiate prion strains derived from PrP (Bessen and Marsh, 1994) and tau (Sanders et al., 2014). We thus determined a “fingerprint” for each strain based on the regions of tau protected from digestion by pronase (Figure 3.1C). This assay suggested that while cell lines with different inclusion morphologies always propagated different strains, inclusion morphology alone could not discriminate all strains. For example, despite appearing equivalent by microscopy, many speckled strains featured different limited proteolysis fingerprints.

**Tau strains show unique seeding profiles in dividing cells and neurons.** Next, we examined the ability of the individual strains to seed natively folded tau using a tau RD P301S split-luciferase complementation assay (Mirbaha et al., 2015; Sanders et al., 2014). When saturating concentrations of lysate from each strain were introduced directly into the cytoplasm of dividing cells, induction of luminescence ranged from 0.3 to 80-fold increase in seeding at saturation vs. background, termed the “seeding ratio” (Figure 3.1D). We observed no association between inclusion morphology and seeding. For example, two ordered strains were strong seeders (DS10, 14), whereas two others were
weak (DS3, 19). Importantly, the relative seeding abilities of individual tau strains were largely recapitulated when lysates were applied to primary hippocampal neurons expressing Tau RD P301S FRET biosensor proteins (Holmes et al., 2014) (Figure 3.1E). We also observed these relative differences upon expression of full-length 1N4R P301S-YFP in primary hippocampal neurons, suggesting common effects on either truncated or full-length tau. Strains also showed different lag phases in neurons that express full-length tau (Figure 3.1F-G, 3.2B). The five strains that seeded most strongly in the split-luciferase assay (DS5, 6, 9, 14, 15) induced intra-neuronal tau aggregation (Triton-X insoluble, MC1-positive inclusions) within three days. Several of the weakest seeders (DS3, 7, 11, 12, 13, 16, 19) failed to cause tau pathology even after eight days. Moreover, certain strains (e.g. DS6) exhibited slightly longer lag periods than other strains (DS9) with respect to peak seeding, yet reached a high level of pathology five days after addition of cell lysate (Figure 3.1F,G). We observed similar lag phase differences and inclusions in primary cortical neurons (data not shown). Thus, strains feature significant differences in their abilities to trigger aggregation of full-length tau, independent of seed uptake and cell type.

**Seeding activity correlates with toxicity in dividing cells.** To investigate the relationship between seeding activity and toxicity, we first performed a detailed titration of cell lysates (30 pg to 10 µg) from the 18 putatively distinct strains. We determined the EC$_{50}$ and peak seeding ratio for each strain using the tau split-luciferase complementation assay. Strains differentially seeded tau monomer in cell culture as reflected by their peak seeding ratios (Figure 3.3A,B). Different strains displayed >10x range for EC$_{50}$ (DS9: 287 ng; DS3, 4908 ng) (Figure 3.3B). Peak seeding and EC$_{50}$ strongly anti-correlated
(Figure 3.3C), suggesting that peak seeding accurately reflects a strain’s potency in triggering tau aggregation.

We then compared the toxicity of strains to their seeding potential. To determine relative toxicities we generated a cell line (LM10) that expresses a mutant tau RD FRET pair (CFP/YFP) at high levels, and assessed the growth potential of cells propagating various strains after first isolating aggregate-positive cells by FRET FACS (Holmes et al., 2014). Several ordered and mosaic strains (DS2, 3, 10, 19) lost the aggregated state with repeated cell division (“sectored”) (Figure 3.3D). All others stably propagated the aggregated state, but exhibited growth defects relative to LM10 cells that lacked tau aggregates (Figure 3.3E). Strains that sectored, a possible correlate of low seeding, were the least toxic. All three seeding metrics (peak seeding, EC$_{50}$, and inflection point) correlated with inhibition of growth (Figure 3.3F, Figure 3.4A-B). In other words, strains that seeded more efficiently were significantly more toxic to cells expressing high levels of monomeric tau.

To test the possibility that variable levels of insoluble or total tau contributed to the differences in seeding and toxicity across strains, we performed sedimentation analyses to determine the level of soluble, insoluble, and total tau for all 18 strains (Figure 3.3G). While each strain (DS2-19) contained the majority of tau in the insoluble fraction (Figure 3.3G-H), different strains featured variable levels of insoluble (Figure 3.3I) and total (Figure 3.4C) tau. Importantly, however, neither total nor insoluble tau levels correlated with seeding (Figure 3.3J, 3.4D) or toxicity (Figure 3.3K, 3.4E). Thus, structural differences among strains, rather than soluble/insoluble tau levels per se, account for seeding activity and toxicity in dividing cells.
Diversity of pathology induced by tau strains. We hypothesized that to account for variation in tauopathies, individual tau strains should produce a wide array of pathological phenotypes \textit{in vivo}. To test whether individual tau strains can produce such diversity, we inoculated cell lysate from each strain (DS1-DS19) into a tauopathy mouse model (PS19) that expresses 1N4R tau with the FTDP-17-associated P301S mutation from the prion promoter (Yoshiyama et al., 2007) (Figure 3.5A). We subsequently examined the pattern and level of pathology induced eight weeks after inoculation (Figure 3.5B-J).

Pathology varied greatly between strains, and was often, but not always, consistent with seeding activity observed by the tau split-luciferase assay and in primary neuron culture (Figure 3.1D-G). Strains with low seeding activity (DS2, 3, 11, 19) produced a “rare seeding” phenotype \textit{in vivo}, with limited AT8 pathology localized in CA1 of the ipsilateral hippocampus (Figure 3.5G). These strains appear different in terms of their AT8 subcellular localization (soma versus axonal pathology in DS2 and 11), but this may reflect different levels of maturation of tau aggregates (e.g. pretangles and tangles). Several strains induced low, yet consistent tangle-like pathology in CA1 of the hippocampus (DS4, 8, 12, 13, 16, 17) (Figure 3.5H). Interestingly, DS12, 13, and 16, which derive from CBD patient homogenate, induced pathology \textit{in vivo} despite not producing pathology in primary neuron cultures that express full-length tau (Figure 3.1F). This contrasts with DS4, 8 and 17, which derive from recombinant fibrils or AD brain homogenate and readily induced pathology both in cultured primary hippocampal neurons and \textit{in vivo} (Figure 3.5H, 3.2A, B). Strains with the highest seeding activity in culture (DS5, 6, 9, 15) caused widespread tangle-like tau pathology throughout several
hippocampal regions (Figure 3.5I, J). These robust strains also consistently induced the spread of tau pathology to distant regions such as the entorhinal cortex (EC) and contralateral hippocampus (Figure 3.6B).

Several strains caused unique pathology in the hippocampus. DS10 typically produced AT8-positive “dots” in the mossy fiber tracts of the ipsilateral and contralateral hippocampus, while mostly sparing CA1 pyramidal neurons (Figure 3.5C, 3.6C) as was observed in previous work (Sanders et al., 2014). DS14, which shared the same ordered cellular phenotype as DS10 (Figure 3.1B), also produced mossy fiber dots (Figure 3.5D). However, this strain showed higher seeding activity in culture (Figure 3.1D-F), and additionally induced CA1 tangle-like pathology in the ipsilateral and contralateral hippocampus (Figure 3.6C).

DS7 produced “wisps” that resemble neuropil threads (Figure 3.5E) while inducing weaker AT8 pathology in the main axon branches. This contrasted with several other speckled phenotype strains, which primarily induced AT8 pathology in the main axon (Figure 3.5H-I). DS18 produced wisps, mossy fiber dots, and “grains,” which are AT8 positive puncta found throughout the hippocampus (Figure 3.5F). DS18-inoculated mice also developed grain pathology in the contralateral hippocampus and wisps in the EC, indicating these specific phenotypic features can spread to distant regions (Figure 3.6D).

Critically, strains with matching limited proteolysis fingerprints (Figure 3.1C) produced similar histopathology in vivo (DS3 and 19; DS6 and 15; DS12 and 16) (Figure 3.5F, 3.5G, 3.5I). These strain pairs (which may propagate identical tau aggregate conformations) displayed similar seeding activity and toxicity levels, and induced similar
phenotypes in primary neuron culture (Figures 3.1-3.4). Importantly, DS6 and 15 derive from distinct aggregate sources (aged PS19 mice and recombinant fibrils, respectively), indicating that these strain-based phenotypes are conformation-specific rather than source-specific (Figure 3.2A).

**Specific strains reliably induce astrocytic pathology.** Several tauopathies, including CBD and PSP, feature unique glial cell pathology, while others do not feature astrocytic or oligodendrocyte tau inclusions (Kovacs, 2015). While the mechanisms that underlie these patterns are not known, previous work suggested that inoculation of CBD patient lysate into PS19 mice is sufficient to induce astrocytic pathology (Boluda et al., 2015). Interpretation of this result was limited by the potential for individual patient brains to contain multiple strains (Sanders et al., 2014), and inherent limitations in acquiring and characterizing discrete forms of tau from patient samples (Boluda et al., 2015; Clavaguera et al., 2013a). We thus tested whether individual tau conformation(s) could be linked to astrocytic pathology.

Eight weeks after inoculation with DS7 and DS9, we observed tau pathology reminiscent of astrocytic plaques in multiple animals, as noted by small AT8-positive inclusions arranged in ring-like structures (Figure 3.7A) (Yoshida, 2014). Strains that produced higher levels of tau pathology such as DS6 did not show similar plaque pathology at this time point (Figure 3.7A). Co-staining with GFAP and AT8 indicated that these accumulations consist of phospho-tau within or directly adjacent to astrocytes as is typical of astrocytic plaques (Figure 3.7B) (Yoshida, 2014). DS12, 15, 16, and 18 induced a small degree of astrocytic plaque-like pathology at 8 weeks (Figure 3.8A-B). Given these findings, we retrospectively quantified the number of animals with astrocytic
plaque-like pathology after completion of the time course injection experiment described below. DS7 or DS9 inoculations induced astrocytic plaque-like pathology in >85% of mice by eight weeks, and 100% by 12 weeks (Figure 3.8A). The number of plaques in each animal also increased over time. DS4 and DS6 induced this phenotype to a limited degree by 12 weeks, but it was far less robust than the level observed in DS7 and 9 inoculated animals (Figure 3.8A, C).

While two CBD-derived strains showed a small degree of astrocytic plaque-like pathology (DS12, 16), plaques were sparse and present in only one animal per condition at this time point (Figure 3.8A, B). These strains may require a longer incubation time to induce astrocytic plaque pathology, or we may have isolated a specific conformation that does not underlie the astrocytic pathology observed in CBD brains. In summary, we isolated two strains that reliably induce astrocytic plaque pathology in vivo. This phenotype is independent of seeding activity, as DS7 is one of the weakest seeders we have observed by the tau split-luciferase assay and in primary culture (Figure 3.1D-G). Instead, specific tau conformations preferentially and predictably induce astrocytic tau pathology.

**Regional vulnerability to specific strains.** Tauopathies feature accumulation of tau pathology in distinct brain regions, yet the mechanisms that underlie these patterns are not well understood (Arnold et al., 2013). To test whether strains differentially induce pathology in specific brain regions, we inoculated DS1, 4, 6, 7, 9, 10 or 11 into six locations per mouse: sensory cortex (SC), caudate/putamen (CP), visual cortex (VC), hippocampus (Hip), thalamus (Thal), and inferior colliculus (IC) (Figure 3.9A). We chose these strains based on their unique limited proteolysis fingerprints, the different tau
pathology patterns that they induce in the hippocampus (Figure 3.5B-J) and their low (DS7, 11), medium (DS4, 10) or high (DS6, 9) seeding activity in culture (Figure 3.1D, F). Further, DS4 derives from AD and DS11 from CBD brain homogenates. Patients with these diseases have different patterns of tau deposition (Arnold et al., 2013).

After five weeks, we quantified the level of AT8 pathology different strains induced at each injection site in a blinded fashion (Figure 3.9B). All produced hippocampal pathology consistent with the previous injection paradigm (Figure 3.5B-I), illustrating the reproducibility of these phenotypes (Figure 3.9C). Strains with the strongest seeding activity in culture (DS6, 9) produced pathology in every injected region. DS4, a medium-seeding strain, induced moderate pathology in each region except the IC. DS11 pathology was entirely limited to the hippocampus (Figure 3.9B, C).

DS10 again induced pathology specific to the mossy fiber tracts of the hippocampus (Figure 3.10A), with limited pathology in the caudate/putamen and thalamus. Of note, it did not produce any pathology in the injected cortical regions. In contrast, DS7 produced limited AT8 pathology in each targeted brain region (Figure 3.9B, C). The specificity of DS10, despite its strong seeding activity, and promiscuity of DS7, despite its weak seeding activity (Figure 3.1D-G), were remarkable. These studies indicate tropism of certain strains for specific brain regions (mossy fiber tracts, cortical structures, IC) that is independent of simple metrics such as seeding activity. This suggests complex biological interactions that are dictated by strain structure determine regional vulnerability to a given strain.
Strains induce different rates of tau pathology spread along neuronal networks.

Even within a single clinical syndrome, tauopathy patients experience rapid or slow rates of progression (Armstrong et al., 2000; Huang et al., 2014; Mann et al., 1992; Thalhauser and Komarova, 2012). Unique PrP strains show different lag phases and rates of neurodegeneration in animal models of prion diseases (Collinge and Clarke, 2007), suggesting this phenomenon may be linked to specific aggregate conformations. While several factors may contribute to the rate of degeneration observed in tauopathy patients, rapid spread of tau pathology likely accelerates this process. Thus, we tested the relationship between strain characteristics and different rates of spread of tau pathology.

To control for differences in insoluble material, we first quantified the insoluble tau present in lysate from DS1, 4, 6, 7, 9, and 10 prepared for this time course experiment (Figure 3.11A, B). As expected, each strain contained a large amount of insoluble tau. However, we hypothesized that a strain’s ability to seed aggregation of endogenously expressed monomeric tau would primarily determine the rate of spread of tau pathology. We predicted that strains such as DS6 and 9 with high seeding activity in culture and in vivo (Figure 3.1D, 3.9B-C) would produce rapid spread of tau pathology even after inoculation of reduced levels of insoluble tau. To test this hypothesis, we included DS6 and DS9 lysate diluted 1:10 as part of this time course experiment.

We injected cell lysate from each condition into the hippocampus of young PS19 mice and collected brains at 4, 8, and 12 weeks post-injection (Figure 3.11C). While each strain induced tau pathology in the contralateral hippocampus, this occurred at different time points (Figure 3.11D, E). DS6 and DS9 pathology progressed rapidly to the contralateral hippocampus, beginning as early as 4 weeks (Figure 3.11D). DS10 induced
mossy fiber dots and limited CA1 pathology in the ipsilateral and contralateral hippocampus by 8 weeks (Figure 3.11D, E). DS7 wisp pathology also spread to the contralateral hippocampus by 8 weeks (Figure 3.11D).

While dilution of DS6 and 9 decreased the initial level of pathology induced upon inoculation, we observed tau pathology in the contralateral hippocampus at 8 weeks. In contrast, DS4 did not show tau pathology in the contralateral hippocampus until 12 weeks (Figure 3.11D, E). Thus, stronger strains induced more rapid spread of pathology even with a reduced amount of insoluble tau inoculum, presumably due to more efficient seeding and spread of endogenous tau aggregates. Interestingly, we also observed the astrocytic phenotype in DS7, DS9, and DS9 1:10 dilution mice at 8 and 12 weeks. These inoculation experiments were performed with cell lysate prepared specifically for this experiment, further illustrating the reproducibility of this conformation-specific phenotype (Figure 3.7, 3.8, and 3.11D).

To assess AT8 pathology induced by each strain, we performed a blinded analysis of AT8 staining in slices at the level of the locus coeruleus, hippocampal injection site, and caudate/putamen. We averaged AT8 pathology rankings for each region and displayed them as a heat map to visualize the spread of tau pathology (Figure 3.12A). We subsequently created a limited heat-map for each strain that focuses on specific brain regions, several of which developed pathology over time (Figure 3.12B).

