Understanding the role and regulation of TTBK1/2 during neurodegenerative disease progression

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Abstract

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A number of neurodegenerative diseases are characterized by the deposition of abnormally phosphorylated tau or TDP-43 in disease-affected neurons. Recent studies have suggested that phosphorylated species of tau and TDP-43 may be the driver behind neurodegeneration in these diseases. A screen for novel TDP-43 kinases identified the Tau Tubulin Kinases 1 and 2 (TTBK1/2), a pair of kinases that were originally characterized by their ability to phosphorylate tau. The function and regulation of TTBK1/2 in adult brain is unknown and there is no current understanding of what role they may play in disease progression. Preliminary studies show increased expression of TTBK1/2 in FTLD-TDP and ALS brain tissue, indicating that the TTBKs may be misregulated during disease. In this body of work, I followed up on these initial findings. I show that co-expression of TDP-43 and TTBK1 and co-expression of tau with both TTBK1 and TTBK2 in *C. elegans* causes exacerbation of behavioral abnormalities, increased pathological protein phosphorylation, and aberrant neuronal architecture and loss. I also show

that TTBK1 is upregulated and processed in a number of tauopathies including FTLD, PSP, CBD, and Pick's disease. Lastly, I identified an array of proteins that interact with the various domains of both TTBK1 and TTBK2 and may be potential regulators of kinase activity or provide insight into the role that TTBK1/2 play in the brain. These findings have expanded our understanding of the way that TTBK1/2 function and has the potential to lead to the development of targeted therapies for both tau and TDP-43 proteinopathies.

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Dedication

To those who believed they could, so they did

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2 Introduction

Tau and TDP-43 phosphorylation plays a role in neurodegenerative disease

The role of tau in tauopathies

Tau is a microtubule binding protein that functions in tubulin assembly and microtubule stabilization [1, 2]. As well as being present in neuron cell bodies, tau protein is abundant in axons [3] and important for cargo trafficking along the microtubule network within axons [4]. In neurons there is a dynamic mechanism for tau phosphorylation and dephosphorylation that regulates microtubule assembly and stability. When tau is phosphorylated, it has decreased microtubule-binding affinity [5] resulting in destabilized microtubules. Phosphorylated tau is also more resistant to proteolysis [6]. A balance of tau-targeted kinase and phosphatase activity is crucial for microtubule homeostasis and subsequently axonal stability. During aging and disease, the balance of tau phosphorylation is disrupted, leading to accumulation of hyperphosphorylated tau that no longer functions as a microtubule stabilizer. Unbound tau can then associate with other tau species and form soluble oligomers. These tau polymers lead to a cascade of tau fibrillization that ultimately triggers neuronal toxicity and the deposition of aggregated tau [7]. Neurodegenerative diseases characterized by the presence of abnormal tau-containing accumulations are termed tauopathies. They are further sub-classified by the presence and distribution of different tau splice isoform that bare either the three or four repeats of the microtubule-binding domain (3R or 4R). These disorders include Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and Pick's disease, among others.

AD is the most common tauopathy and the fourth leading cause of death in industrialized nations [8]. The symptoms of AD include memory loss and cognitive impairment presenting with decreased language ability, impaired abstract thinking, motor performance, and judgment. These symptoms are the direct consequence of synaptic reduction and neuronal loss and synaptic reduction, originating in the hippocampus and extending out to the cortices [9, 10]. The depletion of synapses in AD can begin 20-40 years prior to diagnosis [11], indicating early stages of disease may progress slowly. The two neuropathological hallmarks of AD are the presence of neuritic plaques (NPs) and neurofibrillary tangles (NFTs). Neuritic plaques form by spontaneous self-aggregation of amyloid-beta (Aβ), the cytotoxic product of amyloid precursor protein (APP) cleavage by the protease BACE-1 [12]. The presence of Aβ peptide precedes and may initiate subsequent tau pathology [13]. NFTs form as a consequence of hyperphosphorylated tau oligomers [14, 15]. In AD, soluble tau oligomers can contain both 3R and 4R tau isoforms [16], and the presence of 4R tau is correlated with a higher likelihood of AD diagnosis [17]. Soluble tau oligomers are the most toxic species in AD [17], and the burden of pathological filamentous tau is strongly correlated with the severity of cognitive impairment [18, 19]. Neurofibrillary tangles may exceed the burden of Aβ and occupy a large volume of disease tissue in severe AD cases. Neurons that are burdened by tau pathology end up losing their cell signaling ability [20]. Furthermore, studies in mouse models of AD show that reduction of tau *in vivo* can ameliorate Aβ toxicity [21-23], demonstrating tau's involvement in mediating amyloid related changes. To date, clinical trials to treat AD have all failed to arrest the course of disease or improve cognition in affected individuals [24]. In particular, numerous clinical trials that specifically target Aβ production or accumulation have been halted because the drugs successfully reduced Aβ burden but were ineffective at treating cognitive impairment [25-27], giving further evidence that Aβ

alone does not account for disease course. Overall these data suggest a significant role for tau in AD.

FTLD, the second most prevalent form of presenile dementia after AD, affects 10-30 per 100,000 individuals between the ages of 45-65 years [28]. FTLD is clinically presents with personality and behavioral changes and language dysfunction [29] caused by the deterioration of both the frontal and temporal lobes of the brain [28]. Roughly 40% of FTLD cases exhibit tau tangle as their primary neuropathology (FTLD-tau). [28]. In FTLD-tau, 3R and 4R tau inclusions [31] have been found in both neurons and glial cells [28], which differs from the neuron exclusive tau inclusions in AD. Mutations in *MAPT*, which encodes for tau, can be causal of FTDP-17, an inherited form of the disease [30], and account for roughly 10% of cases

PSP, CBD, and Pick's disease are rare independent diseases that also exhibit tau pathology in disease-affected brain regions [32]. PSP and CBD are two aging –associated Parkinson's-like syndromes that are characterized by the presence of abnormal 4R tau accumulation in neurons and glia. PSP has a prevalence of only 1 in 100,000 [28] and is diagnosed on the basis of supranuclear gaze palsy, Parkinson's-like movement defects, speech deficits, progressive dementia, and posture instability [28, 33-36]. However, roughly three quarters of pathologically confirmed PSP cases did not present with typical clinical indicators of PSP [37], which emphasizes the difficulties in clinical diagnosis. CBD is diagnosed on the basis of focal cortical deficits and progressive asymmetrical movement disorder, with a prevalence of 3 in 100,000 [28]. Though both PSP and CBD accumulate 4R tau in neurons and glia, the morphology of inclusions are distinct in each disease [38]. CBD manifests with filamentous tau-positive inclusions in the hippocampus, basal ganglia, brainstem, and cerebellum. PSP accumulates both filamentous and tangle-like tau deposits in the cortex, basal ganglia, and

subthalamic nucleus, and additional distinct tufted astrocytes, that contain tau inclusions [39]. As in AD, there is evidence for tau oligomers driving neurodegeneration in PSP [40, 41]. Furthermore, the burden of tau pathology is positively associated with degree of cognitive impairment seen in PSP cases [42]. Pick's disease is a rare form of dementia originally characterized by Arnold Pick in the late 1800s. Pick's disease is defined by the presence of eponymous neuronal inclusions (Pick bodies) that are morphologically distinct from neurofibrillary tangles, present in the cortex and dentate gyrus [44] and are composed of 3R tau [43]. Pick bodies positively label for both ubiquitin and paired helical filamentous tau [45]. Another distinctive pathological feature of Pick's disease is the presence of neuronal swelling [46]. Clinically, Pick's disease has an earlier onset than AD and has a more variable duration (from 2-17 years) [47]. Despite differences in clinical and pathological characteristics of PSP, CBD, and Pick's disease, modified tau inclusions are a prominent feature of these diseases. This suggests that even if the initiating cause of those diseases differs, the mechanism leading to neurodegeneration converge on the generation of pathological tau.

The molecular features of pathological tau

The definition of tauopathies is the presence of aggregated, phosphorylated tau protein. Though heavily debated in the field, evidence points towards phosphorylated oligomeric tau as the primary toxic species in tauopathies. The longest isoform, 4R tau, contains 85 potential phosphorylation sites, located throughout the protein [48], many of which are phosphorylated in disease [49]. Hyperphosphorylated tau can be toxic to cells through several mechanisms. The decreased affinity of phosphorylated tau for microtubules may disrupt cargo trafficking through reduced microtubule stability, leading to axonal impairment and disrupted synapses.

Furthermore, phosphorylated tau is resistant to turnover, which greatly increases the amount of intracellular tau [50, 51]. There is additional evidence that hyperphosphorylation of tau changes the ability of tau to interact with other proteins that are involved in its native function [52-54]. Lastly, hyperphosphorylation increases tau aggregation, though whether this is toxic or protective remains unclear.

Many kinases have been identified as tau modifiers, including glycogen synthase kinase-3 (GSK3β)[55, 56], cyclin-dependent kinase 5 (Cdk5) [57, 58], members of the microtubule affinity-regulating kinase (MARK), cyclic AMP-dependent protein kinase (PKA), calcium/calmodulin-dependent protein kinase (CaMK) families that target serine/threonine phosphorylation sites, and tyrosine kinases such as the SRC and ABL families [48]. More recently, the tau tubulin kinases 1 and 2 (TTBK1/2) were identified as tau kinases able to phosphorylate tau at residues Thr181, Ser202, Ser208, Ser210, Thr231, Ser396, and Ser404 [59-62]. Increased TTBK1/2 activity exacerbates tau toxicity [62, 63], suggesting a role for these kinases in disease initiation or progression.

The role of TDP-43 in ALS and FTLD-TDP

Trans-activating response DNA-binding protein of 43 kDa (TDP-43) is an RNA-binding protein that has essential roles in RNA processing, translation, splicing, and mRNA transport [64, 65]. In normal physiological conditions, TDP-43 is located in the nucleus, but under conditions of stress or neuronal injury, it can be sequestered to the cytoplasm where it forms into stress granules [66-68]. Cytoplasmic TDP-43 may be post-translationally modified by ubiquitination, phosphorylation, or truncation [69, 70], all of which are linked with subsequent neurotoxicity. The TDP-43 protein contains nuclear localization and nuclear export signals, two

RNA recognition motifs, and a C-terminal glycine-rich domain. Mutations in *TARDBP,* which encodes TDP-43, can cause familial amyotrophic lateral sclerosis (fALS) [71-73]. These fALS mutations cluster in the C-terminus of the protein, and have also been identified in a subset of sporadic ALS patients. Furthermore, TDP-43 is the primary protein aggregate found in most cases of both familial and sporadic ALS cases as well as in roughly half of all FTLD cases (FTLD-TDP) [74]. In addition to ALS and FTLD-TDP, TDP-43 inclusions are a secondary pathology seen in a number of other neurodegenerative diseases including AD, Parkinson's disease, Huntington's disease, and chronic traumatic encephalopathy [75-78].

Pure ALS and FTLD-TDP exist on opposite ends of a neurodegenerative disease spectrum, with both ALS and FTLD-TDP characterized by aberrantly processed, ubiquitinated, and phosphorylated TDP-43, and the diseases share clinical, neuropathological, and genetic features, which regularly occur concurrently [65, 79]. In roughly half of ALS cases, there is evidence of cognitive impairment and ultimately 15% of ALS patients also get diagnosed with FTLD [80]. Similarly, about 40% of FTLD cases display motor dysfunction and 15% result in a diagnosis of motor neuron disease [81]. These observations support the hypothesis that FTLD and ALS likely stem from similar sources of dysfunction. The recent discovery of *C9ORF72* mutations as the most common genetic cause of both ALS and FTLD provides further linkage between the two diseases. Mutations in *C9ORF72* are expansions of an intronic hexanucleotide repeat of the sequence GGGGCC within the C9ORF72 gene [82, 83]. C9ORF72 mutation carriers exhibit TDP-43 disease pathology, yet the mechanism of pathological progression of C9ORF72 mutation carriers is still poorly understood. Recent studies suggest RNA toxicity and disruption of translation mechanisms may be involved [84].

ALS, the most common adult onset motor neuron disorder, affects 1-2 per 100,000 individuals worldwide [85]. Symptoms include the progressive loss of motor function, leading to muscle atrophy and death [79]. A majority of ALS cases (90%) are sporadic with only a small subset of cases display a pattern of familial inheritance (10%). The most common disease-causing genetic mutations are found in *C9ORF72*, *SOD1*, *TDP-43*, and *FUS* [86]. Despite a large number of clinical trials in the past decade, only two drugs are available for the treatment of ALS. Daily administration of the FDA-approved pharmaceutical drug Riluzole merely prolongs median survival by several months [87]. Another drug, Edaravone, an antioxidant developed to treat ischemic stroke, modestly slows the rate of ALS progression in a subset of patients also being treated with Riluzole. Edaravone was most effective among patients that had a definite or probable diagnosis of ALS and were only symptomatic for less than two years [88]. There remains no significant disease-modifying treatment for ALS.

On the other side of the TDP-43 disease spectrum is frontotemporal lobar degeneration with TDP-43 pathology (FTLD-TDP), which has an age of onset of roughly 60 years and a mean duration of eight years [31]. Familial inheritance occurs in about half of FTLD-TDP cases [28] and is associated with mutations in *GRN*, *TARDBP* , *VCP* and *C9ORF72* [82, 89-91]. In about 40% of cases, there is some motor dysfunction similar to that seen in ALS [81]. Clinically, FTLD-TDP may present with behavior abnormalities, progressive non-aphasia, or semantic dementia [31]. As the name implies, the neuropathological hallmarks of FTLD-TDP are atrophy of the frontal and temporal lobes, caused by massive neuron loss and reactive gliosis in those regions. In most cases of FTLD-TDP, molecular pathology is characterized by inclusions of TDP-43, though a small subset of cases present with FUS inclusions [92].

Although the mechanism for TDP-43 toxicity is unclear, phosphorylation of TDP-43 is a consistent feature in all forms of TDP-43 proteinopathy [74, 93]. Studies have also shown that wild-type and mutant TDP-43 levels are linearly related to toxicity [94] and elevated TDP-43 levels are associated with a more aggressive course of neurodegeneration [95] however, TDP-43 inclusions alone are not sufficient for toxicity [96]. It was previously demonstrated that phosphorylation of serine residues 409/410 causes neurotoxicity and shortens lifespan in a *C. elegans* model expressing pan-neuronal human TDP-43 [97]. Based on these findings, phosphorylated TDP-43 may be the toxic species responsible for triggering cell death. There are only four kinases, CDC7, CK1, TTBK1 and TTBK2 identified that can phosphorylate TDP-43 [97-99]. However, it is not yet known whether one or several of these are the major driver of TDP-43 phosphorylation in disease.

The Tau-Tubulin Kinases

The Tau Tubulin Kinase Family

Tau tubulin kinase 1 and tau tubulin kinase 2 (TTBK1/2) belong to the casein kinase superfamily. They were originally characterized as kinases that phosphorylate tau bound to microtubules and were shown to play a role in tau phosphorylation in AD [58-60]. Recent work has demonstrated that TTBK1 and TTBK2 directly phosphorylate TDP-43 at S409/410 *in vitro* and can promote TDP-43 phosphorylation *in vivo* [97]. Furthermore, we showed that a reduction in TTBK1/2 levels protects against TDP-43 phosphorylation. We also showed that TTBK1/2 colocalize with phosphorylated TDP-43 in ALS and FTLD cases [97]. These data suggest that the TTBKs have the potential to initiate or contribute to both tau and TDP-43 disease courses.

The TTBKs contain a highly conserved homologous dual-kinase domain (88% identity, 96% similarity between human TTBK1 and TTBK2) that can phosphorylate serine, threonine, and tyrosine residues, but distinct regulatory domains [100]. The N-terminal kinase domain structure of TTBK1 has been elucidated [101, 102], and predictive modeling of TTBK2 suggests that the kinase domains of both proteins are functionally identical [103]. To date the roles and regulatory pathways of both TTBK1 and 2 in the brain remain elusive. A BLAST search targeted against the TTBK2 regulatory domains produced no significant similarities to other known protein species, pointing towards a unique regulatory mechanism for TTBK2, independent of TTBK1 regulation [103]. There is evidence for proteolytic processing of the TTBKs into numerous small fragments ranging in size from 25-100kDa [104] suggesting a complex native protein regulation mechanism may exist.

Tau Tubulin Kinase 1

TTBK1 is solely expressed in the CNS and at low levels in testes [100]. Its function is largely unknown, although recent evidence points towards a role in synaptic vesicle formation through phosphorylation of synaptic vesicle protein 2A (SV2A) [105]. Structurally, TTBK1 contains a kinase domain, and a regulatory region that uniquely contains a 39-amino acid polyglutamate stretch [100]. TTBK1 is upregulated in Alzheimer's disease (AD) brain [104] and co-localizes to phospho-tau in pretangles [106]. Two independent GWAS studies in Han Chinese and Spanish populations identified single nucleotide polymorphisms of TTBK1 associated with reduced risk of AD [107, 108]. Another study identified a *de novo* variant of TTBK1 as a potential cause of childhood onset schizophrenia [109]. These findings suggest that TTBK1 plays an essential role in the brain both during development and in aging. Transgenic mouse

lines expressing full-length human TTBK1 display age-dependent impairments similar to what might be seen in AD pathology. These transgenic mice exhibited learning impairments, neurofilament aggregation, microgliosis, altered CDK5/p35 activity, and decreased expression of NMDA receptors [104]. A double-transgenic mouse model of mutant FTLD-causing tau and human TTBK1 showed increased accumulation of oligomeric tau and enhanced motor neuron loss, suggesting a direct role of TTBK1 in accelerating tau-related neurodegeneration [110].

Tau Tubulin Kinase 2

TTBK2 is ubiquitously expressed throughout many tissues including liver, skeletal muscle, pancreas, heart, and brain [60, 100]. TTBK2 was originally isolated from bovine and mouse brain samples and characterized as a kinase targeting tau residues Ser208 and Ser210 [60, 61]. Recent publications have demonstrated a required role for TTBK2 in the initiation of ciliogenesis through the regulation of microtubule dynamics [111]. TTBK2 is known to interact with EB1, EB3, and Cep164 during cilia formation [112, 113]. Specifically, TTBK2 phosphorylates Cep164 when it is recruited to the basal body of a nascent cilium and regulates the removal of CP110, a suppressor of ciliogenesis [112, 114]. Additionally, TTBK2 has been implicated in sodium-coupled transporter regulation, which provides insight into the role it may play in non-neuronal tissues [115, 116].

Mutations in *TTBK2* cause an autosomal dominant form of spinocerebellar ataxia, type 11 (SCA11), a very rare progressive degenerative disease characterized by changes in gait, speech, and eye movements. The SCA11 causative TTBK2 mutations cause frame shifts that lead to a premature stop codon that results in truncated transcripts and depleted TTBK2 levels [117]. TTBK2 depletion in SCA11 significantly reduces kinase activity levels and stimulates

localization of TTBK2 to the nucleus [118]. Homozygous transgenic mice carrying the SCA11 mutation in *TTBK2* exhibit neurodevelopmental abnormalities and are embryonic lethal [118]. These data suggest that TTBK2 is essential for proper neuronal function throughout development and in neuronal maturation. The symptoms of SCA11 are distinct from the common symptoms of ciliopathies, which range from retinal degeneration and renal complications to cerebral malformation [119]. SCA11 pathologically presents with tau pathology, similar to what is seen in AD [117], likely caused upregulation of TTBK1 to compensate for the loss of TTBK2 activity. Differential TTBK2 expression has also been associated with cancer [120]. This gives further evidence for the regulatory role TTBK2 likely plays aside from cilia formation in other tissues.