DS10 once again displayed marked neuronal specificity, with strong pathology only in the mossy fiber tracts (Figure 3.12A, B). Blinded analysis also confirmed that DS4 exhibits slower kinetics than DS6 or 9 even when the latter strains are diluted 10-fold.
DS4 did not develop strong pathology outside the ipsilateral hippocampus until 12 weeks after injection, while these stronger strains showed AT8 staining in distant brain regions by 8 weeks. DS7 induced robust pathology at the injection site as observed previously (Figure 3.5, 3.9). Despite this, the spread of DS7 pathology was relatively slow, and appeared limited to the hippocampus (Figure 3.12A, B). Thus, despite inducing pathology in several regions upon direct injection (Figure 3.9B, C), DS7 has limited ability to spread pathology to distant regions. In other words, its tropism is broad, but its potential for propagation is low.

DS6 and 9 spread rapidly to specific brain regions after 4 weeks. DS6 strongly targeted the ipsilateral retrosplenial cortex and LC, while DS9 pathology spread most strongly to the ipsilateral entorhinal cortex and thalamus. Despite these initial differences, by 8 weeks the patterns of pathology induced by these strains largely resembled one another, and developed within regions that have strong axonal connections to the hippocampus (Figure 3.12B). While diluted lysate of DS6 and 9 induced lower levels of pathology at early time points, by 12 weeks the pathology spread rapidly and followed similar patterns to that of undiluted lysate. Further, despite having lower burdens of total insoluble tau, these diluted lysates induced pathology that spread faster and farther than DS4, 7 or 10 (Figure 3.12B).

Finally, we performed a seeding assay on tissue from the ipsilateral and contralateral hippocampus, thalamus, and sensory cortex at eight weeks post injection as a second metric of tau pathology. While the split-luciferase assay is quantitative and very useful for lysates with high seed titers, the FRET-based biosensor system we have previously reported is more sensitive and has a larger dynamic range (Holmes et al., 2014). We
transduced homogenized brain regions into the FRET biosensor cell line and quantified the level of FRET produced after two days in culture. We have observed that all seeding activity measured after inoculation of DS9 into the hippocampus of tau knockout mice dissipates by approximately six weeks (data not shown). Thus, any signal identified at this time point likely derives from aggregation of endogenous tau expressed in this mouse line.

Several strains displayed robust seeding in the ipsilateral hippocampus (DS4, 6, 9, 10, and 1:10 diluted DS6 and 9). While each of these strains spread to the contralateral hippocampus, DS4 showed distinctly less contralateral seeding activity as a percentage of the ipsilateral hippocampus (Figure 3.12C). Further, DS6 and 9 showed variable yet significant seeding activity in the ipsilateral thalamus. The level of seeding observed in DS1 inoculated mice is consistent with the level observed in this mouse model at 4-5 months of age, suggesting this signal is due to spontaneous aggregation of endogenous tau that normally occurs within this model (Holmes et al., 2014).

DS7 showed remarkably low seeding activity despite displaying robust AT8 pathology. This is consistent with the finding that DS7 cell lysate produces very low seeding activity in the tau split-luciferase assay, despite a high amount of aggregated tau (Figure 3.1D, 3.3G-I). This suggests the conformation of the aggregates induced in this mouse model is likely similar to the conformation of the original inoculum. The high seeding activity observed in DS10-inoculated mice, despite this strain only targeting mossy fiber tracts of the hippocampus, is also concordant with initial findings in the split-luciferase seeding assay (Figure 3.11E, 3.12C). These results are consistent with the different rates of spread observed by AT8 immunohistochemistry for DS4 versus DS6 and 9, while providing a
separate metric to quantify the spread of pathology for strains such as DS10. Taken together, we have observed clear strain-specific patterns of local pathology, local seeding, regional vulnerability, and rates of spread throughout brain networks. These data are most consistent with the idea that aggregate structure alone is sufficient to dictate several important aspects of tau pathology.
3.6 DISCUSSION

Overview of findings. Based on isolation of 18 putatively distinct tau prion strains in vitro, we have tested whether specific tau fibril conformations can account for several critical neuropathological features that are used to discriminate tauopathies. We characterized these strains by various biochemical and biological metrics, including induced morphology of cellular inclusions, seeding activity in dividing cells and primary neurons, detergent solubility, cellular toxicity, and limited proteolysis (Figure 3.1-3.4). We separately inoculated all 18 strains into the hippocampus of a transgenic mouse that expresses full-length human tau harboring the P301S mutation (PS19 mice). We observed several distinct patterns of tau accumulation in cell bodies, axons, and dendrites in the hippocampus, amidst larger patterns of induced pathology (Figure 3.5-3.6). Some strains (DS7 and 9) produced astrocytic tau plaques similar to those described in individuals with corticobasal degeneration (Figure 3.7-3.8), but most did not. To test for regional vulnerability, we injected 6 strains into 6 different brain regions, and waited 5 weeks to evaluate pathology. Many induced pathology in all regions (DS6, 7, and 9), while others exhibited restricted patterns in which very little or no pathology occurred despite direct exposure of neurons to a particular strain (DS4, 10, 11) (Figure 3.9-3.10). We next tested whether strains exhibited unique rates of propagation through the brain. In this case we observed some correlation with in vitro parameters: strains with high seeding activity tended to spread more rapidly through the brain, with one important exception—strain DS10—which only spread to the contralateral mossy fiber tracts of the hippocampus (Figure 3.11-3.12). Within the limits of our experimental system, these observations suggest that distinct tau prion strains can account for many of the features observed in
human tauopathies. This experimental system also allows one to make predictions that derive from the biochemical properties of a given strain (e.g. high seeding strains induce rapid, robust spread even upon a 1:10 dilution, as predicted in culture). More importantly, the characterization of unique strains in vitro using reductionist cell-based assays provides a critical step to determine the relationship between tau prion structure and biological activity.

**limitations of this experimental approach.** It is impossible at this time to directly control strain production in vivo using transgenic mice, or to propagate tau strains faithfully and indefinitely in vitro without using a cultured cell system. This work relies on a cell model that expresses a truncated form of tau with a fluorescent protein tag. However, this imperfect system has allowed us to propagate distinct tau prion strains indefinitely in cultured cells. Likewise, in animal models, we utilized an imperfect model that expresses full-length human tau (1N4R) that contains a disease-associated mutation (P301S) (Yoshiyama et al., 2007). While this obviously deviates from sporadic tauopathy that occurs in most cases, it has enabled us to rapidly and reliably induce unique tau pathology based on local inoculation. We cannot exclude the possibility that inoculated tau prions themselves are moving throughout the brain and inducing pathology based on local uptake rather than true trans-neuronal propagation (Rey et al., 2013). Nonetheless, our observations speak to a disease mechanism whereby strain-specific differences govern seeding, propagation, and specific regional vulnerability.

**the utility of studying isolated strains.** Most evaluations of prion activity in vivo involve injection of material purified from the brains of patients with different syndromes, or the production of fibrils that have distinct ultrastructural morphologies.
Both approaches are potentially confounded by heterogeneity of fibrillar species. Our prior work has indicated that human tauopathy brains, even those carefully defined by histopathology, exhibit enormous diversity of strains within and between individuals (Sanders et al., 2014). Thus, syndromes that appear to be clinically and neuropathologically identical are potentially quite distinct in terms of their strain composition. This presents obvious caveats when attempting to define strains present in human disease based principally on inoculation of purified aggregates into mice. In the case of fibril production in vitro, a multiplicity of structures will typically form. In this study, we isolated several unique tau strains from recombinant tau fibrils (Figure 3.2A). Further, as fibril preps are repeatedly amplified this population will gradually change in its composition. This has been clearly illustrated by recent studies, in which a single preparation of a-synuclein fibrils gradually changes from one dominant structure to another (Guo et al., 2013). In this work, we have stably propagated strains with specific biochemical properties indefinitely in a rarified culture system based on expression of tau RD with two disease-associated mutations fused to YFP. This has allowed us to work with stable strain preparations that most likely represent unitary structures, and has further enabled us to make predictions about their in vivo phenotypes based on properties observed in culture.

**Prion strains characterized in vitro.** Scrapie prion strains, described over 40 years ago, have distinct, yet reproducible patterns of incubation time, neuropathology, and behavioral phenotypes (Collinge and Clarke, 2007). Strains are distinct amyloid structures that faithfully replicate in a living system, and produce well-defined pathology. Thus, if a strain is identified, it is possible to predict incubation time and resultant
pathology (Collinge and Clarke, 2007). Recently, the term “strain” has been used widely
to refer to distinct neuropathology that results from injection of various tau or synuclein
preparations into mouse models (Bousset et al., 2013; Guo et al., 2013). Without isolation
of a single structural conformer that is stably replicated and produces a reliable
phenotype, these “strains” have limited mechanistic, therapeutic, or diagnostic
significance, as it is difficult to study them in a reductionist system or make biological
predictions based on a particular structure.

In this work we characterized multiple tau strains from recombinant, mouse, and patient
sources in vitro and in vivo. We began by isolating strains based on stable mother-
daughter maintenance in clonal cells. We then determined their seeding efficiency using a
quantitative split-luciferase reporter assay. We also determined the relative solubility of
each strain, and its toxicity when introduced into a reporter cell line with relatively high
tau RD-YFP expression. We observed no correlation between seeding activity and
insoluble protein, while toxicity correlated well, albeit imperfectly with seeding activity.
Finally, we probed strain structure using limited proteolysis. This provided the most
definitive differentiation among strains in tissue culture. The characterization of multiple
tau prion strains in vitro allowed us to directly correlate their effects in vivo. Seeding
activity correlated best with induction of local and distant pathology, but this was highly
imperfect, indicating a major role for other strain-specific parameters. With further, more
detailed study, we hope to link specific structural characteristics to various steps in
pathogenesis, i.e. to discern a “logic” that predicts biological effects. For example, cell-
type specificity (or at least preference) might be based on differential strain binding
affinities to heparan sulfate proteoglycans (Holmes et al., 2013a). Likewise, post-
translational modifications of monomeric tau within a target cell might render it more or less vulnerable to conversion by a specific strain.

**Distinct cellular pathologies in vivo.** Tauopathies are defined histopathologically by several criteria, especially the pattern of intracellular tau accumulation: neurofibrillary tangles, Pick bodies, threads, grains, axonal puncta, etc. (Kovacs, 2015). The presence or absence of specific glial pathology also contributes to the definition (Yoshida, 2014). Although we have readily observed patterns of pathology reminiscent of those described in patients, we have not attempted to link the patterns observed in humans to those in P301S mice, which express only a single isoform of mutant tau. Instead, we wish to emphasize how conformational differences in tau prion strains are sufficient to create an enormous diversity of patterns: neurofibrillary tangle-like pathology, soma versus axonal accumulation, grain-like structures, dendritic and axonal terminal deposits that resemble threads, and astrocytic plaque-like pathology. Further, three strain pairs (DS3/DS19; DS12/16; and DS6/15) that displayed similar biochemical features and limited proteolysis patterns produced similar patterns of neuropathology in vivo (Figure 3.5-3.6). These strains served to internally validate the methods we developed, and highlight the close relationship of strain type to induced pathology.

**Rates of propagation in vivo.** Neurodegenerative diseases progress at different rates for unknown reasons. Similar to previous observations regarding PrP prion strains (Legname et al., 2006), our data indicate that the characteristics of an individual tau prion strain are sufficient to dictate the rate at which pathology spreads throughout the nervous system. Seeding activity correlates with this phenomenon, but cannot explain it in isolation. Instead, the rate of spread appears to reflect a unique interaction of specific strains with
vulnerable cells. For example, strain DS10 seeds very strongly \textit{in vitro}, but fails to propagate pathology outside of the hippocampal mossy fibers. Likewise, DS6 seeds very strongly \textit{in vitro}, but exhibits a longer lag phase as it spreads to specific brain regions such as the entorhinal cortex versus the retrosplenial cortex. In contrast, DS9 showed more rapid spread to the entorhinal than the retrosplenial cortex. Taken together, our results suggest that the rate of propagation must be strongly influenced by raw seeding potential, i.e. the ability to convert monomeric tau upon direct introduction to the cytoplasm via lipofectamine, but also the ability of a given strain to spontaneously enter and replicate within a vulnerable cell.

\textbf{Distinct patterns of glial pathology.} Glial pathology occurs in certain tauopathies but not others (Kovacs, 2015). The underlying cause of these differences is unknown, and has been attributed to different tau isoforms, or other factors, but has not been predictably recapitulated in mouse models. We observed that certain strains produce AT8-positive pathology in patterns reminiscent of astrocytic plaques described in tauopathies, with localization of phospho-tau inclusions along the processes of GFAP-positive astrocytes (Yoshida, 2014). We cannot attribute these effects to tau seeding activity or toxicity, as we observed these phenomena in DS7 (low seeding, low toxicity) and DS9 (high seeding, high toxicity). Given that inoculates were identical with the exception of tau structure, and the transgenic mouse model expresses only a single tau isoform, we conclude that tau prion strains themselves dictate the resultant glial pathology.

\textbf{Distinct regional vulnerabilities.} The explanation of regional, or “selective” neuronal vulnerability in neurodegenerative diseases has long confounded investigators. Importantly, PrP prion strains have been observed to account for differential regional
involvement of the brain (Collinge and Clarke, 2007). Within the limits of our experimental system, our identification and characterization of distinct tau strains has allowed us to test whether aggregate structure itself defines regional vulnerability. We have found striking strain-specific regional differences, both in the pattern of spread from a single hippocampal inoculum, and also in vulnerability to a direct injection. These effects reliably tracked to individual tau strains, and were independent of the inoculum dose itself, which might have been expected to account for vulnerability. For example, 10-fold dilution of a potent strain, DS9, produced patterns of spreading pathology very similar to a full dose. Further, low-dose DS9 spread at a faster rate than a full dose of DS4, even though the initial “seed burden” of DS9 was less. By contrast, strain DS10 has relatively high seeding activity yet it selectively targets the mossy fibers of the hippocampus, and does not convert tau in several other brain regions even after direct inoculation.

We are not the first to observe that different tau fibril preparations produce unique patterns of pathology. Groups have inoculated tau purified from tauopathy patient brains or bulk brain homogenate (Boluda et al., 2015; Clavaguera et al., 2013a), and two groups have inoculated unique synuclein preparations (Bousset et al., 2013; Guo et al., 2013; Peelaerts et al., 2015). However, no prior studies can attribute these effects to a single, well-characterized strain or structure, or make predictions about the behavior of a strain in vivo from the biochemical properties of that inoculum. Given that we can now link specific pathology patterns to single tau prion strains, the experiments described here should enable new approaches to define how the structural characteristics of prions dictate neuronal vulnerability.
Implications for diagnosis and therapy. There is no effective therapy for any tauopathy, and precise diagnosis of tauopathy syndromes currently depends on histopathology. Our prior work suggests that this approach may have limitations, since we observed different strain composition patterns even in brains that were considered identical based on histopathology (Sanders et al., 2014). If distinct tau strains propagate between cells, this predicts that certain therapeutic antibodies or small molecules that target tau will be more effective against particular strains, and that, as for PrP, strain evolution may confound efforts to target a specific strain type (Ghaemmaghami et al., 2011; Giles et al., 2010; Li et al., 2010; Oelschlegel and Weissmann, 2013). The ability to create and propagate distinct strains in vitro presents a unique opportunity to create antibodies that target them more precisely. Further, with knowledge that distinct tau prion strains may underlie and predict the development of unique syndromes, the development of methods to identify and discriminate clinically relevant strains will greatly enhance diagnostic accuracy.