The Therapeutic Potential of the TTBKs

As previously described, many age-related neurodegenerative diseases are characterized by the presence of either phospho-tau or phospho-TDP. Despite numerous clinical trials aimed at tauopathies such as AD and TDP-43 proteinopathies such as ALS, therapies for these indications remain elusive. To effectively treat such disorders, therapeutics would ideally target a process that occurs early in disease. Furthermore, any drug administered should have very limited potential to affect other pathways that are essential for healthy human function. An ideal drug target would therefore be one that is specifically expressed in disease-relevant tissues and is non-essential for survival. Because the TTBKs are able to phosphorylate both tau and TDP-43, they make attractive candidates for inhibition since they have the potential to treat numerous neurodegenerative disorders. TTBK1 in particular is a good candidate for targeted therapeutics as its expression is largely limited to the CNS, whereas TTBK2 is more ubiquitously expressed, is essential for viability in mice, and haploinsufficiency of TTBK2 is directly linked to disease.

Yet, in order to create effective therapeutics, we must first gain a better understanding of how the TTBKs are governed within the nervous system, a question this dissertation aims to address.

Using *C. elegans* as a model for neurodegeneration

Caenorhabditis elegans (C. elegans) has been a useful model for the study of basic science questions since its first characterization by Sydney Brenner in 1974 [121]. The utility of the nematode worm lies in the ease of culture, simple genetic analysis, and the abundance of resources made available by prior research. Specifically, for the study of neurodegenerative disease, it is important to have an organism that contains functional neuronal tissue and shows observable phenotypic changes during aging. The *C. elegans* nervous system contains only 302 neurons; the neuronal connectome has been mapped and the circuitry is well defined [122-124]. Due to the predictable neurodevelopment and synaptic morphologies, short lifespan, and the ability to visualize live neurons *in vivo, C. elegans* makes a favorable and convenient model for studying neuronal death in neurodegenerative disease.

Since the 1990s several labs have begun to utilize *C. elegans* to better understand the mechanisms of neurodegenerative disease progression. Common disease modeling efforts involve the expression of disease-implicated human transgenes in healthy *C. elegans* backgrounds in order to observe the effect of protein expression on aging and other *C. elegans* phenotypes such as motility and neuronal loss. The Kraemer lab has previously created and characterized a number of these neurodegenerative models expressing transgenic human tau or TDP-43 protein [125, 126]. Strains that will be referenced throughout the dissertation are outlined in the table below. These strains exhibit changes in protein levels, protein modifications, alterations to lifespan, and neuronal degeneration and death [62, 125, 126]. These features are

characteristic of the human neurodegenerative disease course, which makes *C. elegans* a useful model for the study of protein influences on neurodegeneration.

Table 1: Kraemer lab Tau and TDP-43 *C. elegans* Strains

Name	Protein	Construct	Phenotypes
CK1044	Tau (Wild-Type); Low-Expression	aex::Tau wt (4R1N), myo-2::GFP	Largely resembles N2 (wild-type) movement and lifespan, mild pTau
CK144	Tau (Wild-Type); High-Expression	aex::Tau wt (4R1N), myo-2::GFP	Uncoordinated, shortened lifespan, apparent pTau
CK410	TDP-43 (Wild-Type)	snb-1::TDP-43, myo-2::ds RED	Slightly uncoordinated, mild shortened lifespan, nearly absent pTDP-43
CK423	TDP-43 (M337V)	snb-1::TDP-43 (M337V), myo-2::ds RED	Uncoordinated, shortened lifespan, apparent pTDP-43
CK426	TDP-43 (A315T)	snb-1::TDP-43 (A315T), myo-2::ds RED	Severely uncoordinated, shortened lifespan, apparent pTDP-43

Current Gaps in Knowledge

Given the increasing evidence that TTBK1/2 likely play a role in neurodegenerative processes, further research is needed to better understand how these proteins act both natively and in disease. To date, there has been no experimentation looking into the toxicity of TTBK1/2 kinase overexpression *in vivo*. Preliminary studies demonstrate increased expression of TTBK1/2 in FTLD-TDP brain tissue, indicating that both TTBK1 and TTBK2 may be misregulated during disease. One focus of my research was to elucidate whether TTBK1/2 misregulation contributes to disease progression. Additionally, I sought to determine the role that TTBK1/2 play in disease

by creating an *in vivo* model in *C. elegans* and characterizing subsequent effects on behavior, protein phosphorylation, lifespan, and neurodegeneration. There is evidence that TTBK1 is normally proteolytically processed into distinct shorter protein fragments. The roles that aberrantly processed TTBK1 fragments play in disease progression remains unknown. To better understand this process, I worked to identify disease relevant processing patterns of TTBK1 in human postmortem tissues. Lastly, it is still unclear which proteins interact with TTBK1 and TTBK2 in neuronal populations. A more complete picture of how TTBK1/2 function in the brain in both normal and disease states will allow us to identify additional key protein interactions and processing of the TTBK1/2. These findings have the potential to lead to the development of targeted therapies for both tau and TDP-43 proteinopathies.

3 Pathological phosphorylation of tau and TDP-43 by TTBK1 and TTBK2 drives neurodegeneration

Abstract

Background: Progressive neuron loss in the frontal and temporal lobes of the cerebral cortex typifies frontotemporal lobar degeneration (FTLD). FTLD sub types are classified on the basis of neuronal aggregated protein deposits, typically containing either aberrantly phosphorylated TDP-43 or tau. Our recent work demonstrated that tau tubulin kinases 1 and 2 (TTBK1/2) robustly phosphorylate TDP-43 and co-localize with phosphorylated TDP-43 in human postmortem neurons from FTLD patients. Both TTBK1 and TTBK2 were initially identified as tau kinases and TTBK1 has been shown to phosphorylate tau epitopes commonly observed in Alzheimer's disease and other tauopathies.

Methods: To further elucidate how TTBK1/2 activity contributes to both TDP-43 and tau phosphorylation in the context of the neurodegeneration seen in FTLD, we examined the consequences of elevated human TTBK1/2 kinase expression in transgenic animal models of disease.

Results: We show that *C. elegans* co-expressing tau/TTBK1, tau/TTBK2, or TDP-43/TTBK1 transgenes in combination exhibit synergistic exacerbation of behavioral abnormalities and increased pathological protein phosphorylation. We also show that *C. elegans* co-expressing tau/TTBK1 or tau/TTBK2 transgenes in combination exhibit aberrant neuronal architecture and neuron loss. Surprisingly, the TTBK2/TDP-43 transgenic combination showed no exacerbation of TDP-43 proteinopathy related phenotypes. Additionally, we observed elevated TTBK1/2 protein expression in cortical and hippocampal neurons of FTLD-tau and FTLD-TDP cases relative to normal controls.

Conclusions: Our findings suggest a possible etiology for the two most common FTLD subtypes through a kinase activation driven mechanism of neurodegeneration.

Background

Frontotemporal lobar degeneration (FTLD) is a progressive neurodegenerative disease clinically diagnosed by evidence of personality and behavioral changes and language dysfunction [81]. Following Alzheimer's disease (AD), FTLD is the second most prevalent form of presenile dementia affecting 10-30 per 100,000 individuals between the ages of 45-65 years. In general, FTLD features atrophy of the frontal and temporal lobes resulting from neuron loss [28]. FTLD is sub-classified into three major pathological subtypes based on the presence of aggregated protein deposits of either TDP-43 or tau inclusions, with a small subset of cases exhibiting FUS-related pathology [28]. FTLD-TDP accounts for roughly 50% of cases whereas FTLD-tau accounts for approximately 45% of cases [28]. FTLD-TDP presents with aberrantly processed, ubiquitinated, and phosphorylated TDP-43 in neuronal inclusions and dystrophic neurites. FTLD-tau, on the other hand, is characterized by hyperphosphorylated aggregates of tau, which form tangles and pick bodies in neurons, glia, and neurites. Several mutations in *MAPT* reduce tau's affinity for microtubules and increase its aggregation rate and can cause FTLD-tau [127].

Phosphorylation of TDP-43 at serine residues 409 and 410 is a consistent pathological feature in ALS and FTLD-TDP [74]. Phosphorylation of TDP-43 reduces TDP-43 protein turnover, increases cellular mislocalization of TDP-43, drives protein aggregation, and promotes neurodegeneration [93, 97, 126, 128, 129]. Likewise, hyperphosphorylated tau protein is a hallmark of several neurodegenerative disorders including AD, progressive supranuclear palsy

(PSP), corticobasal degeneration (CBD), and FTLD. Phosphorylated tau has been implicated in the formation of toxic tau aggregates that promote neurodegeneration [14, 15, 125, 130-132]

The kinases TTBK1 and TTBK2 have been implicated in a number of neurodegenerative diseases. TTBK1 protein is increased in AD and influences the aggregation of tau [59, 133]. Furthermore, transgenic mouse lines expressing full-length human TTBK1 exhibit age-dependent detriments consistent with neurodegeneration, including learning impairment, neurofilament aggregation, microgliosis, altered CDK5/p35 activity, and decreased expression of NMDA receptors [104]. A double-transgenic mouse model expressing FTLD mutant tau and human TTBK1 shows increased accumulation of oligomeric tau and enhanced motor neuron loss, suggesting a direct role of TTBK1 in accelerating tau-related neurodegeneration [110]. Mutations in TTBK2 cause spinocerebellar ataxia type 11, a disorder exhibiting both loss of Purkinje cells and widespread deposition of tau [117]. TTBK2 plays an essential role in the initiation of ciliogenesis during embryonic development through the regulation of microtubule dynamics [112-114]. TTBK1 is solely expressed in the CNS and reproductive tissues, whereas TTBK2 is ubiquitously expressed throughout many tissues including liver, skeletal muscle, pancreas, heart, and brain [100].