Conclusion. Useful scientific models allow testable predictions, and can explain a diversity of observed phenomena in nature. In this case, we propose a very simple idea: tau prion strains as defined in reductionist systems can account for enormous diversity of neuropathology. In other words, it appears possible to explain in large degree the range of phenotypes observed in tauopathies based principally on the tau prion structure(s) that underlie them. This study has not determined tau prion strain structure beyond differentiation by proteolytic digestion—we can discriminate, and thus characterize different species, but cannot draw specific structure/function correlations with regard to replication rate, cell biology, and neuroanatomy. Nonetheless, we propose that the
concept of tau prion strains as defined by specific conformations will have enormous power to elucidate the structural determinants that underlie and predict the pathological patterns of diverse human tauopathies.
3.7 FIGURES

Figure 3.1. Generation and characterization of a tau prion strain library. (A) A monoclonal HEK293 line stably expressing tau RD P301L/V337M (“LM”)–YFP (Clone 1/DS1) was treated with diverse sources of fibrillar tau seeds, and 90 monoclonal lines that stably propagated tau inclusions were derived and characterized by the indicated metrics. 18 strains were differentiated based on their unique properties in the indicated assays. (B) Several tau inclusion phenotypes were identified in the monoclonal tau strains: mosaic (magenta), ordered (blue), speckles (red), threads (orange), and disordered (brown box). With the exception of the mosaic phenotype, these inclusion phenotypes were stably propagated to daughter cells over months of division. A negative control cell line (diffuse) featured soluble tau (green box). (C) Limited proteolysis using pronase was used to differentiate the protected fibrillar cores in individual tau strains. Unique “fingerprints,” along with other metrics, were indicative of structurally distinct tau prion strains. (D) A tau RD P301S split-luciferase assay demonstrates that individual strains have large differences in their abilities to seed the aggregation of monomeric tau when introduced directly into the cytoplasm of dividing cells. Seeding ratio indicates relative luminescence relative to sham treatment. Biological quadruplicates with saturating quantities of lysate were averaged. Error bars represent S.E.M. for biological quadruplicates. (E) Differences in seeding abilities between strains are replicated in primary neurons. Primary hippocampal neurons expressing tau RD P301S-CFP and tau RD P301S-YFP were treated with lysates derived from various strains. 96-hours later, neurons were fixed, and the percentage of cells featuring seeded aggregates was determined by FRET flow cytometry. Error bars represent S.E.M. for biological
quintuplicates. (F) Relative seeding abilities for strains are preserved in primary neurons expressing full-length tau. Primary hippocampal neurons expressing 1N4R tau P301S-YFP were exposed to lysates from each strain and the extent of seeding was semi-quantitatively determined at various time points (D = number of days) based on the extent of visible YFP puncta (0-5: 0 = none, 5 = abundant inclusions). Strains showed variable lag times and extent of seeding, which correlated with their ability to seed in the split-luciferase complementation assay. (G) Strains differentially induce the formation of insoluble tau aggregates in primary neurons. Triton-X was used to remove soluble tau and primary neurons were stained for conformationally altered tau (MC1) five or eight days following seeding. Strains showed significant differences in their abilities to seed further aggregation in neurons, which paralleled differences in the split-luciferase complementation assay.
Figure 3.1
Figure 3.2. Origin of strains and primary neuron seeding. (A) 18 putative tau strains propagated on a monoclonal HEK293 background (Clone 1/DS1) were derived from diverse recombinant protein, transgenic mouse, and patient sources as indicated in the table. Some strains were passaged from cell lines previously characterized. Strains are color-coded based on their associated inclusion morphology. (B) Strains showed variability in their ability to seed the formation of insoluble tau aggregates in primary hippocampal neurons. Lysates from individual strains were added to primary neurons. Eleven days later, neurons were fixed and Triton-X was used to extract soluble tau. Accumulation of conformationally altered tau was assessed by MC1 staining. Strong seeders in the tau split-luciferase complementation assay produced more insoluble tau pathology in neurons.
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<td>Rec Fibrils</td>
<td>N/A</td>
</tr>
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<td>Clone 9</td>
</tr>
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<tr>
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<td>CBD3 (Disordered)</td>
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<td>CBD3 (Speckles)</td>
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<td>CBD3 (Disordered)</td>
</tr>
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<td>N/A</td>
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<td>AD1 (Speckles)</td>
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</tr>
<tr>
<td>19</td>
<td>Ordered</td>
<td>Rec Fibrils</td>
<td>N/A</td>
</tr>
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</table>

**Abbreviations:**
- Rec fibrils = recombinant tau fibrils
- AGD = argyrophilic grain disease
- AD = Alzheimer’s disease
- CBD = corticobasal degeneration
- CTE = chronic traumatic encephalopathy

**HeK Morphology**
- Diffuse (1)
- Mosaic (2)
- Ordered (3, 10, 14, 19)
- Speckles (4, 5, 7, 8, 9, 12, 16, 17, 18)
- Threads (6, 15)
- Disordered (11, 13)

**Figure 3.2**
Figure 3.3. Seeding ability but not insoluble tau levels correlates with toxicity in vitro.

(A) Strains showed large differences in their abilities to seed monomeric tau as determined by a tau split-luciferase complementation assay. Indicated amounts of strain lysate were transduced into tau RD P301S split-luciferase cells, seeding ratios relative to sham treatment were determined, and titration curves were plotted using non-linear regression with one-phase decay fit. Error bars represent S.E.M for biological quadruplicates. (B) Based on titration curves in the tau split-luciferase complementation assay, the EC$_{50}$, inflection point, and peak seeding ratio were determined for each strain. The inflection point represents the amount of lysate required to achieve a 50% increase in luminescence relative to sham treatment. (C) Peak seeding significantly correlated with EC$_{50}$s for the strain library in the tau split-luciferase complementation assay. (D) Strains displayed significant differences in toxicity. Strains were transduced in biological triplicates into cells overexpressing both tau RD LM-CFP and tau RD LM-YFP. 72-hours later, equivalent numbers of aggregate-containing (FRET+) cells were sorted for each condition by FRET flow cytometry. For the negative control (DS1), aggregate-negative (FRET-) cells were sorted. Sorted cells were given a week to proliferate in technical sextuplicates. Aggregate-positive (FRET+) and aggregate-negative (FRET-) cells were then quantified by flow cytometry. The presence of FRET- cells in certain conditions reflects the fact that some strains lose the aggregated state with cell division. Error bars represent S.E.M. of biological triplicates. Technical sextuplicates were first averaged for each biological replicate. (E) Aggregate-positive (FRET+) cells were quantified and plotted after one week of growth. Error bars represent S.E.M. of biological triplicates. (F) Toxicity significantly correlates with seeding ability for the strain library. The number of aggregate-positive (FRET+) cells...
for a strain was plotted against its peak seeding ratio in the tau split-luciferase complementation assay. Strains that seed more efficiently are associated with reduced growth of aggregate-positive cells. (G) Sedimentation analysis was performed on the strain library. Lysates were ultracentrifuged and tau as well as a loading control protein (cofilin) were probed in the total, supernatant, and pellet (Tot = total, Sup = supernatant, Pel = pellet). Blots are representative for biological quadruplicates. (H) Sedimentation analysis indicates that all strains feature the majority of tau in the insoluble pellet fraction. Densitometric analysis of tau in the total, supernatant, and pellet fractions was used to calculate supernatant to total ratios (a higher ratio indicates a smaller proportion of tau in the insoluble pellet). Error bars represent S.E.M. of biological quadruplicates. (I) Densitometric analysis of tau in the insoluble pellet fraction suggests variability in the extent of aggregated tau in the various strains. Error bars represent S.E.M. of biological quadruplicates. (J) Despite differences in the quantity of insoluble tau for various strains, this metric does not correlate with seeding ability as reflected by the peak seeding ratio. (K) Quantity of insoluble tau does not correlate with a strain’s toxicity.
Figure 3.3

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<td>603</td>
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<td>1.14 ± 0.04</td>
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<tr>
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<td>1.13 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>4508</td>
<td>10000</td>
<td>1.80 ± 0.25</td>
</tr>
<tr>
<td>4</td>
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<td>30</td>
<td>6.00 ± 0.28</td>
</tr>
<tr>
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<td>370</td>
<td>3</td>
<td>35.33 ± 1.40</td>
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<td>900</td>
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<td>1000</td>
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<tr>
<td>19</td>
<td>1081</td>
<td>3000</td>
<td>1.67 ± 0.07</td>
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*Inflection point is the amount of lysate required to achieve a 50% increase in luminescence relative to sham treatment.
**Figure 3.4. Seeding ability but not total tau correlates with toxicity *in vitro*.** (A) A tau strain’s seeding ability correlates with its toxicity. The number of aggregate-positive (FRET+) cells for a strain was plotted against its associated EC$_{50}$ in the tau split-luciferase complementation assay. Strains that reached their half-maximal seeding at lower protein concentrations were associated with decreased cell growth. (B) The association between greater seeding and toxicity was further strengthened by a significant correlation between cell growth (FRET+ cells) and inflection point, the amount of lysate required for a strain to show significant seeding in the tau split-luciferase complementation assay. (C) Densitometric analysis of tau in the total fraction suggests variability in the extent of total tau in the various strains. Error bars represent S.E.M. of biological quadruplicates. (D) Despite differences in total tau, this property does not correlate with a strain’s peak seeding ratio. (E) There is no correlation between a strain’s total tau and its toxicity as assessed by relative growth of aggregate-positive (FRET+) cells.
Figure 3.4
Tau prion strains induce diverse patterns of hippocampal tau pathology.

(A) Tau strains (10 µg) were injected into the left hippocampus of young PS19 mice (n=3 per condition). Mice were sacrificed 8-weeks post injection. Relevant hippocampal regions are diagramed onto a representative mouse hippocampus (DG, dentate gyrus; mf, mossy fibers; Sub, Subiculum). (B) DS1 injection produced no AT8 tau pathology. Representative images of CA1 and CA3 are displayed for whole hippocampal images for DS1-19. Scale bars represent 50 µm. (C) DS10 produced AT8 positive mossy fiber (mf) “dot” pathology, with limited CA1 pathology as observed previously. (D) DS14 seeded mf dots similar to DS10, as well as tangle-like pathology, indicating that it is a distinct strain despite its other similar features to DS10. (E) DS7 induced “wisps” that resemble neuropil threads, but may fall within axon terminals and the dendritic tree of pyramidal neurons. (F) DS18 pathology included wisps and mf dots similar to DS7 and 10 respectively, as well as “grains” that were found throughout much of the hippocampus. (G-H) Several strains produced different levels of tangle-like AT8 pathology in CA1 and CA3 of the hippocampus. (G) DS2, 3, 11, 19 induced rare AT8 pathology in pyramidal CA1 neurons. The localization of AT8 staining varied in certain cases (DS2 primarily in cell bodies), but this may simply represent different levels of maturity of tangles. (H) DS4, 8, 12, 13, 16, 17 induced slightly stronger tangle-like pathology in CA1 of the hippocampus (“low tangles”). CA3 showed limited or no tangle pathology at this time point. (I) DS5 and 9 produced AT8 tangle-like tau pathology that reached CA3 of the hippocampus as well as CA1 pyramidal cells (“medium tangles”). Tangles appeared relatively consolidated within the soma of neurons. (J) DS6 and 15 display the highest...
level of tangle-like AT8 pathology ("high tangles"). Highly consolidated pathology was observed throughout cell bodies and axons of CA1 and CA3 neurons.

Figure 3.5
Figure 3.6. Strains induce unique tau pathology in various brain regions. (A) AT8 pathology present in the ipsilateral hippocampus of DS1-19. Morphology refers to the original cell line phenotype inoculated into the hippocampus. Scale bars represent 250 µm. (B) Spread of AT8 tau pathology to the contralateral hippocampus and ipsilateral entorhinal cortex in DS5 and 9 (medium tangles), and DS6 and 15 (high tangles). Low levels of DS1 baseline staining can be observed in the ipsilateral EC, and is at the level expected for animals at this age. Scale bars represent 250 µm for the whole hippocampus, and 50 µm for EC. (C) DS10 and 14 show mossy fiber dots in the contralateral hippocampus. DS14 CA1 AT8 tangle-like pathology is much stronger than DS10. Scale bars represent 250 µm for the whole hippocampus, and 50 µm for CA1 and CA3. (D) DS18 shows grains throughout CA1, and wisps in the ipsilateral EC, showing the unique features induced by this strain can spread to distant regions. Scale bars represent 250µm for the whole hippocampus, and 50µm for CA1 and EC.
Figure 3.6

<table>
<thead>
<tr>
<th>HEK Morphology</th>
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<tbody>
<tr>
<td>Diffuse (1)</td>
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<td>Threads (6,15)</td>
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<tr>
<td>Disordered (11,13)</td>
</tr>
</tbody>
</table>

A

B

C

D

Figure 3.6

Ipsi Hippocampus

Contra hippocampus

Contra hippocampus

Contra hippocampus

Ipsi EC

Ipsi EC

Ipsi EC
Figure 3.7. Specific strains induce astrocytic tau pathology. (A) AT8 tau pathology 8-weeks after injection with DS1, 6, 7, or 9. DS1 did not induce tau pathology. DS6, 7 and 9 developed strong AT8 staining in ipsilateral and contralateral hippocampi. DS7 and 9 developed diffuse, circular-shaped accumulations of AT8 staining that did not appear to localize to a neuronal cell body (black arrow heads). Scale bars represent 250 µm for the whole hippocampus, and 50 µm for CA1. (B) Co-staining of AT8 (green) for phospho-tau, GFAP (red) for astrocytes, and DAPI (blue) for cell nuclei. DS1 showed limited GFAP staining, and no AT8 pathology. DS6 showed strong AT8 staining with limited overlap of AT8 staining. DS7 and 9 injected mice showed astrocytic plaque-like pathology that either deposited within or around GFAP-positive processes of astrocytes. Scale bars represent 25 µm for left-most column, and 10 µm for all remaining images.
Figure 3.7
Figure 3.8. Certain strains reliably produce robust astrocytic pathology that increases over time. (A) Quantification of the number of mice that displayed astrocytic tau pathology at 4, 8, or 12 weeks post-inoculation with different strains. Two experimental inoculations were assessed for astrocytic pathology. Greater than 85% of animals inoculated with DS7 or 9 display astrocytic plaque-like pathology by 8-weeks. Other strains do not show any consistent plaque pathology until 12-weeks. (B) Average number of plaques counted in ipsilateral hippocampus of time course inoculation mice. Several other strains begin to develop astrocytic pathology by 12-weeks, but the number of plaques is far lower than that observed in DS7 and 9. (C) Representative images of the astrocytic plaque-like pathology observed in 1/3 animals inoculated with DS12, 15, 16, and 18 at 8-weeks post injection. Scale bars represent 50 µm.
Figure 3.8

A

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</tr>
<tr>
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<td>0/3</td>
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</tr>
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</table>

B

C

Number of Astrocytic Plaques Ipsilateral Hippocampus at 4wks

Number of Astrocytic Plaques Ipsilateral Hippocampus at 8wks

Number of Astrocytic Plaques Ipsilateral Hippocampus at 12wks

Figure 3.8
Figure 3.9. Tau strains preferentially seed pathology in specific brain regions. (A) Six tau strains were injected simultaneously into six brain regions: sensory cortex (SC); caudate/putamen (CP); visual cortex (VC); hippocampus (Hip); thalamus (Thal); inferior colliculus (IC) (5 µg per region). Mice that received DS1 (negative control), 4, 6, 7, 9, 10, or 11 strain injections were kept for 5-weeks post-inoculation before collection and assessment of AT8 tau pathology (n=3 per condition). (B) Strains preferentially induce tau pathology in specific brain regions. Slices that contained the injection sites were stained for AT8 phospho-tau. Each injection site was assessed in a blinded fashion for tau pathology on a 0-3 scale (none, low, medium, high pathology). The level of background AT8 pathology at each injection site was accounted for by subtracting the level of pathology present in DS1 mice within each brain region. A binned heat map was generated to represent the level of pathology observed at the injection site for each strain. (C) Representative images are displayed for each brain region injected with the different tau strains. Scale bars represent 100 µm.
Figure 3.9

**AT8 Pathology at Injection Sites**

<table>
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<tr>
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<th>CP</th>
<th>VC</th>
<th>Hip</th>
<th>Thal</th>
<th>IC</th>
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</table>

The table above shows the pathology at injection sites for different regions (SC, CP, VC, Hip, Thal, IC) across different datasets (DS1 to DS11). The color scale indicates the severity of pathology, with dark blue representing the highest and light blue the lowest intensity.
Figure 3.10. DS10 reliably produces mossy fiber dot pathology. (A) Mice inoculated with DS10 for the regional vulnerability experiments develop mossy fiber dots as expected in the ipsilateral and contralateral hippocampus. Scale bars represent 250 μm for the whole hippocampus, and 50 μm for CA3.