Purified recombinant human TTBK1 and TTBK2 can directly phosphorylate both TDP-43 at S409/410 [97] and tau at S198, S199, S202, and S422 [59] and S208 and 210 [61]. Additionally, both TTBK1 and TTBK2 co-localize with phosphorylated TDP-43 (pTDP) in human postmortem tissues from both FTLD and ALS cases [97] and phosphorylated tau (ptau) in AD cases [133]. Because TTBK1/2 phosphorylate both TDP-43 and tau, a common pathway could be involved in the initiation of FTLD. While TTBK1/2 directly phosphorylate TDP-43 and

tau *in vitro,* it remains unclear how TTBK1/2 activity *in vivo* influences disease onset and progression.

To examine how TTBK1/2 contribute to both TDP-43 and tau phosphorylation, we analyzed their effects on lifespan, proteostatic function, and neurodegeneration in the context of tau or TDP-43 transgenic animal models. We also examined the expression of TTBK1 and TTBK2 in post-mortem human brain. We demonstrate that TTBK1/2 kinase expression leads to significant neurodegenerative phenotypes in our transgenic tau and TDP-43 models and is also a consistent feature of FTLD-tau and FTLD-TDP.

Methods

C. elegans *strains*

The N2 (Bristol) strain of *C. elegans* was used for all experimental controls and maintained as described [121]. Strains were maintained at 20°C on OP50 seeded nematode growth media (NGM). All experiments were performed at room temperature unless otherwise designated. Construction and characterization of TDP-43 transgenic (tg) (CK410) and tau (high-expression) (CK144) lines used were described previously [126]. Tau (low-expression) (CK1044) tg strains were generated by introducing wild type human full length 1N4R splice isoform tau cDNAs driven by the pan neuronal *aex-3* promoter (*Paex-3*::Tau) into the *C. elegans* genome.

TTBK1 and TTBK2 kinase domain (hTTBK1-cat and hTTBK2-cat) worms were constructed by introducing human TTBK1 or TTBK2 cDNA encoding the kinase domain, driven by the pan neuronal *rgef-1* promoter (*Prgef-1*::hTTBK1cat, *Prgef-1*::hTTBK2cat) (TTBK1:CK1051; TTBK2:CK646, CK645) into the *C. elegans* genome. *Prgef-1*::hTTBK1cat was microinjected

into N2 at a concentration of 30ng/µl with an *elt-2*::mCherry coinjection marker at a concentration of 25ng/µl. *Prgef-1*::hTTBK2cat was microinjected into N2 at a concentration of 50ng/µl with an *elt-2*::mCherry coinjection marker at a concentration of 25ng/µl. For all transgenic strains, extrachromosomal arrays were then integrated by exposing animals to a dose of 4000 Rad Gamma rays and subsequently outcrossed back into the N2 background at least twice. TTBK1 and TTBK2 strains used were CK1051, CK645, CK646. Strain CZ1200 [134], which carries an integrated *Punc25::GFP* transgene marker in GABAergic motor neurons, was a generous gift from Dr. Y. Jin. CK1051 and CK646 were crossed with CK1044, CK144, and CK410 to generate homozygous double transgenic *C. elegans*. CK646/CK1044 and CK1051/CK144 double transgenic were subsequently crossed with CZ1200 to produce triple transgenic lines with GFP marked GABAergic neurons.

Radial locomotion assay

Behavior was assessed by placing 15-20 age-matched (L4) *C. elegans* at the center of a 150mm NGM plate supplemented with 5x peptone (5xPEP), with a uniform OP50 bacterial lawn. After one hour of free movement at room temperature, the radial distance traveled from the origin by each animal was measured. Distance from the origin traveled per unit of time was expressed in micrometers per second to give a radial velocity. The assay was performed in triplicate by an observer blinded to genotype and statistical analyses were performed using GraphPad Prism software.

Lethality assay

CK144 was crossed with CK646 or CK645 and made homozygous for either the tau or hTTBK2-cat transgene to create the F1 population. From a single F1 parent, each F2 progeny was isolated onto an individual plate and the subsequent F3 offspring were scored for transgene expression. Animals that did not survive past L2 were characterized as larval lethal. Animals that survived into adulthood but did not produce progeny or died prior to egg laying were classified as adult sterile. A chi-squared analysis was performed to assess significance.

Neurodegeneration assays

Strains with *Punc25*::GFP-tagged (GABA)-ergic motor neurons were generated by crossing to the reporter strain CZ1200. Strains were staged to day 1 of adulthood and immobilized on a 2% agarose pad with 0.01% sodium azide. Live VD and DD GABAergic neurons were assessed under fluorescent microscopy on DeltaVision Elite (Applied Precision, Issaquah, WA) imaging system using an Olympus 60x oil objective. The number of live neurons, number of dorsal cord gaps, and percentage of neurons with aberrantly branched neuronal commissures were scored. Statistical significance was analyzed by performing a One-way ANOVA with a Tukey's post-hoc test using GraphPad Prism statistical software.

Lifespan analysis

C. elegans were synchronized to L4 stage from a timed egg lay on NGM plates at 20°C. Lifespan plates were prepared from 30mm NGM plates that were seeded with 10X concentrated OP50 and treated with 10mg/ml 5-fluorodeoxyuridine (FUDR). One hundred animals per strain were assayed at 25°C. Animals were checked daily for signs of movement by observing

locomotion and pharyngeal pumping. Towards the end of life, animals were tapped lightly with a platinum pick to look for a response. Animals that failed to respond were scored as dead and removed from the plate. Animals that died of bursting, bagging, or mishandling were censored from the data. Statistical significance was analyzed by performing a Chi-squared analysis with a Mantel-Cox test using GraphPad Prism software.

Immunoblotting

Mixed-stage populations of *C. elegans* were grown on 150mm 5XPEP plates, washed with M9 buffer, and frozen with liquid nitrogen. Protein lysates were prepared by sonication of frozen *C. elegans* pellets in lysis buffer (10 mM Tris-HCL pH 7.5, 5 mM EDTA, 10% sucrose) at 70% amplitude for 10 seconds, repeated three times. The lysate was loaded and resolved on precast 4-15% gradient SDS-PAGE gels (BioRad) and transferred to PVDF membrane (Bio-Rad Immun-blot PVDF membrane) at 100 volts for 32m. Human TDP-43 was detected with the commercially available monoclonal antibody ab57105 (Abcam, 1:2500) directed at human TDP-43 amino acids 1-261. TDP-43 phosphorylated at S409/S410 was detected by a commercially available monoclonal antibody (Cosmobio, Catalog # TIP-PTD-M01, 1:1000). Total Tau was detected with a pan-tau polyclonal antibody rb17025 (V. Lee lab, 1:3000) [135]. Tau phosphorylated at T181 was detected by AT270 (Thermo Scientific; 1:15000). Tau phosphorylated at S202 was detected by CP13 (1:500), a generous gift from Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY). Tau phosphorylated at T231 was detected by AT180 (Thermo Scientific, 1:2000). Tau phosphorylated at S396/404 was detected by PHF-1 (P. Davies lab, 1:2000) [136]. Load controls were detected by probing for β-Tubulin as previously described [125].

Human post-mortem brain lysate was prepared by homogenization of tissue in lysis buffer (50mM HEPES pH 7.5, 1mM EDTA, 150mM NaCl, 10% Glycerol, 0.1% Triton X-100, 1mM PMSF, 1 protease inhibitor pellet (Roche cOmplete Mini)) followed with a 10 second sonication at 50% amplitude using a Branson Sonifier with micro tip. Total protein lysate was loaded in 5XSDS buffer (5% SDS, 200mM DTT, 50mM Tris pH 6.8, 5mM EDTA, 50% sucrose, 0.05% Bromophenol Blue) and resolved on a precast 4-15% gradient SDS-PAGE gel (BioRad) at 200V and transferred to PVDF membrane (Bio-Rad Immun-blot PVDF membrane) at 100V for 30m. TTBK1 was detected with the commercially available polyclonal antibody directed at N-terminal amino acids 240-270 of human TTBK1 (Abcam, ab103944, 1:1000). TTBK2 was detected with the commercially available antibody directed at the N-terminus (Abcam, ab67839).

Post-mortem human tissue

We obtained de-identified samples of *postmortem* tissue from the University of Washington Alzheimer's Disease Research Center (ADRC) Neuropathology Core (PI, Dr. C. Dirk Keene) after receiving human subjects approval (University of Washington human subjects division approval: HSD# 06-0492-E/A 01). FTLD cases were selected on the basis of having an autopsy-confirmed diagnosis of FTLD-tau or FTLD-TDP. Control samples were from neurologically healthy control participants, who were of a similar age and were confirmed to be negative for neuropathologic changes of FTLD-tau or FTLD-TDP using routine and immunohistochemical assays. Frontal cortex (prefrontal middle frontal gyrus) and hippocampus (at the level of the lateral geniculate nucleus) samples were dissected at the time of autopsy in coronally sliced brains fixed approximately 3 weeks in 10% neutral buffered formalin according

to routine protocols. Samples were processed and embedded in paraffin according to standard protocols.

Immunohistochemistry and Immunofluorescence

Formalin-fixed, paraffin-embedded human brain tissue samples were sectioned by the University of Washington Alzheimer's Disease Research Center neuropathology core (Seattle, WA) onto standard charged glass microscope slides. Primary antibodies used for immunohistochemistry were anti-TTBK1 (Abcam, 1:100) and anti-TTBK2 (Abgent, 1:200). In order to minimize variability, sections from all cases (normal and affected subjects) were stained simultaneously for each antibody. Briefly, 5 μm sections from the frontal cortex and hippocampus were deparaffinized in xylene, rehydrated through graded alcohols, and an antigen retrieval step consisting of autoclaving sections in citrate buffer (1.8 mM citric acid/ 8.2 mM sodium citrate) was performed. Sections were treated for endogenous peroxidases with 3% hydrogen peroxide, blocked in 5% milk, incubated with primary antibody overnight at 4^{o}C, followed by biotinylated secondary antibody for 45 minutes at room temperature. Finally, sections were incubated in an avidin-biotin complex (Vector's Vectastain Elite ABC kit, Burlingame, CA) and the reaction product was visualized with 0.05% diaminobenzidine (DAB)/0.01% hydrogen peroxide in PBS. Specificity of these antibodies has been previously shown [97]. Immunohistochemistry photomicrographs were taken with a digital camera and imported into Adobe Photoshop for mounting. To optimize visualization of staining, photomicrographs were modified when necessary by adjusting brightness and contrast.

For double label immunofluorescence experiments, sections were co-immunostained with TTBK1 or TTBK2 and pathological tau as detected by pT231 specific monoclonal antibody

AT180 (ThermoScientific). AlexaFluor 647 goat anti-mouse and 568 goat anti-rabbit secondary antibodies (Molecular Probes) were used and autofluorescence was quenched with 0.1% Sudan Black. Microscopy was performed on a Delta Vision microscope (GE, Inc) using a 60x or 100x oil immersion objective, a sCMOS camera, and 2x2 binning. Image analysis was performed using softWoRx 6.0 Beta software (GE, Inc). Human brain samples stained for AT180 and TTBK1 or TTBK2 were imaged on a Leica TCS SP5 II confocal microscope using a 63x oil immersion objective. Colocalization analysis of confocal images was conducted in ImageJ 1.51n using Coloc 2.

Results

Human TTBK1 and TTBK2 kinase domain transgenic C. elegans *are behaviorally normal.*

To understand whether TTBK1 and TTBK2 kinases play a direct role in neurodegeneration, we constructed transgenic (Tg) *C. elegans* lines expressing kinase catalytic domains of human TTBK1 (hTTBK1-cat) or TTBK2 (hTTBK2-cat) under the pan-neuronal promoter *rgef-1* by micro-injecting a plasmid transgene (P*rgef-1*::hTTBK1 and P*regef-1*::hTTBK2). The resultant extrachromosomal transgenic strains were exposed to gamma radiation to create stable genomically integrated transgenic lines. Two lines of each hTTBK1-cat and hTTBK2-cat were then characterized for behavior using a radial locomotion assay. We observed that both hTTBK1-cat lines exhibited hyperactive locomotion when compared with wild type animals (Supplemental Figure 1a). We hypothesize the motor hyperactivity is a result of excess kinase activity. Neither TTBK2-cat strain was significantly different from non-transgenic *C. elegans*. One of each hTTBK1-cat and hTTBK2-cat line was selected for further characterization and used for the remainder of experiments. We measured the lifespan of

hTTBK1-cat, but saw no significant difference in its median or maximum lifespan as compared to wild-type *C. elegans* (Supplemental Figure 1b). Similarly, TTBK2-cat had no significant changes in median or maximum lifespan as compared to wild-type controls (Supplemental Figure 1c). Furthermore, ablation of the active site of the hTTBK1_mut transgene did not modify tau toxicity as measured by motor function (Supplemental Figure 1d).

Co-expression of TTBK1 or TTBK2 with tau causes behavioral abnormalities, aberrant phosphorylation, and shortened lifespan.

TTBK1 was originally characterized as a tau kinase and shown to directly phosphorylate tau at Ser198, Ser199, Ser202, and Ser422 *in vitro* [59]. To test whether TTBK1 driven phosphorylation of tau influences tauopathy phenotypes, we crossed our hTTBK1-cat Tg line with *C. elegans* strains expressing either low or high levels of wild-type human tau (isoform 1N4R). High expression tau Tg worms exhibit a variety of tau-dependent phenotypes including impaired movement, age-dependent neurodegeneration, and accumulation of detergent insoluble phosphorylated tau [125]. The tau(high) Tg line expresses approximately four-fold higher levels of tau than the tau(low) Tg line. Because the tau(high) worms already exhibit a strong phenotype [125], we chose to assess phenotypic changes in a tau(low) line. Tau(low) Tg *C. elegans* do not display significant differences in locomotion as compared to non-transgenic animals, whereas tau(high) Tg animals do exhibit significant impairment. We observed a significant reduction in locomotion velocity as measured by radial dispersion in both hTTBK1-cat;tau(low) (70.7% reduction) and hTTBK1-cat;tau(high) (69.4% reduction) compared to the respective tau transgenes alone (Figure 1a-b). We also observed a significant increase in both total tau and phosphorylated tau at Thr181, Ser202, Thr231, and Ser396/404 in hTTBK1-cat;tau(low) and hTTBK1-cat;tau(high) strains (Figure 1 c-n).

To assess whether TTBK2 catalytic activity could drive tauopathy related phenotypes, we crossed hTTBK2-cat Tg *C. elegans* lines with tau(low) and tau(high) Tg lines. We asked whether TTBK2-cat activity could modulate tau-induced behavioral defects and increase protein phosphorylation. We found that hTTBK2-cat expression exacerbates behavioral defects as indicated by a significant 50.6% reduction in radial motor velocity in our tau(low) Tg lines (Figure 2a). Additionally, we saw a significant increase in total tau and significant increases phosphorylated tau at Thr181, Ser202, Thr231, and Ser396/404 (Figure 2 b-g).

Figure 1: Expression of hTTBK1-cat causes neurodegenerative phenotypes in a tau background.

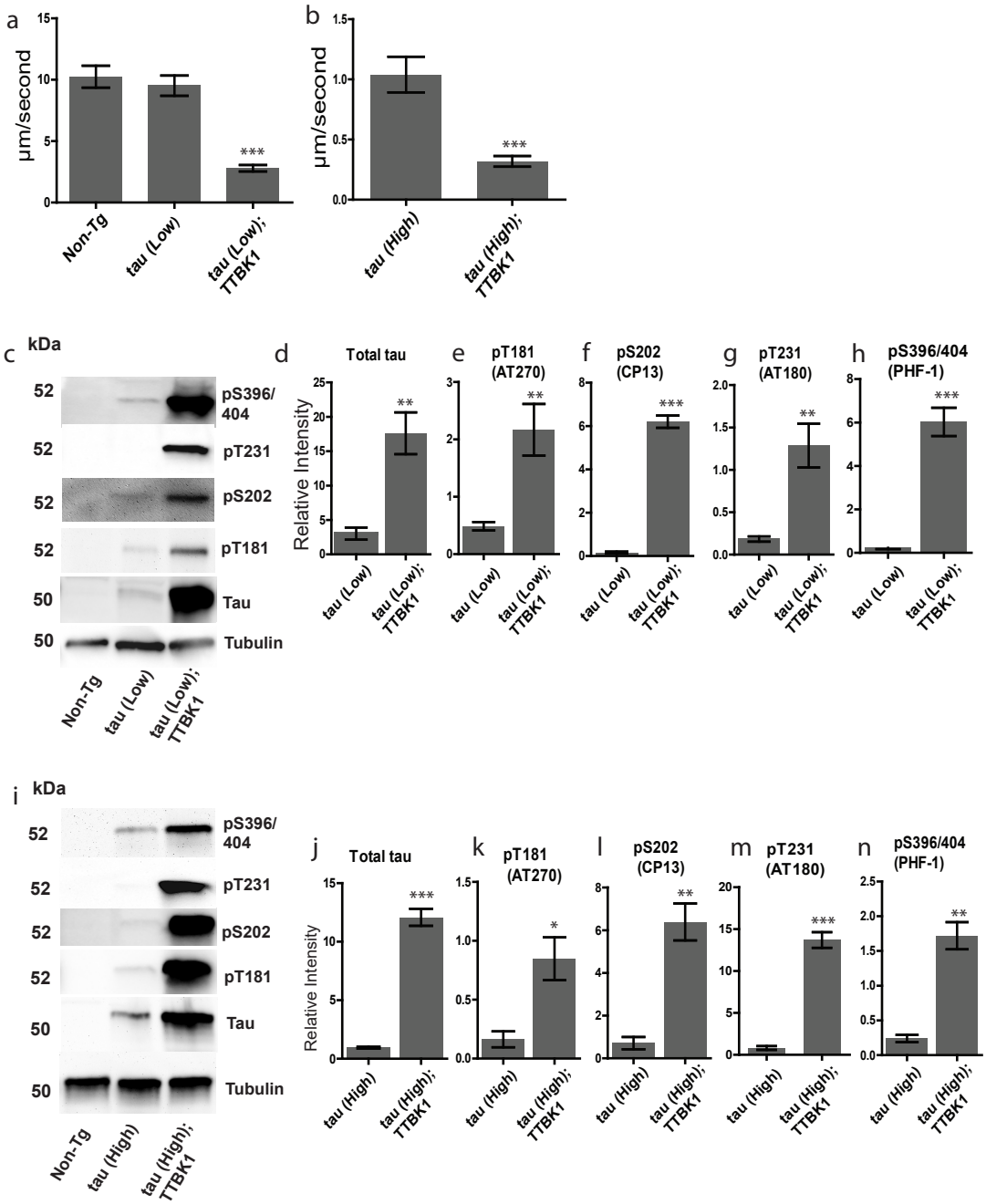


Figure 1 Legend: (**a)** Staged hTTBK1-cat;tau transgenic L4 larvae exhibit significantly decreased radial velocity relative to tau(low) and (**b**) tau(high) transgenic animals. Animals were measured for the linear distance traveled from a central reference point over 1 h, $N > 70$ for each genotype. Significance was determined using an unpaired T-test. $P < 0.0001$ versus tau(low) and $P < 0.0001$ versus tau(high). **c** hTTBK1-cat;tau(low) transgenic animals have increased total tau and ptau relative to tau(low) animals. Bar graphs represent four independent replicate immunoblots of (**d**) Total tau, (**e**) pThr181, (**f**) pSer202, (**g**) pThr231, and (**h**) pSer396/404. Graphs are plotted in relative intensity. Significance was determined using an unpaired T-test. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). **i** hTTBK1-cat;tau(high) transgenic animals have increased total tau and ptau relative to tau(high) animals. Bar graphs represent four independent replicate immunoblots of (**j**) Total tau, (**k**) pThr181, (**l**) pSer202, (**m**) pThr231, and (**n**) pSer396/404. Graphs represent densitometry analysis and are plotted in relative intensity. Significance was determined using an unpaired T-test. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)