A  
<table>
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<th>Ipsi Hippocampus</th>
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<th>Contra Hippocampus</th>
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</thead>
<tbody>
<tr>
<td>DS10</td>
<td></td>
<td></td>
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</tbody>
</table>


Figure 3.11. Strains induce different rates of tau pathology spread. (A)

Sedimentation analysis was performed on cell lysate used for this time course inoculation experiment. Each strain contains a large amount of insoluble material (T, total; S, soluble; I, insoluble). Western blot analysis of insoluble tau was performed on three biological replicates. For each experiment, the soluble fraction was loaded at 2x the concentration of the total and insoluble fractions. A coflin loading control was performed on the blots to verify the same amount of cell lysate was added for each strain. (B) The level of insoluble tau present in each strain was quantified by measuring the mean grey value of the insoluble tau western blot band. Samples were normalized to the mean grey value of coflin in the total cell lysate fraction. An ANOVA shows strains have significantly more insoluble tau than DS1. A two-way t-test demonstrates DS10 and DS4 do not contain significantly different levels of insoluble tau (ns for P > 0.5; * for P ≤ 0.05; ** for P ≤ 0.01). Error bars represent S.E.M. of biological triplicates. (C) Strains were inoculated into the hippocampus of young PS19 mice (n=6 per condition per time point). DS6 and 9 lysate diluted 1:10 in HEK293 cell lysate was also injected as part of this experiment (n=4-5 per condition per time point). Mice were collected at 4, 8, or 12 weeks and brain slices were stained for AT8-positive tau pathology. (D) Representative images of ipsilateral and contralateral CA1 are displayed for each strain at 4, 8 and 12 weeks post-injection. Tau pathology spreads to the contralateral hippocampus at different time points. Diluted DS6 and 9 lysate showed faster spread than concentrated DS4, and more robust spread than DS7 and 10 at 8-weeks post-injection. Scale bars represent 50 µm. (E) Spread of mossy fiber dot pathology occurs by eight weeks in DS10 mice. Dot pathology
appears to eventually develop in DS4 mice, but that spread appears to be delayed compared to DS10.
Figure 3.11
Figure 3.12. Conformation affects the rate and pattern of spread of tau pathology.

(A) Slices from mice injected with each strain at each time point were stained for AT8 pathology. Tau pathology was quantified in a blinded fashion on a 0-3 scale, and averaged for each location within a given condition (n=6 per condition). A continuous heat map was generated. Regions are listed on the x-axis, and conditions/time points are on the y-axis. (B) Limited heat maps were generated from the above data set (Figure 3.12A). Ipsilateral (Ip) and contralateral regions were included to assess pattern and rate of spread of pathology (retrosplenial cortex, RS; entorhinal cortex, EC; sensory cortex, SC; thalamus, Thal; CA1 of hippocampus, CA1; locus coruleus, LC; subiculum, Sub). Time points are arranged in order from earliest (4-weeks) to latest (12-weeks). Diluted DS6 and 9 lysates were also displayed (DS6 1:10 and DS9 1:10). (C) Homogenized tissue from the hippocampus, thalamus, or sensory cortex of mice 8-weeks after inoculation with strains was applied to tau biosensor cell lines. After 48-hours, cells were collected and flow cytometry was performed to quantify the level of seeding activity in each region by integrated FRET density (IFD = percent FRET-positive cells*median fluorescent intensity of FRET positive cells). DS4 showed lower spread of seeding activity to the contralateral hippocampus at 8-weeks. DS10 displayed high seeding activity despite limited AT8 pathology, while DS7 showed low seeding activity despite high AT8 pathology. DS6 and DS9 showed seeding activity in ipsilateral thalamus. A one-way ANOVA with Bonferroni correction for multiple comparisons was performed between ipsilateral DS1 and every other sample within a given region. (* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001). Error bars represent S.E.M., n = 4-5.
Figure 3.12
Figure 3.13. Summary of mice used in inoculation experiments. List of experimental mice discussed in this study, including sex, age at time of surgery, and age at time of tissue collection.

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Chapter 4: Conclusions and Future Directions
4.1 PREFACE

This chapter contains reference to unpublished data (see Appendix) that will be used in a future manuscript:


Note: In section 4.4, three tau aggregate-associated proteins are termed “Protein A”, “Protein B”, and “Protein C”. See Appendix, which is redacted for one year to protect ongoing research, for identity of these proteins.
4.2 SUMMARY OF RESULTS

Over the past decade, extensive data has been amassed, which supports the hypothesis that fibrillar aggregates (amyloids) spread along neuronal networks to drive the progression of neurodegenerative diseases (Guo and Lee, 2014; Sanders et al., 2016; Walker and Jucker, 2015). However, the mechanisms that underlie the phenotypic diversity observed in the tauopathies (e.g. Alzheimer’s disease, Pick’s disease, corticobasal degeneration) and other common proteopathies have remained elusive. How is it that one pathogenic protein fibril (e.g. tau) can drive such diverse clinical outcomes? Decades of research have suggested why such phenotypic diversity exists in the prionopathies (e.g. Creutzfeldt-Jakob disease). The prion protein (PrP) can form a multitude of amyloid structures that stably replicate with remarkable fidelity over tremendously long time scales in animal models of prion disease (Collinge and Clarke, 2007). Based on simple polymorphism at the level of amyloid structure, diverse syndromes can result, each having its own characteristic incubation period, regions of the brain affected, and cell types featuring pathogenic accumulations of PrP prions (Collinge and Clarke, 2007; Gambetti et al., 2011). In my dissertation work, I asked whether self-propagating tau prion strains are similarly responsible for the phenotypic diversity observed in the tauopathies, a category of diseases that features over a dozen major neurodegenerative syndromes (Lee et al., 2001) (Figure 4.1). I reasoned that for this to be the case, tau must form numerous different conformations (i.e. prion strains) that replicate themselves with fidelity through living systems over time scales that would approach those seen in the gestation of patient diseases. Moreover, these strains must
result in distinct experimental tauopathies that re-capitulate unique features of human diseases (e.g. selective vulnerability of different cell types and brain regions). Further, I hypothesized that different tauopathies should be able to be differentiated based on the tau amyloid conformations (strains) that they feature.

In order to test these hypotheses, I developed a reductionist approach to study tau aggregate biology, generating a dividing, monoclonal human cell line (DS1) that stably expresses a truncated version of tau fused to a YFP reporter (Chapter 2) (Sanders et al., 2014). These cells never spontaneously develop tau aggregates. However, when one adds recombinant tau amyloids (but not other fibrils such as α-synuclein and amyloid beta) to the media, tau-YFP within the cells rapidly converts from a soluble to an aggregated state. The aggregated state then becomes self-propagating, inherited as an epigenetic trait by daughter cells. Similar epigenetic inheritance of the aggregated state had previously been reported for PrP, (Taraboulos et al., 1990a), Sup35 (Krammer et al., 2009), and SOD1 (Münch et al., 2011) prions, indicating that mammalian cells feature a remarkable ability to replicate fibrillar amyloids. Next, I isolated numerous monoclonal lines that featured aggregates, and identified two clones (DS9 and DS10) that appeared distinct based on their patterns of tau aggregate accumulation (DS9: small juxtanuclear aggresome with nuclear speckles; DS10: large juxtanuclear aggresome with no nuclear speckles). These inclusion morphologies remained unaltered following six months of cell division. Further analyses (limited proteolysis, sedimentation analysis, seeding efficacy, aggregate size, toxicity) confirmed that each cell line stably replicated a different aggregate structure.
But to be true prion strains, phenotypes must be independent of the original cellular context. A stringent test of this is to passage individual conformations into naïve cells or through living animals to determine whether associated characteristics are maintained.

Were DS9 and DS10 *bona fide* prion strains? Identical phenotypes were reproduced after introduction of denatured (boiled) lysates into naïve DS1 cells. More amazingly, in collaboration with Sarah Kaufman, I found that these strains produced different patterns of tau pathology in transgenic mice and maintained their associated characteristics following intracerebral passage through three generations of mice. Immunoprecipitated full-length tau from the third generation of mice was inoculated into naïve DS1 cells, and 12 clones were blindly isolated for both DS9 and DS10 conditions. For each strain, in 11 of 12 cases, newly derived clones recapitulated the parent strain’s distinctive inclusion morphology, limited proteolysis fingerprint, and seeding efficiency. To summarize this portion of the dissertation (Chapter 2), I found that tau is capable of forming at least two amyloid structures (prion strains) that stably propagate over months of cell division, are resistant to harsh denaturation, and maintain their properties following serial passage *in vivo*. Thus, it is conceivable that such long-term, stable information transfer via tau amyloid strains could contribute to the development of human tauopathies, which progress over years or decades.

There are over a dozen neurodegenerative tauopathies and each affects distinct brain regions and exhibits discrete patterns of tau aggregate accumulation in neurons and glia (Lee et al., 2001). Moreover, within individual syndromes, patients may develop neurodegeneration that progresses at very different rates (Armstrong et al., 2000; Huang et al., 2014; Mann et al., 1992; Thalhauser and Komarova, 2012). Could tau amyloid
polymorphism account for these diverse clinical features? In collaboration with Sarah Kaufman, I examined the degree to which tau can form diverse amyloid conformations and their resulting biological consequences (Chapter 3). I inoculated my monoclonal cell line lacking tau aggregates (DS1) with diverse recombinant, transgenic mouse, and patient sources of tau aggregates to generate hundreds of monoclonal lines that stably propagate tau aggregates. Based on numerous assays (inclusion morphology, limited proteolysis fingerprint, sedimentation analysis, seeding efficiency, toxicity), a panel of 18 putatively distinct tau prion strains (DS2 to DS19) was established. I determined that there was a strong correlation between a strain’s seeding efficiency and toxicity in vitro, which was independent of the amount of insoluble tau that it featured. In other words, conformation alone likely accounts for differential toxicity. It appears that the faster a strain replicates (i.e. adds monomer to the free ends of fibrils), the more deleterious it is for a cell. Next, we inoculated the panel of 18 strains into the hippocampi of transgenic mice and found eight different patterns of tau aggregate accumulation. Seven putative strains, which featured the most divergent properties by various in vitro and in vivo metrics, were examined in additional longitudinal, in vivo experiments. Several strains were unique in causing astrocytic pathology, and this was independent of the amount of neuronal pathology they induced. Moreover, when inoculated into a series of brain regions, strains caused different degrees of pathology in each location. In other words, specific regions of the brain feature selective vulnerability to different tau amyloid conformations. Finally, we found that strains spread at different rates along neuronal networks to distant locations of the brain. Seeding ability in vitro predicted this propensity to spread. To summarize, it appears likely that simple differences at the level
of tau aggregate structure are sufficient to account for diverse criteria (inclusion morphologies, selective cell and network vulnerability, rates of progression) that differentiate human tauopathies (Chapter 3).

Finally, I asked whether individual tauopathies could be differentiated based on strain composition (Chapter 2). I inoculated cells expressing tau-YFP with immunoprecipitated tau from the brains of 29 patients with 5 different tauopathies (Alzheimer’s disease, corticobasal degeneration, Pick’s disease, argyrophilic grain disease, progressive supranuclear palsy). Numerous monoclonal lines were isolated per patient and a separate observer blindly scored them based on tau inclusion morphology, a proxy for unique tau strains. I found that Alzheimer’s disease, corticobasal degeneration, and Pick’s disease were relatively homogenous but distinct from each other based on strain composition, whereas argyrophilic grain disease and progressive supranuclear palsy were significantly more diverse. This suggested that some tauopathies likely feature a dominant tau prion strain, which spreads throughout neuronal networks to drive disease progression. Contemporary work from two other labs, which used inoculation paradigms and compared tau lesion profiles in patients and treated mice, supported this hypothesis (Boluda et al., 2015; Clavaguera et al., 2013a). However, it should be noted that in our cell reporter system, specific inclusion morphologies have been associated with several distinct strains (see Chapter 3). Moreover, the reporter cell line can only detect strains that it can stably propagate. This combined with the fact that some patients feature multiple strains suggests that individual tauopathies may be defined by a population or “cloud” of strains that evolve over time (Bateman and Wickner, 2013; Collinge and Clarke, 2007). Nevertheless, it is likely that improved assays will be able to differentiate
human tauopathies based on their strain composition, which will have major implications for how we diagnose and treat this incurable class of neurodegenerative diseases.
4.3 TOWARD A STRUCTURAL UNDERSTANDING OF TAU PRION BIOLOGY

In my dissertation work, I created a panel of 18 putatively distinct tau prion strains propagated on the same monoclonal human cell line background (Chapter 3). These strains have very different biological consequences for the cell and for the organism. For example, in dividing cells, strains are differentially trafficked to distinct locations, resulting in unique distributions of tau aggregates in the nucleus and cytoplasm. In transgenic mice, certain strains preferentially affect different regions of neurons and cause different degrees of pathology in specific regions and cell types (e.g. astrocytes). Moreover, strains seed monomeric tau with efficiencies that span several orders of magnitude, resulting in very different rates of spread in vivo. The biophysical basis for these differences is completely unknown.

A structural understanding of what differentiates our panel of tau prion strains is essential to making further progress toward linking tau amyloid conformation and phenotype. This problem can be tackled on several different fronts, each of which will benefit from improved protocols for purifying tau aggregates from cell lines. In work outside the scope of this dissertation, I developed a protocol to purify tau aggregates to approximately ~50% purity by taking advantage of their relative size and detergent insolubility (Figure AP.1). We can now grow individual strains in large flasks to isolate micrograms of tau fibrils putatively featuring a single amyloid structure. Currently, we are using size exclusion chromatography and fluorescence-activated cell sorting (FACS) techniques to improve purity and yield. We hypothesize that the tremendous size and stability of
micron-sized tau aggresomes, which pellet along with nuclei at 250xg, will allow us to isolate these structures using a FACS protocol similar to that used for fluorescently labeled inflammasomes (Franklin et al., 2014). This project is currently ongoing with methods being optimized by a new graduate student in the lab.

Following the optimization of purification methodologies, numerous experiments are planned to examine the structure and biophysical characteristics of various tau aggregate conformers. Initial efforts will aim to determine structural differences between our most well characterized strains, DS4, DS6, DS7, DS9, DS10, and DS11, which have strikingly divergent properties in vitro and in vivo. First, we plan to look at ultrastructural features of aggregates using electron microscopy, determining whether there are disparities in width and periodicity, similar to differences that have been described for tau fibrils isolated from different tauopathy brains (Crowther, 1991; Crowther and Wischik, 1985; Lee et al., 2001) and those generated from unique sources in vitro (Morozova et al., 2013). Next, we will perform a series of experiments to test whether the biophysical model proposed by Weissman (yeast prions) (Tanaka et al., 2006; Toyama et al., 2007) and Prusiner (PrP prions) (Colby et al., 2009; Legname et al., 2006), which posits that there is a direct correlation between a conformer’s fragility and its seeding propensity, is generalizable to intracellular mammalian prions such as tau. We will examine the fragility of our strains by taking advantage of sonication protocols previously used by our lab to differentiate tau conformers formed in vitro (Frost et al., 2009b). We hypothesize that strains that seed more efficiently (e.g. DS6, DS9) will fragment more readily than those that seed poorly (e.g. DS7, DS11) as determined by sedimentation analysis and EM-based fibril length measurements before and after sonication. We also plan to use a
guanidinium hydrochloride (GdHCl) denaturation assay, which I previously developed to differentiate tau conformers formed in vitro (unpublished data), to examine the stability of our various strains. Together, these experiments will determine whether differential frangibility of tau fibril conformers drives the rate of pathology progression in vivo or whether a more complex interplay of fibril growth (Safar et al., 2015) and co-factor (e.g. chaperone) recruitment (Frederick et al., 2014; Stein and True, 2014a; 2014b) should be considered.