Figure 2: Expression of hTTBK2-cat causes neurodegenerative phenotypes in a tau background

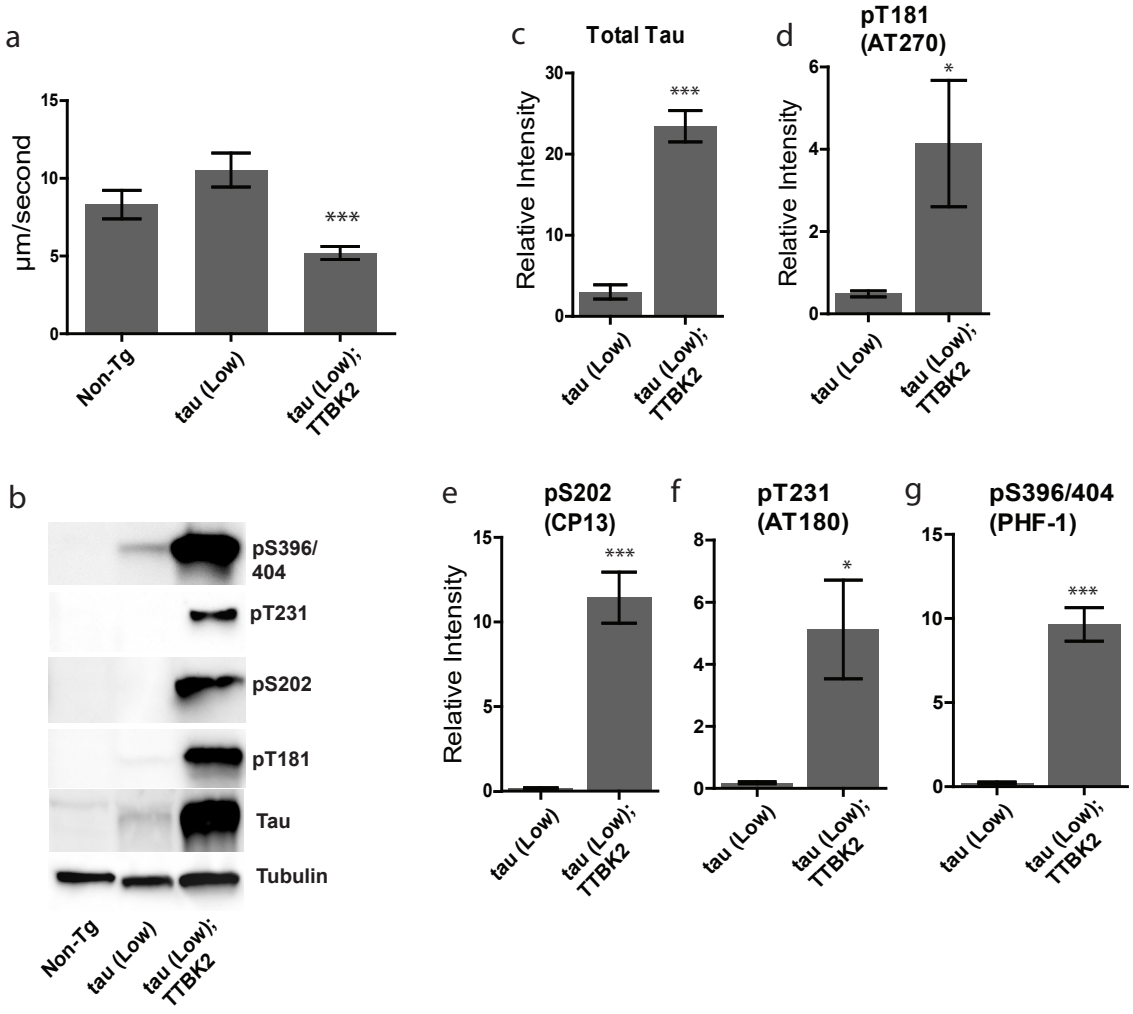


Figure 2 Legend: (**a)** Staged hTTBK2-cat;tau(low) transgenic L4 larvae exhibit significantly decreased radial velocity relative to tau(low) and (**b**) tau(high) transgenic animals. Animals were measured for the linear distance traveled from a central reference point over 1 h, $n > 75$ for each genotype. Significance was determined using an unpaired T-test. $P < 0.0001$. (**b**) hTTBK2-cat;tau transgenic animals have increased total tau and ptau relative to tau animals. Bar graphs represent four independent replicate immunoblots of (**c**) Total tau, (**d**) pThr181, (**e**) pSer202, (**f**) pThr231, and (**g**) pSer396/404. Graphs represent densitometry analysis and are plotted in relative intensity. Significance was determined using an unpaired T-test. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)

Given the motor behavior and protein phosphorylation phenotypes in our double transgenic hTTBK1-cat;tau and hTTBK2-cat;tau *C. elegans*, we wanted to test whether there was an effect on longevity. Tau worms lived to a median age of 14 days post-development, whereas hTTBK1-cat;tau worms lived to 12 days post-development and hTTBK2-cat;tau worms lived to 11 days post-development. This equates to a significant 14% reduction in lifespan for hTTBK1-cat;tau and a significant 21% reduction in lifespan for hTTBK2-cat;tau transgenic animals (Supplemental Figure 2a). These findings suggest the tauopathy modification is strong enough to limit lifespan as a consequence of synergy between TTBK1 or TTBK2 and tau.

Co-expression of high levels of tau and hTTBK2 causes lethality.

When crossing hTTBK2-cat with tau(high) Tg lines, we were unable to obtain a homozygous hTTBK2-cat;tau(high) population. To determine whether the two transgenes were synthetic lethal in combination, we counted the observed phenotypes of progeny in comparison to expected Mendelian ratios. We generated lines homozygous for tau(high) and heterozygous for hTTBK2-cat (tau(high)/tau(high); hTTBK2-cat/+). These animals were allowed to self-fertilize and the resultant F2 progeny were scored for the presence of a fluorescent co-injection marker to infer the genotype of the F1 parent (hTTBK2-cat/hTTBK2-cat, hTTBK2-cat/+, or +/+). Only 3.6% (6/165) of progeny were homozygous for both tau and TTBK2-cat, compared to the expected Mendelian outcome of 25% for segregation of a single genetic trait in the F1 generation (Table 1). Of those individual populations that were homozygous, 100% of subsequent progeny were dead as larvae. Additionally, we observed that 28.5% (47/165) of F1s were dead as larvae and 19.4% (32/165) of F1s that reached adulthood died prior to egg laying or were sterile. To confirm the results, we repeated the experiment with a second independent

hTTBK2 line, and made the parental genotype homozygous for hTTBK2-cat and heterozygous for tau(high) (hTTBK2-cat/hTTBK2-cat; tau(high)/+). Similar to our previous results, only 7.1% (18/254) of F1 individuals were homozygous for both hTTBK2-cat and tau(high) expression with 100% of subsequent F2 progeny observed dead as larvae. Additionally, 18.1% (46/254) of F1s were dead as larvae and of those that developed to adulthood, 11.8% (30/254) were sterile (Table 1). Based on these results, we conclude that TTBK2 kinase activity causes synthetic lethality when co-expressed with high levels of human tau.

Table 1: Expression of TTBK2-cat is embryonic lethal in a high-expression tau homozygous background

(Homozygous for TTBK2)	Observed	Expected
tau +/+	6 (3.6%)	41 (25%)
tau +/−	30 (18.2%)	83 (50%)
tau −/−	50 (30.3%)	41 (25%)
Larval Dead	47 (28.5%)	0 (0%)
Adult Sterile	32 (19.4%)	0 (0%)
Total	165	
(Homozygous for tau)	Observed	Expected
TTBK2 +/+	18 (7.1%)	63.5 (25%)
TTBK2 +/−	99 (39.0%)	127 (50%)
TTBK2 −/−	61 (26.5%)	63.5 (25%)
Larval Dead	46 (18.1%)	0 (0%)
Adult Sterile	30 (11.8%)	0 (0%)
Total	254	

Co-expression of TTBK1 or TTBK2 with tau causes neurodegeneration.

To determine whether hTTBK1-cat;tau(high) and hTTBK2-cat;tau(low) lines exhibit normal embryonic neuronal development of GABAergic motor neurons, we crossed our hTTBK1-cat;tau(high) and hTTBK2-cat;tau(low) transgenic animals to a strain carrying a fluorescent GABAergic neuronal reporter driven by the glutamic acid decarboxylase (GAD) promoter (P*unc25*::GFP) [134]. Using this reporter, we can assess neuron loss and axonal integrity in living animals using fluorescence microscopy. We scored living GFP-tagged neurons in hTTBK1-cat;tau(high) and hTTBK2-cat;tau(low) developing larvae. We found that all transgenic *C. elegans* appear grossly developmentally normal and all expected GABAergic neurons were present and structurally sound at larval stage L1 (Supplemental Figure 3a-f). Additionally, we observed that expression of hTTBK1-cat or hTTBK2-cat does not affect GFP expression levels (Supplemental Figure 3g).

Previous evidence suggests phosphorylated tau is increased in neurodegenerative diseases and correlates with the formation of toxic tau aggregates [15, 131, 132]. We tested whether TTBK1/2 driven increases in tau phosphorylation cause neuron loss using the lines described above. In our hTTBK1-cat;tau(high) animals, we observed a significant decrease in live GABAergic neurons in day 1 adult worms. Live hTTBK1-cat;tau(high) animals lost on average 20% (3.8/19) of neurons as compared to 12.6% (2.4/19) neurons lost in tau animals by day one of adulthood (Figure 3a,d). We also assessed whether neuronal connectivity degenerates in these animals by counting gaps in the continuity of the dorsal nerve cord. Whereas a non-transgenic animal presents no degeneration of the nerve cord in early adulthood, we found that hTTBK1-cat;tau(high) animals had significant disruption of nerve cord continuity with on average 7.0 dorsal cord gaps, as compared to tau(high) alone with an average of 2.6 gaps (Figure 3 b, e).