The experiments outlined above would provide broad insights into the relationship between fiber stability and rates of seeding and spread. Ultimately, however, we would like to develop an appreciation of different tau amyloid conformations at an atomistic level in order to better understand why these structures drive very different biological outcomes. Amyloid polymorphism can result from differences in beta-strand peptide composition (i.e. portion of protein that forms fibrillar core), alignment of beta-sheets (parallel vs. antiparallel), and whether identical amino acids on different monomers interact (in register vs. out of register) (Toyama and Weissman, 2011; Tycko, 2015). Although x-ray crystallography has given us an appreciation of the packing arrangements of amyloidogenic hexapeptides from model amyloids (Nelson et al., 2005; Sawaya et al., 2007), this technique has not been amenable to studying the full-length versions of these proteins, which likely contain amino acid stretches critical to encoding conformational diversity. Rather, much insight into amyloid structure has been gleaned from solid-state nuclear magnetic resonance spectroscopy (ssNMR) studies (Tycko, 2015), including recent work that determined potential structures for the amyloid beta fibrils derived from the brains of different AD patients (Lu et al., 2013). However, this method requires
synthesizing proteins with $^{15}$N and $^{13}$C residues at specific sequences and milligram quantities of protein (Tycko, 2015). Thus, in order to use ssNMR to differentiate our fibril conformers, the structures will have to be templated onto synthetic peptides in vitro using successive seeding reactions, an experimental set-up comparable to protein misfolding cyclic amplification (Saborio et al., 2001) and RT-QuIC (Wilham et al., 2010). Such serial seeding reactions are prone to generating new amyloid conformations, independent of the source material, which evolve with time (Guo et al., 2013; Meyer et al., 2016; Paravastu et al., 2008; Qiang et al., 2011; Tycko, 2015). Thus, stringent controls (e.g. EM determination of periodicity and width of fibrils, limited proteolysis fingerprints, seeding profiles, etc.) will need to be included to ensure that the end structures are representative of the original strains generated in cells. To circumvent confounding by-products that could conceivably arise in serial amplification reactions, long-term ssNMR structural studies of tau prion conformers should aim to make use of newly developed in-cell NMR techniques, directly templating synthetic tau containing $^{15}$N and $^{13}$C residues with strain lysate (Frederick et al., 2015; Theillet et al., 2016).

However, such experiments will require further refinement of current protocols and external collaborations.

Alternative methods that require lower quantities of protein will also afford insights into tau prion strain structures. We plan to subject FACS-purified tau aggregates to pronase (i.e. limited proteolysis) and will then use mass spectrometry to determine how the protected amyloid cores differ between strains. In addition, UT Southwestern features state-of-the-art cryo-EM core facilities. Currently, we are in discussions with a new faculty recruit about using single-particle electron cryomicroscopy to differentiate tau
strains at atomic resolution. Overall, numerous complementary studies, both internal and
with collaborators, will allow tremendous insights into the structural rules that govern tau
prion strain biology. This should allow the rational design of compounds, dyes, and
antibodies that target and/or differentiate specific tau fibril structures. Moreover, we hope
to ultimately decipher the fundamental logic that determines why strains replicate at
different rates and affect different regions of the cell and brain.
4.4 TAU STRAINS AS A TOOLKIT FOR PROBING DIVERSE ASPECTS OF INTRACELLULAR PRION BIOLOGY

The trans-synaptic spread of intracellular prions such as tau requires specific events: escape from a pre-synaptic terminal, adherence to a post-synaptic membrane, endocytosis, escape into the cytosolic compartment, conversion of soluble monomer to the aggregated state, and replication/fragmentation to continue the “prion life cycle” (Sanders et al., 2016). Further, fibrillar aggregates must be actively cleared by the cell, since in transgenic mouse models of proteopathies, amyloidogenic inclusions disappear once expression of the associated protein is abrogated (Mallucci et al., 2007; Polydoro et al., 2013; Walker et al., 2015; Yamamoto et al., 2000). Finally, individual amyloid conformations (strains) must differentially “infect” specific networks, cell types, and regions of the cell to account for histopathological observations in patients and mouse models of disease (Boluda et al., 2015; Clavaguera et al., 2013a; Sanders et al., 2014). The tau strain library documented in this dissertation affords tremendous opportunities to glean mechanistic insights into the cellular biology of intracellular prion metabolism (i.e. its “life cycle”) (Figure 4.2). We plan to use biased (based on known pathways and proteomic results) and unbiased genetic and pharmacological screens to begin to interrogate these mechanisms.

Of the steps of the prion life cycle, uptake has been most well characterized. Previously, we established that heparan sulfate proteoglycans (HSPGs) are essential for recombinant tau aggregate uptake and seeding in vitro and in vivo (Holmes et al., 2013a). Entry into the cell depends on trimeric and larger tau assemblies triggering a critical mass of HSPGs
to actively stimulate fluid-phase macropinocytosis (Mirbaha et al., 2015). Further, in unpublished work, we have determined that different patterns of HSPG sulfation are critical for the uptake of amyloid beta, α-synuclein, and tau fibrils (Stopschinski, Holmes, et al., in preparation). We plan to test whether this is also the case for tau strains. It is conceivable that different cell types and regions of the brain display selective vulnerability to distinct strains based on their sulfated HSPG profiles. In order to test this hypothesis, we will examine whether seeding of a tau aggregation biosensor cell line (Furman et al., 2015; Holmes et al., 2014) by various tau strains (DS4, DS6, DS7, DS9, DS10, DS11) is differentially inhibited by heparin with specific sulfation profiles (fully sulfated, 2-O desulfated, 6-O desulfated, and N-desulfated heparin). If there are differences, we will confirm the results via gene editing, knocking out a panel of genes that regulate HSPG sulfation using CRISPR/Cas9 technology (gRNA constructs generated by B. Stopschinski). Ultimately, we will validate interactions using carbohydrate arrays (Stopschinski, Holmes, et al., in preparation) and purified tau aggregate conformers (see Section 4.3). This work will represent a first step toward determining potential mechanisms that contribute to strain-cell selective vulnerability.

Dividing mammalian cells propagate fibrillar amyloids indefinitely to daughter cells (Krammer et al., 2009; Münch et al., 2011; Sanders et al., 2014). It is unknown whether this is a passive process (i.e. shearing forces in cytoplasm contribute to further fragmentation and seed generation) or whether disaggregation machinery exists analogous to Hsp104 in yeast to actively generate new seeds (Chernoff et al., 1995; Glover and Lindquist, 1998; Lee et al., 2003; Shorter and Lindquist, 2004). Further, the mechanisms that cause distinct tau strains to accumulate in specific regions of the cell are
unknown (Clavaguera et al., 2013a; Sanders et al., 2014). Finally, whether different proteins and pathways contribute to the degradation of distinct strains remains unexplored. I have begun to investigate these questions in work that is outside the scope of this thesis, by coupling tau aggregate interactome data with targeted CRISPR/Cas9 genetic screens (see Appendix). Taking advantage of the size and insolubility of juxtanuclear aggresomes (Figure AP.1), I purified tau aggregates from DS9 and DS10 cells and identified 79 candidate proteins that putatively associate with tau amyloids (Figure AP.2). 33 of these 79 proteins were involved in the ubiquitin-proteasome system or selective autophagy/vesicular trafficking, pathways critical for the clearance of misfolded proteins and insoluble aggregates (Schreiber and Peter, 2014). Four of these proteins have previously been linked to genetic forms of ALS/FTD and multisystem proteopathy. Surprisingly, I also identified four SR-rich proteins, which likely play a role in constitutive RNA splicing (Figure AP.2). 9 (out of 9 tested) of the 79 hits were confirmed to co-localize with DS9 and DS10 tau aggregates by immunocytochemistry, whereas a panel of other proteins not identified in the proteomic analysis (n=11) failed to co-localize (Figure AP.3-6). Many of the identified proteins also co-localized with full-length tau aggregates (Figure AP.7) and huntingtin polyQ inclusions (Figure AP.8). Overall, co-localization analysis confirmed the power of the proteomic approach to identify proteins that associate with intracellular amyloids. However, it must be noted that the approach failed to identify striking interactome differences between DS9 and DS10 aggregates.

Next, I designed a targeted CRISPR-Cas9 screen (n=25 proteomic hits, n=9 negative controls) to examine whether the most abundant proteins in tau aggregates affected their
maintenance, trafficking, turnover, or seeding into naïve cells. First, I demonstrated that
the designed protocol was effective at knocking out a variety of endogenous proteins
(Figure AP.9A-D). Knockout of the panel of genes failed to identify any one protein that
is essential for aggregate maintenance or seeding into naïve cells (data not shown).
However, knockout of two proteins (Protein A and Protein B) altered aggresome
architecture (Figure AP.9). Knockout of Protein B, but not master regulators of
autophagic pathways, greatly extended tau aggregate lifespan (Figure AP.9). This
suggests that Protein B plays a pivotal role in tau aggregate metabolism (Figure AP.9).
Currently, another graduate student is examining whether knockout of proteins that
interact with Protein B affect tau aggregate turnover or maintenance of the aggregated
state. Further, I identified an SR protein (Protein C) that is essential for DS9 tau
aggregate entry into splicing speckles (Figure AP.10). Future studies will expand the
breadth of knockouts and will examine whether specific proteins are critical to tau
aggregate turnover in non-dividing cells (e.g. primary neurons or induced pluripotent
stem cells with tau aggregates), where aggregate dilution does not occur by cell division.

In collaborative and future work, we also plan to use unbiased chemical and
CRISPR/Cas9 genetic screens to interrogate steps of the proteopathic seed life cycle.
Recently, several labs have made critical advances in generating platforms that make use
of CRISPR/Cas9 technology to allow for unbiased genome-wide screens in mammalian
cells (Gilbert et al., 2014; Shalem et al., 2014). Each of these platforms utilizes stable
Cas9 (or catalytically dead Cas9) expression in a dividing cell population, along with a
lentivirus-encoded library of pooled guide RNAs that are stably inserted into the genome
to drive knockout (Shalem et al., 2014), knockdown (Gilbert et al., 2014), or upregulation
(Gilbert et al., 2014) of individual genes at a single cell level. With a sufficiently large cell population, genome-wide coverage can be achieved with multiple guide RNAs targeted against every gene. A stimulus is applied and cells containing a specific phenotype (e.g. fluorescence, viability) are selected. Deep sequencing is then performed to determine gRNAs that are enriched in the selected population relative to cells prior to stimulus application. To date, these screens have been successful in determining critical regulators of cell death (e.g. by certain chemotherapeutic drugs) (Shalem et al., 2014).

We have developed several cell assays that are amenable to such screens (Furman et al., 2015; Holmes et al., 2014). In unpublished work, I created a tau RD-CFP/YFP FRET cell line that stably propagates the DS9 strain (LM39-9). This cell line can be differentiated from a parent line (LM39-1), which lacks tau aggregates, by measuring single cell FRET using a flow cytometer (Furman et al., 2015; Holmes et al., 2014). We can use FACS to sort cells containing aggregates from those that lack inclusions based entirely on FRET signal. We will use a genome-wide knockout (GeCKO) screen (Shalem et al., 2014) to determine whether there are essential proteins for maintenance of the aggregated state, selecting for cells that decrease in FRET following infection with specific gRNAs.

Second, we will use a tetracycline-regulated tau aggregate FRET line (OFF11::DS11, unpublished) to determine proteins whose knockout phenocopies that of Protein B (see Figure AP.9). We will turn off tau expression for 8 days and will then re-initiate expression for two days. We will then use FRET flow cytometry to isolate cells that contain aggregates, thus enriching the population for gRNAs that prolong aggregate lifespan. This screen should elucidate key regulators of tau aggregate turnover in dividing cells and may determine the pathway in which Protein B acts. Third, we will perform a
viability screen that makes use of LM10, a cell line overexpressing tau RD-CFP/YFP, which features 100% lethality when exposed to DS9 lysate (Figure 3.2). We will expose LM10 cells to DS9 lysate, select for cells that contain aggregates by FACS, and will then identify gRNAs that prevent cell death. Future work will repeat these screens using CRISPRa (upregulation) (Gilbert et al., 2014). Finally, the Stanley Prusiner lab (UCSF) in collaboration with Daiichi Sankyo is pursuing a drug discovery program to identify chemicals that inhibit seeding of DS1 by DS9 lysate (Woerman et al., 2015). Several promising compounds have been identified and target drugs are currently being tested in transgenic mouse models of tauopathy (Yoshiyama et al., 2007) to determine whether or not they slow the progression of pathology. Together, these unbiased screens should begin to elucidate critical regulators of tau aggregate seeding, maintenance, turnover, and toxicity.
4.5 IMPLICATIONS FOR DIAGNOSTICS, THERAPEUTICS, AND OTHER PROTEOPATHIES

Based on this dissertation work, it is now clear that specific tauopathies (e.g. Alzheimer’s disease, corticobasal degeneration, Pick’s disease) can be differentiated based on tau strain composition (Chapter 2) (Sanders et al., 2014). However, the methodology required to categorize specific patients is time-intensive and laborious, demanding weeks of cell culture and the selection of numerous monoclonal lines (Sanders et al., 2014). We are now developing assays that are more quantitative and high-throughput. As a first step, a post-doctoral scholar (Jenny Furman) and graduate student (Jaime Vaquer-Alicea) have generated a panel of monoclonal cell lines that express various tau RD point mutants with FRET pair tags (unpublished). Individual cell lines respond very differently to unique strains. For example, mutant tau in line A is seeded strongly by DS9, whereas mutant tau in line B is seeded more robustly by DS7. Remarkably, using biological triplicates from our strain library, individual conditions can be binned based on how they induce tau aggregation in the panel of lines. In other words, we can determine the identity of a strain purely based on how the cell panel responds to its lysate. Based on this data, we determined that DS12 and DS16 are in fact identical strains, whereas all others in the panel are unique. This cell panel assay is now being utilized to group patients based on strain composition. Thus far, it can reliably identify CBD and AD patients, which further supports the hypothesis that distinct tau strains cause these diseases (Boluda et al., 2015; Clavaguera et al., 2013a; Sanders et al., 2014). Generation of additional cell lines expressing various truncated versions and isoforms of tau may allow further differentiation of patients. Finally, our lab is pioneering epitope-based antibody mapping
approaches to differentiate tau conformers in biological samples. Together, we believe that such techniques will prove to be superior to traditional histopathological approaches in grouping patients based on the causative pathogen (i.e. tau prion strain) of their disease. Ultimately, we envision coupling such assays to sensitive methods (Atarashi et al., 2011; Colby et al., 2007; Wilham et al., 2010) of seed amplification (e.g. PMCA and RT-QuIC approaches) to diagnose patients using fluid samples (e.g. CSF, blood).

It is also important to emphasize that the findings of this dissertation have important implications for how tauopathies are treated. We believe that our data strongly supports the validity of the prion hypothesis in patients, as a trans-cellular templating mechanism would most parsimoniously explain how different patients could feature dissociable but homogenous strain populations throughout their brains. Therefore, future therapeutics should target tau in the extracellular space to prevent the trans-cellular spread of tau amyloids and the resulting progression of neurodegeneration. Antibodies (Asuni et al., 2007; Boutajangout et al., 2011; d’Abramo et al., 2013; Funk et al., 2015; Yanamandra et al., 2013; 2015) and heparinoids (Holmes et al., 2013a) should be effective in this regard. Nevertheless, we also wish to emphasize that not all tau aggregates are created equal. It is likely that strains will respond differently to distinct therapeutics. For example, an antibody that is effective at clearing an AD tau strain may not be as effective at removing a CBD tau strain due to their unique amyloid structures. There is precedent for this in prion disease models, where certain strains are cleared by specific drugs leading to the emergence of drug-resistant strains (Ghaemmaghami et al., 2011; 2009; Giles et al., 2010; Li et al., 2010). In other words, prion strains are prone to Darwinian evolution based on selection pressures (Li et al., 2010; Oelschlegel and Weissmann, 2013;
Weissmann et al., 2011). Thus, therapies that target diverse tau aggregate species should be favored over those that target individual conformers. Finally, we envision that it will be possible to develop different PET imaging agents to differentiate unique tauopathies (Okamura et al., 2014; 2013; Ossenkoppele et al., 2016; Schöll et al., 2016; Schwarz et al., 2016). Unpublished work indicates that current tracers identify AD tau tangles but not many other types of tau aggregates. We believe that this is due to the fact that different tau strains arise in different diseases and animal models of tauopathy. Future studies should attempt to identify specific tracers that respond to different strains. Finally, we wish to highlight that techniques developed to probe phenotypic diversity in the tauopathies are likely relevant to the study of other proteopathies. Synucleinopathies and TDP43opathies also feature tremendous diversity in clinical presentation (Halliday et al., 2011; Van Langenhove et al., 2012). In fact, work from the Prusiner lab and unpublished work from our lab suggests that multiple systems atrophy and Parkinson’s disease may be caused by different α-synuclein strains (Prusiner et al., 2015; Woerman et al., 2015). Thus, the concepts underscored in this dissertation likely have broad implications for the development of therapeutics and diagnostics for all non-infectious neurodegenerative proteopathies.
4.6 CONCLUDING REMARKS

The prion hypothesis is a unifying mechanistic paradigm that accounts for diverse features of sporadic neurodegenerative diseases (Prusiner, 2013; Sanders et al., 2016; Walker and Jucker, 2015). Not only does it elucidate why fibrillar protein aggregates spread hierarchically along neuronal networks in diverse syndromes, but it also explains how a single pathogenic protein can give rise to distinct diseases. In this dissertation, I have built the foundations for linking tau amyloid structure to clinical phenotype through the generation of diverse tau prion strains. Based on simple yet to-be-defined differences at the level of tau amyloid conformation, strains affect specific regions of individual cells as well as different cell types, which likely contributes to the characteristic neuropathological phenotypes observed in distinct tauopathies. Moreover, it appears likely that individual strains have the ability to affect dissociable networks to drive clinical phenotypes. Ultimately, a structural understanding of the tau amyloid conformations that drive distinct tauopathies (Figure 4.1) and mechanistic interrogation of their biological effects (Figure 4.2) will enlighten therapeutic and diagnostic interventions for this class of neurodegenerative diseases.
4.7 FIGURES

Figure 4.1. Strains: linking structures and disease phenotypes. Different self-propagating tau amyloid conformations (i.e. strains) drive unique phenotypes that span from the cell to the clinic. Well-characterized tau strains, which feature structural differences that have yet to be elucidated, are deposited in specific patterns in individual cells. In humans, this is reflected by the unique patterns of tau aggregate deposition in neurons and glia that are characteristic of each individual tauopathy. Via unknown mechanisms, specific cell types and regions of the brain are preferentially affected. The work described in this doctoral dissertation represents a starting point for determining how a specific amyloid conformation can drive a unique experimental tauopathy. Ultimately, this will allow a further appreciation of the individual tau amyloid structures that drive specific phenotypes in human diseases.
Figure 4.2. Tau strains as a toolkit for probing diverse aspects of aggregate biology.

The ability to propagate an array of tau prion strains on the same monoclonal background will open up many avenues of future research. Currently, we are developing more sensitive and quantitative assays for differentiating strains derived from patient specimens. We are actively improving our aggregate purification protocols, with the ultimate goal of determining structural variations between our strains. Specifically, we seek to determine why it is that certain fibril conformations spread at such different rates and why they are trafficked into distinct inclusion types. Elucidation of the tau aggregate interactome (see Appendix) is guiding targeted screens, which seek to determine mechanisms of tau aggregate degradation and disaggregation. Finally, we will be employing unbiased genetic and drug screens to determine whether there are active mediators of tau aggregate inheritance, toxicity, degradation, and seeding.
Appendix: Characterization of the Tau Aggregate Interactome
AP.1 PREFACE

This chapter contains unpublished data and text that will be used in a future manuscript:


Author contributions: D.W.S. and M.I.D. designed the experiments. D.W.S. collected and analyzed the data. J.V.-A. performed DNAJC7 Western blots and has performed additional experiments not included in this write-up. A.S. and L.T.G. performed immunohistochemistry on patient samples. H.M. performed mass spectrometry. I.S., F.U.H., and M.S.H. performed complementary SILAC studies (data not included). M.I.D. supervised the work. D.W.S. wrote the text.
In mammalian cells, tau amyloids stably propagate distinct fibrillar structures (“prion strains”) to daughter cells indefinitely. However, it is unknown whether there is a mammalian amyloid replication machine, similar to the Hsp104 chaperone system in yeast, which allows for the efficient inheritance of amyloids (“seeds”). Further, the mechanisms of tau aggregate clearance in cells and in vivo are unclear. In this study, we establish an unbiased, mass spectrometry approach to identify proteins that co-purify with micron-sized, insoluble tau aggregates (“aggresomes”) in dividing mammalian cells. We identify 79 candidate proteins that putatively associate with tau amyloids and use immunocytochemistry to confirm 9 of these hits. We report preliminary studies using CRISPR-Cas9 to knockout 25 of the top hits. We were unable to identify a single non-essential protein necessary for tau aggregate inheritance. However, DNAJC7 and SQSTM1 knockout both have major effects on the organization of tau aggregates. Using a tetracycline-regulated tau aggregate cell line, we demonstrate that DNAJC7 is critical for tau aggregate clearance. Mechanistic studies of DNAJC7’s role in tau aggregate metabolism are in progress. Identification of the pathological tau aggregate interactome will inform future studies examining mechanisms underlying tau aggregate metabolism in non-dividing cells and in vivo.
Prions are higher-order assemblies of protein that are capable of communicating information stably by template-based propagation of a specific fibrillar conformation (Prusiner, 1998; Sanders et al., 2016). These self-amplifying structures may be pathological, leading to progressive and fatal neurodegenerative diseases (Walker and Jucker, 2015), or physiological, allowing the inheritance of beneficial traits, as in the case of *Saccharomyces cerivisiae* (yeast) (Halfmann et al., 2012; Holmes et al., 2013b; True and Lindquist, 2000; True et al., 2004). Remarkably, the prion form for any given protein is not a single structure. Rather, individual prions can take a variety of forms or “strains” that link structure to syndrome in animals or severity of phenotype in yeast (Collinge and Clarke, 2007; Toyama and Weissman, 2011). In mice, prion strains can replicate their associated fibrillar structures with remarkable fidelity, leading to distinct incubation times, histopathological profiles, and behavioral phenotypes, which maintain themselves even after years of passage (Collinge and Clarke, 2007). In cells and mice, tau prion strains are especially robust, maintaining their structures over months of cell division and passage through multiple generations of transgenic mice (Sanders et al., 2014). Whether there are active mechanisms that govern the continued replication of specific prion strains in mammalian systems is unclear.

In yeast, the mechanisms that govern the inheritance of the aggregated or prion state have been well defined. The Hsp104 chaperone protein forms a two-tiered, hexameric complex that interacts with other co-chaperones to thread fibrils through its central pore, further fragmenting (or “disaggregating”) them into smaller assemblies in an ATP-dependent
fashion (Chernoff et al., 1995; Glover and Lindquist, 1998; Lee et al., 2003; Shorter and Lindquist, 2004). These smaller assemblies are then capable of “seeding” further aggregation by growth of monomer on their free ends, thus amplifying the prion state (Collins et al., 2004a). Notably, there is no mammalian homologue to this protein. However, recent work has suggested that three-component chaperone systems are capable of disaggregating both amorphous and fibrillar aggregates in vitro (Gao et al., 2015; Nillegoda et al., 2015; Rampelt et al., 2012; Shorter, 2011). To date, there is no clear evidence that the same machinery (or analogous systems) fragment fibrillar aggregates in mammalian cells.

Further, it is also now clear that fibrillar aggregates are capable of being degraded in mammalian systems. In inducible mouse models of neurodegenerative diseases, fibrillar deposits will be cleared once expression of the associated protein is turned off (Mallucci et al., 2007; Polydoro et al., 2013; Walker and Jucker, 2015; Yamamoto et al., 2000). Protein degradation can occur via the ubiquitin-proteasome system, chaperone-mediated autophagy, or selective autophagy (Schreiber and Peter, 2014). The latter appears to be the most likely scenario by which these fibrillar assemblies are actively degraded due to their immense size. Intriguingly, mutations to proteins that regulate selective autophagy of ubiquitin aggregates cause familial forms of several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and multisystem proteopathy (Cirulli et al., 2015; Deng et al., 2011; Johnson et al., 2010; Laurin et al., 2002; Maruyama et al., 2010). This suggests that failure to degrade fibrillar aggregates is sufficient to cause specific neurodegenerative syndromes. Although much work has been performed delineating the mechanisms by which selective autophagy of
ubiquitinated cargo occurs (see Khaminets et al., 2016) for Review), additional work is required to identify all the major proteins that recognize and mark seeding-competent prions for degradation. Furthermore, whether all fibrillar assemblies are degraded using the same pathway requires elucidation.

In the present work, we performed an unbiased, mass spectrometry study of proteins that co-purify with micron-sized tau aggregates (“aggresomes” (Johnston et al., 1998)) derived from two monoclonal tau prion strains to gain insights into fundamental mechanisms of tau aggregate metabolism, trafficking, and degradation. We show by immunocytochemistry that proteins identified by this method do in fact co-localize with tau aggregates. We use CRISPR-Cas9 to knockout top hits from our analysis and find that no single protein is essential to inheritance of the prion state by dividing mammalian cells. We identify a co-chaperone, DNAJC7, which affects the organization of tau aggresomes and influences the rate at which tau seeds are cleared. Further work will determine the mechanisms by which this poorly characterized chaperone influences tau aggregate metabolism.
AP.4 MATERIALS AND METHODS

Molecular cloning. With the exception of gRNA constructs, all plasmids used were described previously (Sanders et al., 2014). The pCRISPRv2 plasmid, which contains both Cas9 and a site to insert gRNAs, was a kind gift from the Feng Zhang lab (Massachusetts Institute of Technology). For each gene selected for knock-out, six separate twenty base-pair Guide RNAs (gRNAs) were designed according to previously published protocols and datasets (Shalem et al., 2014). Forward and reverse oligonucleotides containing the gRNA, a PAM site, and BsmBI overhangs were generated (Integrated DNA Technologies). Oligonucleotides were then sub-cloned into the pCRISPRv2 vector according to the following protocol. 5 µg pCRISPRv2 was digested with 3 µL FastDigest BsmBI (Fermentas) and backbone DNA was purified using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer’s protocol. Meanwhile, forward and reverse oligonucleotides were annealed at a concentration of 10 µM by placing them at 95°C and dropping the temperature by 5°C per minute until 25°C was reached. Annealed oligonucleotides were then diluted 1 to 100 in molecular grade water. Next, annealed oligonucleotides were ligated into BsmBI-digested pCRISPRv2 by combining 50 ng digested product with 1 µL annealed oligonucleotide (diluted 1:100) and 1 µL Quick Ligase (New England Biolabs) in a total volume of 10 µL. Ligation reactions were then transformed into Stbl3 bacteria (Invitrogen) according to manufacturer’s protocol, and individual colonies were amplified and selected. Correct insertion of gRNAs was confirmed by DNA sequencing.

Cell culture. HEK293T and HEK293 cell lines were cultured in complete media: Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) with 1% penicillin/streptomycin
(Gibco) and 10% fetal bovine serum (HyClone). Cell lines were maintained in a humidified incubator at 37°C, 5% CO₂. For washing cells, Dulbecco’s phosphate buffered saline (Life Technologies) was used. Trypsinization for passaging was achieved using 0.05% Trypsin-EDTA (Life Technologies).

**Lentivirus production.** Lentivirus was generated as described previously (Araki et al., 2004; Sanders et al., 2014). For production of pCRISPRv2 gRNA lentivirus, six gRNA plasmids were first pooled at equivalent DNA concentrations. HEK293T cells were plated at 1x10⁶ cells/well in a 6-well plate. 18 hours later, cells were co-transfected with three plasmids: PSP (1200 ng), VSV-G (400 ng), and FM5 or pCRISPRv2 (400 ng), after incubating the DNA mix with 250 µL OptiMEM (Gibco) and 7.5 µL TransIT-293 (Mirus) for 15 minutes. 48 hours later, media was collected from the dying cells. The media was spun at 250xg for 5 minutes to remove dead cells and debris. Lentivirus-containing supernatant was then stored at -80°C until addition to cells.

**Stable HEK293 cell line generation.** Monoclonal Tau RD (amino acids 244-372 of the 2N4R isoform of tau) P301L/V337M-EYFP HEK293 cell lines (DS1, DS9, DS10, OFF1::DS10) were generated and described previously (Sanders et al., 2014). To generate additional polyclonal lines, HEK293 cells were plated at 50,000 cells/well in a 12-well plate. 24 hours later, 1 mL media containing lentivirus (FL tau 4R1N P301S or Huntingtin Exon1 Q72-EYFP) was added to the wells. For both polyclonal lines, cells were grown for 72 hours in the presence of virus. Cells were then passaged into a 6-well plate and grown to confluency, followed by subsequent passage and amplification in a 10 cm dish. Once confluent, cells were stored in liquid nitrogen until use.
To generate monoclonal lines, the polyclonal Htt Exon1 Q72-EYFP line was diluted sparsely in 10 cm dishes, so that there were fewer than 25 cells per dish. Next, cells were grown for a week until they formed discrete, visible colonies. Cloning cylinders (Bel-Art Products) were then used to pick single colonies, and these were passaged into wells of a 12-well plate. Upon confluency, the lines were passaged into 6-well plates. A monoclonal Htt Exon1 Q72-EYFP line that stably propagated polyQ aggregates to daughter cells (Q72E) was amplified to confluency in a 10 cm dish and was stored in liquid nitrogen until use.

**Immunocytochemistry.** Monoclonal cell lines were passaged from 10 cm dishes to coverslips in 24-well plates and were grown for 48 hours. Media was removed and cells were fixed with either 300 µL 3% PFA (most ICC experiments) or 300 µL ice-cold methanol (100%) (MLF2, VCP ICC) for 15 minutes. Fixative was removed and replaced with PBS. Cells were then permeabilized with 0.25% Triton-X in PBS for 15 minutes. Permeabilized cells were blocked with blocking buffer (0.1% Triton-X, 10% Normal Goat Serum, PBS) for 60 minutes at room temperature. Primary staining was performed overnight in blocking buffer at 4°C with the following antibodies: Rb α-Coilin (1:200, H-300 sc32860), Mo α-SAFB (1:500, 6F7 ab8060), Mo α-FBL (1:100, 38F3 ab4566), Mo α-SMN1 (1:200, 2B1 sc-32313), Mo α-PSF/SFPQ (1:100, Sigma P2860), Rb α-SRRM2 (1:2000, ab122719), Mo α-phospho-SR/Sc35 (1:2000, ab11826), Mo α-PML (1:50, sc966), Rb α-Pinin (1:25, ProteinTech 18266-1-AP), Mo α-Ubiquitin (1:100, Enzo FK2), Mo α-SQSTM1 (1:50, ab56516), Rb α-NBR1 (1:25, ProteinTech 16004-1-AP), Mo α-TRAFD1 (1:50, ThermoScientific MA5-17190), Mo α-MLF2 (1:50, B-6 sc-166874), Rb α-VCP (1:25, H-120 sc-20799), Rb α-DNAJC7 (1:50, ProteinTech 11090-1-AP), Mo α-
SRSF1 (1:100 sc-36532), Rb α-SRSF11 (1:100, Atlas HPA008762), Rb α-ACIN1 (1:50, CST 4934), Rb α-SRSF7 (1:100, Sigma HPA043850), Rb α-snRNP70 (1:50, EMD Millipore 06-1297), or Mo α-Tau (1:200, HJ9.3). Cells were washed three times with 0.1% Triton-X in PBS and were then counter-stained with goat α-mouse or goat α-rabbit AlexaFluor-546 (1:400, Life Technologies) for 1 hour in the dark at room temperature. Cells were washed three additional times with 0.1% Triton-X in PBS and nuclei were stained with 1:1000 DAPI in 0.1% Triton-X for 10 minutes in the dark. DAPI solution was replaced with PBS and coverslips were mounted on Prolong Gold Antifade reagent (Life Technologies), sealed with nail polish, and placed at 4°C prior to confocal analysis.

**Immunohistochemistry.** Patient tissue sections were dewaxed in fresh xylene using three 10-minute washes. Sections were then dehydrated by serial treatment with 100% ethanol then 96% ethanol then 80% ethanol. Next, sections were blocked with BloxAll (Vector Laboratories) for 15 minutes followed by three PBS washes. Antigen retrieval was performed using citrate buffer and autoclaving for five minutes. Sample were then blocked for lipofuscin using 0.8% Sudan Black in 100% ethanol. Additional blocking was performed with 5% milk/PBS (with 0.05% Tween). Primary staining was performed overnight with mouse α-tau (1:500, CP13, gift from Peter Davies) and rabbit α-SRRM2 (1:200, ab122719) in blocking buffer. After three additional washes, secondary staining was performed with horse α-mouse Dylight-488 (1:300, Vector Laboratories) and goat α-rabbit Dylight-488 (1:300, Vector Laboratories). Sections were washed four additional times and were then mounted with VECTASHIELD HardSet (Vector Laboratories). Slides were placed at 4°C prior to confocal analysis.
Confocal microscopy. For confocal microscopy, a Zeiss Axiovert 200M microscope was coupled to a Zeiss LSM 5 PASCAL system. For the collection of all images, a pinhole size of 0.8 µm was used.