Lastly, in our double transgenic hTTBK1-cat;tau(high) worms, there was strong evidence for aberrant axonal branching. In non-transgenic animals, healthy neurons do not form branches. To quantify this observation, we counted the number of live neurons that exhibited abnormal branching. Our hTTBK1-cat;tau(high) line had on average 31% of GABAergic motor neurons with aberrant branching per worm as opposed to the tau worms, which only had 10% of the same neurons displaying aberrant branching (Figure 3c, f).

To investigate the role of TTBK2 in tau-mediated neurodegeneration, we also assessed whether hTTBK2-cat;tau(low) animals exhibited increased neuronal loss, dorsal cord degeneration, and altered axonal architecture. We found that in our hTTBK2-cat;tau(low) models there was also a significant loss of GABAergic motor neurons. The hTTBK2-cat;tau(low) animals had lost on average 15% of their GABAergic neurons by day one of adulthood while animals that expressed tau alone lost on average 7% neurons. Furthermore, we observed a significant increase in dorsal cord degeneration of our double transgenic line (2.3 gaps) in comparison to those expressing tau alone (1.1 gaps) as well as significantly more aberrant axonal branching in our hTTBK2-cat;tau(low) lines (21%, 4 branched neurons) versus the tau line (5%, 0.89 branched neurons) (Figure 4a-f). Together, these data suggest increased neurodegeneration and neuronal dysfunction driven by TTBK1 and TTBK2 in tau transgenic *C. elegans*.

Figure 3: Expression of hTTBK1-cat causes tau-dependent neuron loss and axonal abnormalities

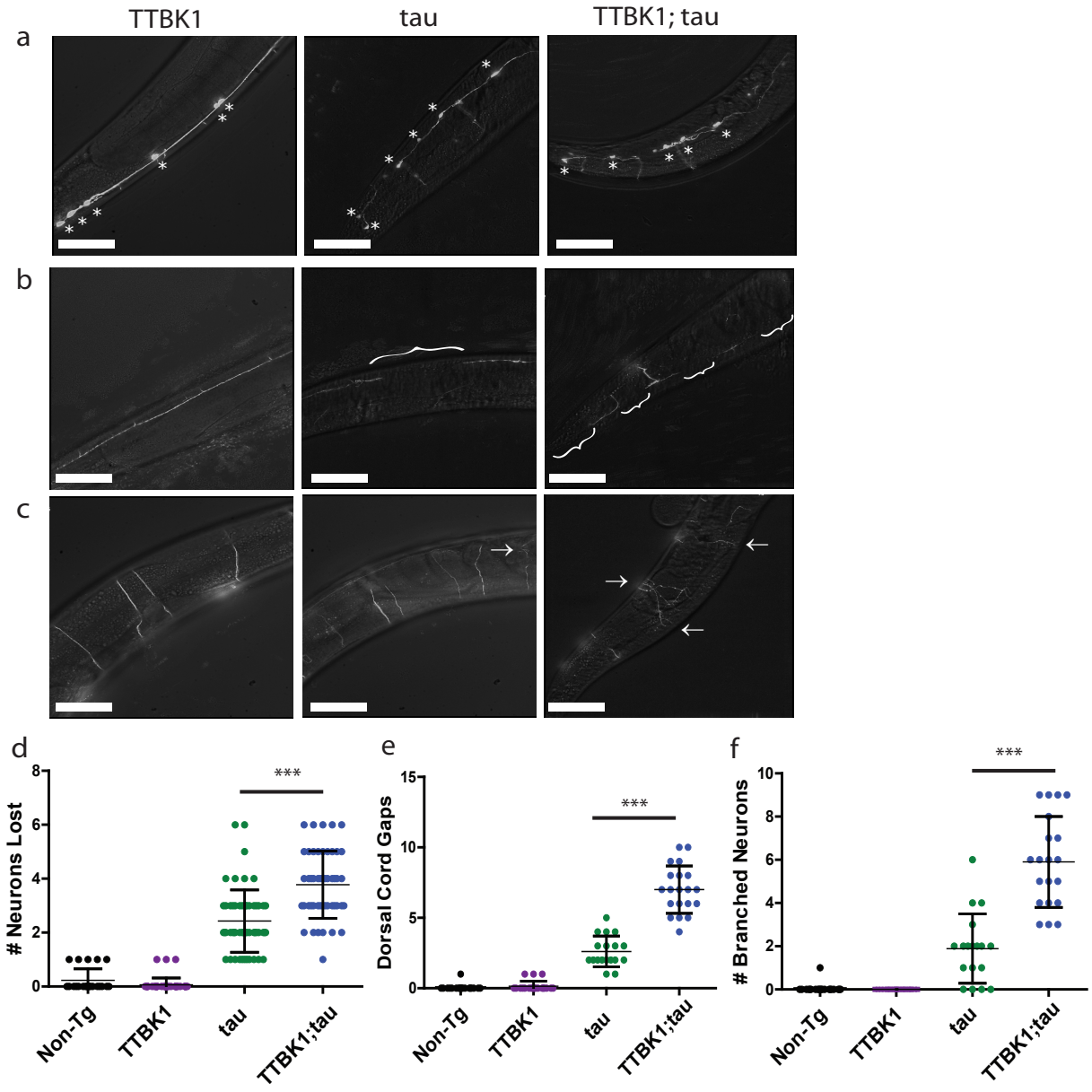


Figure 3 Legend: GFP-labeled D-type GABAergic motor neurons were observed in vivo in live day 1 adult transgenic *C. elegans*. **a** Fluorescent images of GABAergic live neurons in the posterior region. Each live neuron is marked with an asterisk. **b** Fluorescent images of GABAergic dorsal cord. Gaps in dorsal cord are marked with brackets (**c**) Fluorescent images of GABAergic axonal commissures. Aberrantly branched commissures are marked with arrows. **d** Number of neurons lost for each worm is plotted. GFP-labeled controls lost an average of 0.22 neurons per animal ($n = 22$). hTTBK1-cat Tg animals lost an average of 0.06 neurons ($n = 46$). Tau Tg animals lost an average of 2.4 neurons ($n = 56$). hTTBK1-cat;tau Tg animals lost an average of 3.8 Neurons ($n = 49$). $P < 0.001$ for tau versus hTTBK1-cat;tau. **e** Number of dorsal cord gaps for each worm is plotted. hTTBK1-cat Tg animals had an average of 0.14 gaps ($n = 21$). Tau Tg animals had an average of 2.6 gaps ($n = 18$). hTTBK1-cat;tau Tg animals had an average of 7.4 gaps ($n = 10$). $P < 0.001$ for tau versus hTTBK1-cat;tau (**f**) Number of aberrantly branched commissures for each worm is plotted. hTTBK1-cat Tg animals had an average of 0 aberrantly branched neurons ($n = 20$). Tau Tg animals had an average of 1.9 aberrantly branched neurons ($n = 18$). hTTBK1-cat;tau Tg animals had an average of 5.9 aberrantly branched neurons ($n = 20$). $P < 0.001$ for tau versus TTBK1-cat;tau. Significance was determined using a one-way analysis of variance with Tukey's multiple comparison test among strains. Scale bar = 50 μm

Figure 4: Expression of hTTBK2-cat causes tau-dependent neuron loss and axonal abnormalities