Western Blots. OFF1::DS10 cell pellets were thawed on ice and lysed in PBS with 0.05% Triton X-100 and a cOmplete mini protease inhibitor tablet (Roche) by triturating 10x and incubating at 4°C for 15 minutes. Sequential 5-minute centrifugations were then performed at 500xg and 1000xg to clarify the lysate. A Bradford assay (Bio-Rad) with BSA standard curve was performed and protein concentrations were normalized to 4 µg/µL with addition of lysis buffer. 10 µg of total protein was denatured in 2x sample buffer (2.5 µL sample, 2 µL BME, 5.5 µL PBS, 10 µL 2x Laemmli buffer) with the aid of five minutes of boiling. 13 µL of each sample was loaded onto a NuPAGE 10% Bis-Tris gel (Life Technologies). Gels were run at 150 V for 60 minutes. Protein was transferred to Immobilon P (Millipore) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked for 1 hour in 5% milk/TBS-T. Membranes were then probed with either Mo α-SQSTM1 (1:500, ab56516), Mo α-TRAFD1 (1:1000, ThermoScientific MA5-17190), or Rb α-DNAJC7 (1:1000, EPR13349 ab179830) overnight at 4°C. The next day, membranes were washed four times with TBS-T and counter-probed with goat α-mouse HRP (1:4000, GE Healthcare) or goat α-rabbit HRP (1:4000, Jackson Immunotherapy) for 1.5 hours. Membranes were washed four additional times with TBS-T and finally with plain TBS. Membranes were exposed to ECL Prime Western Blotting Detection System (Fisher Scientific) for 3 minutes and were developed using a digital Syngene imager.
Identification of aggresome-associated proteins by mass spectrometry. DS1, DS9, and DS10 were grown to confluency in two T300s per condition. Cells were harvested, pelleted, and washed, prior to storage as 0.5 T300 pellets (four total) at -80°C. For each condition, three pellets were thawed on ice, and each was lysed by trituration in 1 mL ice-cold PBS with 0.25% Triton-X and a dissolved protease inhibitor tablet (cOmplete Mini, Roche) followed by a 15 minute incubation on ice. Aggresomes and nuclei were collected by centrifuging at 1000xg for 15 minutes followed by re-suspension in 400 µL lysis buffer. An Omni-Ruptor 250 probe sonicator was then used at 30% power for thirty 3-second pulses to partially dissolve the pellets. Samples were centrifuged at 250xg for 5 minutes, and the supernatant was set aside as Fraction B. Pellets were re-homogenized in an additional 400 µL lysis buffer and sonication and centrifugation was repeated. The final supernatant was added to the previous Fraction B (800 µL volume total). A Bradford assay (Bio-Rad) with BSA standard curve was performed and the protein concentrations were calculated for the nine fractions. Protein concentrations were normalized to 1.1 µg/µL. 72 µL of 10% sarkosyl was added to 650 µL of each sample in ultra-centrifuge tubes (Beckman Coulter) and samples were rotated end-over-end at room temperature for one hour. Samples were then spun at 186,000xg for 60 minutes, supernatant was set aside, and pellets were washed with 1 mL lysis buffer prior to an additional 30 minute 186,000xg spin. Final pellets were re-suspended in 30 µL 2% SDS/2% BME/PBS by boiling and trituration. 5 µL of Fraction B supernatants and pellets were loaded onto NuPAGE 10% Bis-Tris gels (Life Technologies) and were run at 150 V for 60 minutes. Gels were washed 1x with water and were then stained with SimplyBlue
SafeStain (Life Technologies). Images of gels were captured using a digital Syngene imager.

For LC-MS/MS-based detection of proteins, 20 µL re-suspended Fraction B pellets were run 1 cm onto an Any kD Mini-Protean TGX gel (Bio-Rad) followed by Coomassie Blue staining. Whole lanes were excised using ethanol-washed razor blades and gel samples were cut into 1 mm$^3$ chunks. Gel pieces were reduced with DTT and alkylated with iodoacetamids (Sigma-Aldrich) and were then digested overnight with trypsin (Promega). Next, excised proteins were subjected to solid-phase extraction cleanup with Oasis HLB plates (Waters). The processed samples were then analyzed by LC-MS/MS using a Q Exactive mass spectrometer (Thermo Electron) coupled to an Ultimate 3000 RSLC-Nano liquid chromatography system (Dionex). Samples were injected onto an 180 µm i.d., 15-cm long column packed with reverse-phase material ReproSil-Pur C18-AQ, 1.9 µm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Peptides were eluted with a gradient from 1-28% buffer B (80% (v/v) ACN, 10% (v/v) trifluoroethanol, and 0.08% formic acid in water) over 60 minutes. The mass spectrometer was capable of acquiring up to 20 MS/MS spectra for each full spectrum obtained. Raw mass spectrometry data files were converted to a peak list format and analyzed using the central proteomics facilities pipeline (CPFP), version 2.0.3 (Trudgian and Mirzaei, 2012; Trudgian et al., 2010). Peptide identification was performed using the X!Tandem (Craig and Beavis, 2004) and open MS search algorithm (OMSSA) (Geer et al., 2004) search engines against the human protein database from Uniprot, with common contaminants and reversed decoy sequences appended (Elias and Gygi, 2007). Fragment and precursor tolerances of 20 ppm and 0.1 Da were specified, and three miscleavages were allowed.
Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met was set as a variable modification. Label-free quantitation of proteins across samples was performed using SINQ normalized spectral index Software (Trudgian et al., 2011). Finally, Spectral counts were added across triplicates. Proteins with a spectral count of greater than 5 in DS9 and/or DS10, but not identified in DS1 were reported, as were proteins with at least two-fold enrichment in DS9 or DS10 relative to DS1.

**Seeding of Full-Length Tau Aggregation.** Polyclonal HEK293 cells overexpressing untagged Full-Length Tau 4R1N P301S (FL PS) were generated and plated in 24-well plates at 120,000 cells/well. 18-hours later, cells were transduced (lipofectamine-2000) with either 50 µg of DS1 or DS9 lysate. 24 hours later, cells were re-plated into 6-well plates and grown to confluency. On Day 4, cells were re-plated 1:40 onto coverslips in 24-well plates. 48 hours later, cells were fixed and stained for tau and indicated proteins.

**Knockout of Endogenous Proteins using CRISPR-Cas9.** Lentivirus encoding Cas9 and indicated pooled gRNAs was produced (see Lentivirus Production). OFF1::DS10 or DS9 cells were plated at 5000 cells/well in 96-well plates. 18 hours later, 150 µL of media was removed and replaced with 150 µL lentivirus supernatant. 72 hours later, cells were passaged 1:8 into new 96-well plates and virus addition was repeated. 72 hours later, cells were passaged into 24-well plates for maintenance. Experiments examining the effect of knockouts were performed at two to three weeks following the first addition of lentivirus. For lethal knockouts, cells stopped growing or died within ten days following lentivirus addition. Knockout of overexpressed tau RD-YFP protein was achieved within a week in >99% of cells. Toxicity (VCP) and YFP knockout controls
were performed in all experiments as a proxy for sufficient viral titer to achieve high efficiency of knockout.

**Flow Cytometry.** Single cell YFP fluorescence measurements were performed using a MACSQuant VYB (Miltenyi).

**“On-Off-On” Tau Aggregate Degradation Time Courses.** OFF1::DS10 cells were treated with two rounds of indicated pooled gRNA lentivirus (see Knockout of Endogenous Proteins using CRISPR-Cas9) and were maintained in 24-well plates to assess viability. Two weeks later, a time course examining the decay of tau aggregate seeding activity in the various non-lethal knockouts was performed as follows. Confluent 24-wells were re-suspended in 1 mL media and 3.5 µL cells were re-plated into 200 µL total volume in 96-well plates. Tau RD-YFP expression was turned off using 30 ng/mL doxycycline for 1 day, 2 days, 3 days, 4 days, or 5 days. After five days, cells reached confluency and were passaged onto coverslips. 48 hours later, at which point tau RD-YFP expression reached its maximum, cells were fixed and cells containing or lacking inclusions were manually counted. Six replicates of 150+ cells were counted per condition and averages were calculated.

**Statistical analysis.** One-way analysis of variance with Bonferroni’s multiple comparison test was used to assess statistical significance relative to non-target controls.
AP.5 RESULTS

Identification of putative tau aggresome-associated proteins. In previous work, we created a monoclonal human embryonic kidney cell line (DS1) that overexpresses an aggregation-prone, truncated version of tau with two patient-derived mutations and a fluorescent YFP protein tag (Tau RD-YFP) (Sanders et al., 2014). These cells never form intracellular tau aggregates unless first exposed to preformed fibrillar tau seeds. Remarkably, once formed, tau aggregates become self-amplifying and propagate the prion state to daughter cells indefinitely (Sanders et al., 2014). Moreover, we found that tau could form discrete strains (DS9 and DS10), which replicated themselves with tremendous fidelity over months of cell division, and display dissociable biochemical and biological properties (Sanders et al., 2014). These strains feature accumulation of tau in different regions of the cell (DS9: nuclear speckles and a small cytoplasmic aggresome; DS10: a single, large aggresome) and display significant differences in their ability to seed further aggregation of monomeric tau (DS9: high, DS10: low). Limited proteolysis revealed that these divergent properties were likely due to different structural properties of the propagated tau aggregates. However, the mechanisms by which these structures drove divergent phenotypes were unclear. We hypothesized that examining the interactomes of these two strains would not only be informative in this regard, but would also reveal potential pathways by which tau aggregates are disaggregated, degraded, and trafficked.

Thus, we developed a protocol to purify tau aggregates and their associated proteins, taking advantage of the immense size and insolubility of micron-sized juxtanuclear
aggresomes (Figure AP.1A). Coomassie Blue staining demonstrated that this method resulted in significant enrichment of tau RD-YFP in the insoluble pellet relative to the soluble supernatant (Figure AP.1B). We thus sent this material (biological triplicates for each condition) for analysis by mass spectrometry, using the background parent line lacking tau aggregates (DS1) as a negative control. We identified 1530 proteins present in at least one of the samples. In most cases, spectral counts associated with specific proteins were similar between the conditions (data not shown). However, we noted that a subset of proteins was only present in DS9 and/or DS10 analyses but not that of DS1. To identify putative tau aggresome-associated proteins, we set an arbitrary cutoff for these proteins as a spectral count >5 in DS9 or DS10 (Figure AP.2A). We also examined proteins that were >2-fold enriched in DS9 or DS10 insoluble fractions relative to DS1 (Figure AP.2B). Both lists featured a preponderance of proteins whose canonical functions were in pathways of ubiquitin-dependent protein degradation (n=33 of 79), including the ubiquitin-proteasome system and selective autophagy/vesicular trafficking. Intriguingly, four of these proteins (SQSTM1, CHMP2B, UBQLN2, VCP) have been implicated as being causative to specific neurodegenerative syndromes (Deng et al., 2011; Johnson et al., 2010; Laurin et al., 2002; Parkinson et al., 2006; Skibinski et al., 2005; Watts et al., 2004). We also identified several SR-rich proteins (SRRM2, Pinin, CWC22, ARGLU1), which likely play a role in constitutive RNA splicing. Many of the identified proteins are uncharacterized or poorly characterized and could potentially play important roles in protein aggregate degradation or processing. For example, the Drosophila homologues of MLF1/MLF2 and DNAJC7 were previously identified as modifiers of polyQ toxicity (Kazemi-Esfarjani and Benzer, 2000; Kim et al., 2005).
Importantly, a separate, parallel SILAC-based analysis of proteins that were enriched in tau aggregates from DS9 to DS10 relative to soluble tau in DS1 (n=126 proteins) identified 25 of the same proteins, including 3 of the 4 proteins associated with familial forms of neurodegeneration (SQSTM1, CHMP2B, VCP), 3 of the 4 identified SR-rich proteins (SRRM2, Pinin, CWC22), all three uncharacterized modifiers of polyQ toxicity (MLF1, MLF2, DNAJC7), and two completely uncharacterized proteins (FAM199X and FAM63A) (Figure AP.2C). Overall, DS9 and DS10 interactomes were similar, although more putative aggresome-associated proteins were identified in the DS10 analysis. However, this may merely reflect the fact that a greater quantity of tau was partially purified from DS10 than DS9 (Figure AP1.B).

**Immunocytochemistry confirms the presence of candidate aggresome-associated proteins in tau inclusions.** Next, we examined whether the putative tau aggresome-associated proteins were truly present in this compartment. We ordered antibodies against 9 of the 25 proteins identified by both parallel mass spectrometry analyses of tau aggregate-associated proteins (Figure AP.2). We performed immunocytochemical co-localization studies in DS1, DS9, and DS10 cells. Seven proteins with putative functions in protein degradation (UBB, SQSTM1, NBR1, TRAFD1, VCP, MLF2, DNAJC7) displayed diffuse distributions in DS1 cells (Figure AP.3). However, in both DS9 and DS10 cells, these proteins displayed heavy co-localization with juxtanuclear tau aggresomes (Figure AP.3, AP.4). DS10 aggresomes featured a greater accumulation of these proteins. We also confirmed that two specific SR proteins (SRRM2 and pinin) were sequestered into tau aggresomes in the cytoplasm, but were exclusively in nuclear speckles in the DS1 negative control (Figure AP.5, AP.6). Importantly, five other SR-rich
splicing proteins (SRSF1, SRSF11, ACIN1, SRSF7, snRNP70) not identified by the proteomic analysis were absent from cytoplasmic tau aggresomes. This highlights not only the specificity of the potential interaction between tau and these proteins, but also the predictive power of the proteomic analysis.

**DS9 nuclear aggregates are present in splicing speckles.** Unlike DS10, DS9 features spherical nuclear aggregates, the identity of which is unknown (Sanders et al., 2014). The nucleus is remarkable in that it features a tremendous diversity of membraneless organelles (Zhu and Brangwynne, 2015). Most, if not all, of these compartments arise by liquid-liquid phase separation, a phenomenon by which proteins with similar RNA- and protein-binding multivalent domains form selectively permissible coacervates (Brangwynne, 2013; Hyman et al., 2014). Thus, we examined whether nuclear tau aggregates co-localize with any of these bodies. As noted previously (Sanders et al., 2014), DS9 nuclear aggregates never co-localize with PML bodies, despite the fact that these organelles are thought to be organizing centers for the degradation of misfolded nuclear proteins (Fu et al., 2005; Guo et al., 2014; Janer et al., 2006) (Figure AP.6C). Similarly, DS9 aggregates did not co-localize with Cajal bodies, nuclear gems, nucleoli, paraspeckles, or nuclear stress bodies (Figure AP.6C). Rather, DS9 aggregates showed strong co-localization with splicing speckles, as marked by a phospho-SR antibody (Figure AP.6C). It is possible that DS9 aggregates deposit to this location due to an interaction between SRRM2 and tau. Notably, CRISPR-Cas9-based knockout of SRRM2 obliterates nuclear tau aggregates (Figure AP.10). However, cytoplasmic DS10 aggregates sequester SRRM2 but do not show re-distribution to nuclear splicing speckles.
(Figure AP.5). Thus, the mechanisms that underlie the differential subcellular distribution of tau aggregates in DS9 and DS10 require further elucidation.

**Immunocytochemistry suggests similarities and differences between tau RD and full-length tau and polyQ aggregate interactomes.** Next, we asked whether proteins identified in the tau RD-YFP aggresome interactome were present in other types of inclusions. That is, were these specific markers of tau aggregates or were they likely to be more general markers of cytoplasmic amyloids? First, we performed ICC on HEK293 cells expressing untagged full-length 4R1N tau P301S, that had previously been exposed to tau seeds (DS9) (Figure AP.7B) or inoculate lacking seeds (DS1) (Figure AP.7A). As expected, cells exposed to DS9 lysate formed tau inclusions in the cytoplasm of the majority of cells (Figure AP.7B). Relative to the canonical, spherical aggresome structures observed in DS9 and DS10, these aggregates were not as dense and ordered. These structures stained positive for SQSTM1 and NBR1, but not TRAFD1, VCP, or MLF2 (Figure AP.7B). SRRM2 was sequestered in significant quantities, thus demonstrating that recruitment of this RNA-binding protein by tau is not an artifact of truncated tau RD expression in the nucleus. Knockout of SRRM2 was found to be lethal to dividing cells (Figure AP.10), which suggested that sequestration of this protein from the nucleus to the cytosol could potentially contribute to the toxicity of tau aggregates.

Therefore, we asked whether SRRM2 is recruited into tau aggregates in the brains of tauopathy patients. We performed dual-color IHC (tau and SRRM2) on tissue sections from patients with several tauopathies: Alzheimer’s Disease, Pick’s Disease, corticobasal degeneration, progressive supranuclear palsy. In all cases, SRRM2 was observed only in the nucleus and never in tau inclusions. Thus, it is unlikely that mislocalization of
SRRM2 from its physiological context in the nucleus to tangles in the cytoplasm is a contributing factor in the etiology of tauopathies. Rather, sequestration of SRRM2 by tau aggregates in HEK293 cells may instead be an artifact of nucleo-cytoplasmic mixing during cell division.

Unlike full-length tau inclusions, huntingtin exon1-Q72 (htt Q72) aggregates are dense and display features of canonical aggresomes (Waelter et al., 2001). Thus, we created a monoclonal cell line that stably propagated htt Q72-YFP aggregates and examined the presence of tau aggresome-associated proteins in these juxtanuclear inclusions. Similar to DS9 and DS10 tau aggresomes, polyQ inclusions stained positive for SQSTM1, NBR1, TRAFD1, VCP, MLF2, and DNAJC7 (Figure AP.8). This suggests that these proteins, and likely many others identified in proteomic analysis of tau aggresomes, play roles in the metabolism and degradation of diverse amyloids. Unlike tau RD and full-length tau aggregates, however, polyQ aggregates did not co-localize with SRRM2, suggesting that specific SR-motif proteins are not globally sequestered by cytoplasmic amyloids.

**Examining the functional consequences of aggresome-associated protein knockout.**

Having confirmed that our proteomic analysis indeed identified *bona fide* aggresome-associated proteins, we next asked whether these proteins play a role in the inheritance of tau aggregates or their degradation. In order to examine these questions, we turned to a monoclonal cell line that propagated the DS10 strain and whose tau expression was under a tetracycline regulated promoter (OFF1::DS10) (Sanders et al., 2014). Similar to DS10 on the DS1 background, these cells feature large juxtanuclear aggresomes and lack nuclear aggregates (Figure AP.9A). First, we asked whether knockout of specific proteins resulted in spontaneous curing of the aggregated state. In other words, did any of the
aggresome-associated proteins that we identified play a similar role to the yeast Hsp104 disaggregate machinery? As it could conceivably take weeks for cells to spontaneously cure in the absence of such machinery and low levels of protein may be sufficient for amyloid disaggregation, we decided to use CRISPR-Cas9 to knockout proteins of interest. We used a lentiviral vector that expressed Cas9 and a gRNA that targeted a gene of interest. For each gene of interest, we cloned and pooled six gRNAs (Shalem et al., 2014) prior to lentivirus production. We then treated OFF1::DS10 with lentivirus supernatant. This method was extremely effective at introducing indels and disrupting expression of an exogenously expressed gene, tau RD-YFP, present in numerous copies (Figure AP9.A). In fact, knockout was “all-or-none” (Figure AP9.B), demonstrating that introduction of a single gRNA into a cell was sufficient to disrupt all copies of the associated gene. Further, by biochemical approaches, we found that this method was effective at reducing levels of select endogenous proteins (SQSTM1, TRAFD1, DNAJC7) by >99% (Figure AP9.D). Next, we used this method to knockout our top 25 candidate genes as well as 6 additional controls in five cell lines: DS1, DS9, DS10, OFF1::DS1, OFF1::DS10. 9 of the genes were essential, as all cells died within a week of lentivirus treatment (Figure AP9.C). This was not dependent on the presence of aggregates in the cells. One month after treatment, spontaneous curing was not observed in any of the aggregate-containing lines (data not shown). Thus, none of the non-essential genes are critical to the maintenance of the aggregated state in dividing mammalian cells.

Was it possible that knockout produced more subtle effects? Two weeks after lentivirus treatment, OFF1::DS10 cells were observed by confocal microscopy. SQSTM1 knockout resulted in less dense aggresomes and more tau present in diffuse accumulations.
throughout the cytosol (Figure AP9.E). Conversely, DNAJC7 knockout resulted in aggresomes that were denser, with decreased quantities of extra-aggresomal tau (Figure AP9.E). No other non-lethal gene knockout produced an observable phenotype. We asked whether knockout of specific genes influenced the clearance of tau seeds in this cell line. We turned off tau expression for 0 to 5 days, re-started expression for 2 days, and counted the percentage of cells with inclusions. ADRM1 knockout resulted in more rapid clearance of tau aggregates (Figure AP9.F, AP9.G). Conversely, DNAJC7 knockout resulted in greatly decreased rates of clearance (Figure AP9.F-H). Knockout of SQSTM1 and other proteins had no effect. Importantly, knockout of control proteins (ATG5, autophagy; LAMP1, chaperone-mediated autophagy) had no effect, thus indicating that selective autophagy does not play a significant role in the clearance of tau seeds in this cell culture system. Rather, dilution of aggregated material by cell division or possibly the ubiquitin-proteasome system is responsible for the clearance of seeds over several days.
**AP.6 DISCUSSION**

Like yeast, mammalian cells feature a remarkable ability to stably propagate the aggregated state to daughter cells as an epigenetic phenotype (Krammer et al., 2009; Ren et al., 2009; Sanders et al., 2014). Similar to yeast, metazoans also feature the capacity to replicate unique fibrillar structures (prion strains) (Krammer et al., 2009; Sanders et al., 2014). However, unlike yeast, a disaggregase system that efficiently generates additional seeds for the replication of fibrillar amyloids has not been defined in mammalian cells. Further, the mechanisms that regulate trafficking and degradation of specific prion strains have not been elucidated. In the present work, we used an unbiased mass spectrometry approach to identify proteins that co-purify with specific tau aggregate structures (prion strains DS9 and DS10) with the goal of identifying critical mediators of prion inheritance, degradation, and trafficking. We identify 79 proteins that putatively target to micron-sized aggresomes, organizing sites for protein aggregate degradation (Johnston et al., 1998). We confirm that 9 of these proteins co-localize with tau aggresomes by immunocytochemistry, demonstrating the power of this analytical approach. By examining the presence of these same proteins in huntingtin polyQ aggresomes, we conclude that most of these proteins appear to generically target to these aggregate organizing centers. Thus, the provided resource will likely be informative to examining the metabolism of diverse amyloids.

Using CRISPR-Cas9, we examined the consequences of knockout of many (n=25) of these proteins. We were unable to find proteins that were essential to the continued inheritance of the prion state. However, we cannot rule out that essential proteins (e.g. VCP) or proteins that transiently interact with smaller aggregates do not play a role in
disaggregation of amyloids. Nevertheless, we identify three proteins whose knockout alters the morphological phenotypes of tau inclusions. SRRM2, an essential SR-motif containing gene that functions in constitutive RNA splicing (Gautam et al., 2015), is necessary for the deposition of DS9 tau aggregates in nuclear splicing speckles. However, SRRM2 is also recruited into DS10 aggresomes, suggesting that differential binding of this protein to different tau aggregate structures is not the critical mediator of the unique inclusion profile patterns observed in specific strains (Sanders et al., 2014). Removal of SQSTM1, a selective autophagy adaptor protein that forms filaments to recruit other autophagy cargo (Ciuffa et al., 2015), disrupts the formation of tau aggresomes, resulting in more diffuse accumulations of aggregated tau. This suggests that the protein likely plays a role in the 3D organization of juxtanuclear aggresomes. Conversely, DNAJC7 knockout results in denser inclusions. Moreover, it greatly impairs the kinetics of tau seed removal. This is in contrast to the knockout of essential proteins for selective autophagy (ATG5) and chaperone-mediated autophagy (LAMP2). This suggests that these pathways are not critical to the clearance of seeds in dividing mammalian cells. Rather, seeds are likely removed by dilution via cell division or possibly the ubiquitin-proteasome system (UPS). Partial knockdown of critical UPS components by shRNA or CRISPRi will be necessary to dissociate these possibilities. Moreover, additional studies will be required to determine the mechanisms by which DNAJC7, a poorly understood co-chaperone that has nonetheless been previously implicated as a moderator of polyQ toxicity (Kazemi-Esfarjani and Benzer, 2000), influences tau aggregate trafficking and degradation. Notably, knockout of other DNAJC proteins (DNAC9 and DNAJC14) do not phenocopy this effect. Current work seeks to examine the effects of knockout of all mammalian
chaperones in these systems to see whether this striking effect is specific to DNAJC7. Moreover, this work will determine whether other chaperone proteins that are not as enriched in aggresomes and may transiently interact with smaller species mediate the inheritance of the aggregated state. Overall, the present work provides a list of proteins that are likely to be critical to tau aggregate degradation and trafficking. Further work must be performed in non-dividing cells to determine the relative roles of these various proteins in tau seed degradation.
Figure AP.1. Partial purification of tau aggresomes. (A) A method was generated to partially purify tau aggresomes from tau RD-YFP cells, with the goal of identifying tau aggregate-associated proteins. (B) Sarkosyl-soluble and sarkosyl-insoluble fractions were run on gels and proteins were stained with SimplyBlue. DS9 and DS10 (contain tau aggregates) but not DS1 (lacks tau aggregates) insoluble fractions featured a significant enrichment of tau RD-YFP. Whole lanes for pellet fractions were analyzed by mass spectrometry (biological triplicates).
A

Identification of Aggresome-Associated Proteins

**DS1, DS9, DS10 Cell Pellets (3 Biological Replicates Per)**

- Lysis in 0.25% Triton-X followed by low-speed spin (1000xg). Keep pellet.
- Probe sonication followed by low-speed spin (500xg). Keep supernatant.
- Add 1% Sarkosyl and extract at RT for one hour.
- Spin at 186,000xg for 1 hour. Separate supernatant and pellet.
- Re-suspend pellet in high SDS buffer with aid of boiling.
- Run gel and perform Coomassie stain.
- Mass spec on pellets. Compare spectral counts of DS1, DS9, and DS10.
- Identify proteins enriched in or only present in DS9 and DS10.

B

Supernatant Fraction | Pellet Fraction
---|---
![Image](image.png)

**MW (kDa)**

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Mass Spec Analysis (Whole Lane)!

**Figure AP.1**
Figure AP.2. Mass spectrometry identifies potential proteins present in tau aggresomes. (A) Spectral counts were combined across biological triplicates to identify proteins present in DS9 and/or DS10 but not DS1 insoluble fractions (arbitrary cutoff = spectral count of > 5). (B) Proteins, whose spectral counts were greater than two-fold increased in tau aggregate-containing lines, were identified. Many of the proteins in both lists function in selective autophagy and the ubiquitin-proteasome system. Unexpectedly, SR-motif containing proteins were also identified. (C) A separate SILAC proteomic analysis of proteins that co-immunoprecipitated with tau aggregates identified many of the same proteins (data not shown).
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**Proteins Only Present in DS9, DS10 (Insoluble Fraction)**

**Proteins Enriched in DS9, DS10 (Insoluble Fraction)**

**Proteins Also Identified in SILAC Proteomic Analysis**

Figure AP.2
Figure AP.3. Verification of putative tau aggresome-associated proteins in DS10.

DS1 and DS10 cells were stained with antibodies raised against proteins putatively present in tau aggresomes: Ubiquitin, SQSTM1, NBR1, TRAFD1, VCP, MLF2, DNAJC7. In all cases, proteins showed co-localization with DS10 aggresomes.
Figure AP.3
Figure AP.4. Verification of putative tau aggresome-associated proteins in DS9. DS9 cells were stained with antibodies raised against proteins putatively present in tau aggresomes: Ubiquitin, SQSTM1, NBR1, TRAFD1, VCP, MLF2, DNAJC7. In all cases, proteins showed co-localization with DS9 aggregates, although in many cases, they showed weaker co-localization than in the case of DS10 aggregates.
Figure AP.4
Figure AP.5. Specific SR proteins are sequestered in DS10 tau aggrosomes. (A) SRRM2 and pinin, two SR-motif containing proteins identified by insoluble proteomics, co-localize with DS10 aggrosomes. (B) Other SR-motif containing proteins (SRSF1, SRSF11, ACIN1, SRSF7, snRNP70) are not sequestered by tau aggrosomes, which further confirms the specificity of the list of proteins identified in the insoluble proteomic analysis to aggrosome-associated proteins.
Figure AP.5
Figure AP.6. DS9 nuclear aggregates are in splicing speckles not other nuclear bodies. (A) SRRM2 and pinin, two SR-motif containing proteins identified by insoluble proteomics, co-localize with DS9 aggresomes. (B) Other SR-motif containing proteins (SRSF1, SRSF11, ACIN1, SRSF7, snRNP70) are not sequestered by DS9 tau aggresomes. (C) ICC with a panel of antibodies (phospho-SR, PML, Coilin, FBL, SMN1, PSF/SFPQ, SAFB) against canonical markers of specific nuclear bodies demonstrates that nuclear DS9 aggregates are present in splicing speckles but not other RNP bodies.
Figure AP.6
Figure AP.7. Full-length tau aggregates do not display the same interactome as tau RD in cells and patients. (A) In unseeded (DS1-treated) full-length tau P301S cells, SRRM2, SQSTM1, NBR1, TRAFD1, and VCP do not re-localize to cytoplasmic puncta. (B) SRRM2, SQSTM1, and NBR1 (but not other putative aggresomes markers), co-localize with seeded (DS9-treated) FL tau P301S aggregates. (C) SRRM2 remains confined to nuclear splicing speckles in both control and tauopathy brains (representative AD sample shown).
Figure AP.7
Figure AP.8. Many tau aggresome-associated proteins co-localize with polyQ aggregates. A monoclonal Huntingtin Exon 1 Q72-EYFP cell line that stably propagates the aggregated state was generated and stained with shown antibodies. All putative aggresome markers (SQSTM1, NBR1, TRAFD1, VCP, MLF2, DNAJC7) co-localize with cytoplasmic polyQ inclusions. An SR-motif protein (SRRM2) did not, confirming the specificity of this marker to tau inclusions in cells.
Figure AP.8
Figure AP.9. DNAJC7 KO inhibits removal of tau aggregate seeds. (A) A monoclonal Tet-regulated tau RD-YFP aggregate line (OFF1::DS10) was exposed to two treatments of the indicated pCRISPRv2 lentivirus, which demonstrated that this method was effective at disrupting multiple copies of an exogenously introduced gene (tau RD-YFP). (B) Flow cytometry shows that knockout of expression is “all-or-none.” (C) CRISPR-Cas9 was used to knockout top candidate genes identified by proteomic analyses as well as several controls in OFF1::DS10 cells. Some of these genes were essential (marked in red) and killed all cells. These genes were also essential to viability of cells lacking tau aggregates (OFF1::DS1), suggesting that knockout of these genes does not cause toxicity through altered tau aggregate metabolism. (D) Western blot was used to show that the method (1 or 2 treatments with pCRISPRv2 lentivirus) was effective at knocking-out the expression of endogenous proteins (SQSTM1, TRAFD1, DNAJC7), without subsequent selection. (E) Of knockouts that were not lethal, only DNAJC7 and SQSTM1 had a significant effect on tau aggregate phenotypes. SQSTM1 KO resulted in less dense aggresomes, with greater amounts of tau diffuse in the cytoplasm. DNAJC7 KO caused a phenotype whereby tau inclusions became denser, with lower quantities of extra-aggresomal tau. (F) Tau expression was turned off for three or five days, and was then re-started for two additional days. Percentage of cells containing inclusions was quantified (n=6 fields, each with >150 cells). Only ADRM1 and DNAJC7 KO had significant effects on tau seed clearance (** = p<0.01, **** = p<0.0001). Error bars represent S.E.M. (G) Tau expression was turned off for the indicated number of days, and was then re-started for two additional days. Percentage of
cells containing inclusions was quantified (n=6 fields, each with >150 cells). Only ADRM1 and DNAJC7 KO had significant effects on tau seed clearance (* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001). Error bars represent S.E.M. (H) Confocal images of select KOs with five days of repressed expression.
Figure AP.9
Figure AP.10. SRRM2 is required for tau aggregate entry into splicing speckles.

DS9 cells were treated with two rounds of NonTarget or SRRM2 gRNA lentivirus and cells were imaged after six days. Nuclear tau aggregates (splicing speckles) were disrupted in SRRM2 knockout cells. Other lethal knockouts did not produce this same phenotype (data not shown).
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