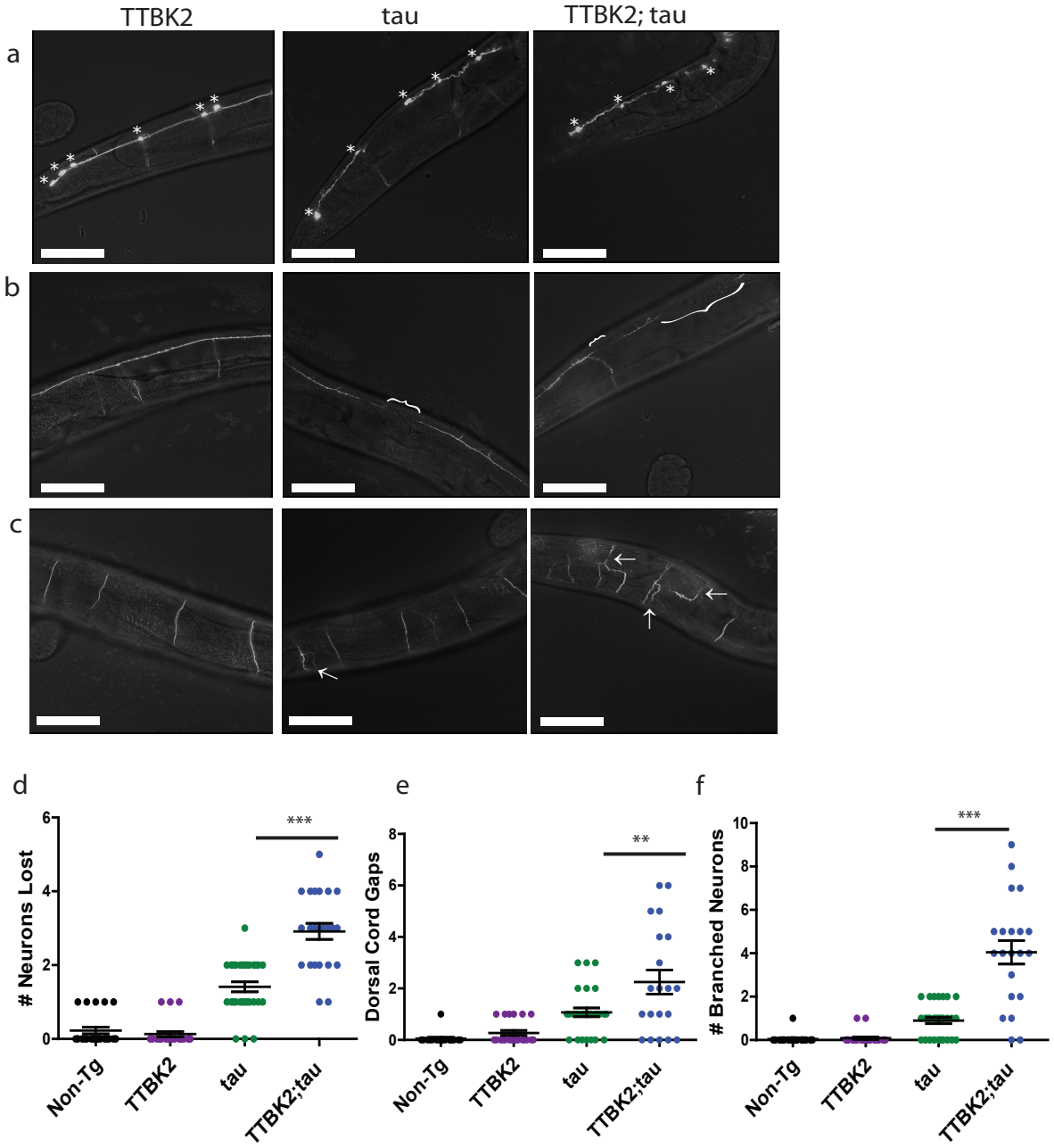


Figure 4 Legend: GFP-labeled D-type GABAergic motor neurons were observed in day 1 adult transgenic animals in vivo. **a** Fluorescent images of GABAergic live neurons in the posterior region. Each live neuron is marked with an asterisk. **b** Fluorescent images of GABAergic dorsal cord. Gaps in dorsal cord are marked with brackets (**c**) Fluorescent images of GABAergic axonal commissures. Aberrantly branched commissures are marked with arrows. **d** Number of neurons lost for each worm is plotted. hTTBK2-cat Tg animals lost an average of 0.13 neurons ($n = 23$). Tau Tg animals lost an average of 1.4 neurons ($n = 29$). hTTBK2-cat;tau Tg animals lost an average of 2.9 Neurons ($n = 23$). $P < 0.001$ for tau versus hTTBK2-cat;tau (**e**) Number of dorsal cord gaps for each worm is plotted. hTTBK2-cat Tg animals had an average of 0.27 gaps ($n = 22$). Tau Tg animals had an average of 1.1 gaps ($n = 28$). hTTBK2-cat;tau Tg animals had an average of 2.3 gaps ($n = 20$). $P < 0.001$ for tau versus hTTBK2-cat;tau (**f**) Number of aberrantly branched commissures for each worm is plotted. hTTBK2-cat Tg animals had an average of 0.09 aberrantly branched neurons ($n = 23$). Tau Tg animals had an average of 0.9 aberrantly branched neurons ($n = 29$). hTTBK2-cat;tau Tg animals had an average of 4.1 aberrantly branched neurons ($n = 21$). $P < 0.001$ for tau versus hTTBK2-cat;tau. Significance was determined using a one-way analysis of variance with Tukey's multiple comparison test among strains. Scale bar = 50 μm

Co-expression of TTBK1, but not TTBK2 kinase domains with TDP-43 causes behavioral abnormalities and increased phosphorylated TDP-43.

Given that human TTBK1 phosphorylates TDP-43 at S409/410 *in vitro* and colocalizes with pathological TDP-43 in both ALS and FTLD post-mortem tissues [97], we wanted to observe the effects of TTBK1 activity on TDP-43 *in vivo.* We crossed our hTTBK1-cat transgenic *C. elegans* to hTDP-43 transgenic models expressing wild-type TDP-43 [126] to generate hTTBK1-cat;TDP-43 transgenic animals. Animals expressing wild-type TDP-43 alone do not exhibit motor defects as compared to non-transgenic animals. We observed a significant decrease in hTTBK1;TDP-43 locomotion as measured by radial dispersion velocity relative to TDP-43 alone (Figure 5a). We then asked whether TTBK1 activity influenced accumulation or phosphorylation of TDP-43. We found a significant increase in both total TDP-43 and S409/410 phosphorylated TDP-43 (Figure 5b-d) in our double transgenic lines. Given the apparent effects on pathological TDP-43 protein accumulation and the impairment of motor phenotypes in our double transgenic hTTBK1-cat;TDP-43 *C. elegans*, we wanted to test whether there was also an effect on longevity. However, we found no significant difference in lifespan when compared to the TDP-43 alone (Supplemental Figure 2b). Likewise, hTTBK1_mut transgenes with a disrupted active site failed to modify TDP-43 toxicity (Supplemental Figure 1d).

We also asked whether expression of hTTBK2-cat in a TDP-43 background produces similar phenotypes by crossing our hTTBK2-cat *C. elegans* line to wild-type hTDP-43 transgenic *C. elegans*. Although there was a slight increase in total TDP-43 and phosphorylated TDP-43 levels, we did not observe a significant change in behavior or phosphorylated TDP-43 accumulation in the hTTBK2-cat;TDP-43 double transgenic (Supplemental Figure 4), demonstrating that hTTBK2-cat likely does not influence TDP-43 toxicity *in vivo* as strongly as hTTBK1-cat.

Figure 5: Expression of hTTBK1-cat causes neurodegenerative phenotypes in a TDP-43 background

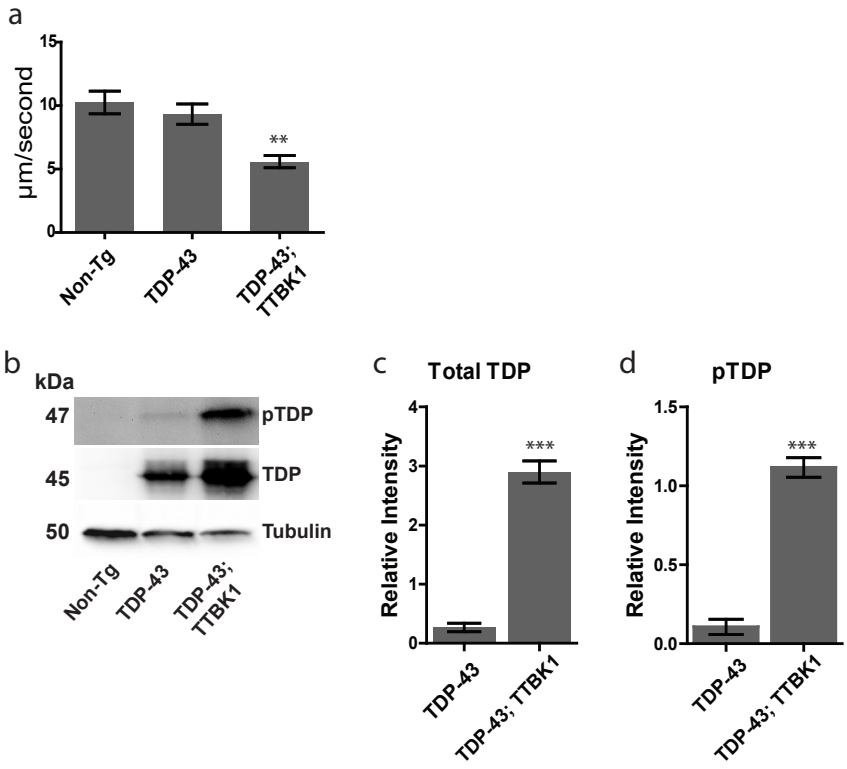


Figure 5 Legend: **a** Staged hTTBK1-cat; TDP-43 transgenic L4 larvae exhibit significantly decreased radial velocity relative to TDP-43 transgenic animals. Animals were measured for the linear distance traveled from a central reference point over 1 h, $N > 90$ for each genotype. Significance was determined using an unpaired T-test. $P < 0.01$ versus TDP-43. **b** hTTBK1-cat; TDP-43 transgenic animals have increased total TDP-43 and pTDP-43 relative to TDP-43 animals. Bar graphs represent six independent replicate immunoblots of (**c**) Total TDP and (**d**) pTDP. Graphs are plotted in relative intensity. Significance was determined using an unpaired T-test. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)

TTBK1 levels are elevated in both FTLD-tau and FTLD-TDP.

We have demonstrated in *C. elegans* that catalytically active TTBK1 and TTBK2 promote tau and TDP-43 phosphorylation [97]. However, it is unknown whether there are changes in the abundance of TTBK1 and TTBK2 in patients with FTLD. In order to assess whether elevation of TTBK1 or TTBK2 occurs in FTLD, we examined TTBK1 and TTBK2 protein levels in human post-mortem brain tissues of both FTLD-tau and FTLD-TDP patients. To measure changes in protein abundance, we performed immunoblot analyses on human postmortem cortical tissue. We found that in both FTLD-tau (N=7) and FTLD-TDP (N=5) cases, there was a visible increase in the levels of proteolytically processed TTBK1 and TTBK2 kinase domain bearing species as well as full length TTBK1 (Figure 6a-d).

To establish the distribution of TTBK1/2 accumulation, we examined TTBK1 and TTBK2 immunoreactivity in the hippocampus and frontal cortex of FTLD-tau and FTLD-TDP cases by immunohistochemistry. Our previous study demonstrated an increase in TTBK1 and TTBK2 immunostaining in the frontal cortex of FTLD-TDP cases [97]. In this study, we examined additional FTD-TDP cases and extended our analyses to include the hippocampus. We again observed an increase in TTBK1 and TTBK2 immunostaining in the frontal cortex of FTLD-TDP cases relative to normal controls (Figures 7b and 8b). In contrast, immunoreactivity in the hippocampus of FTLD-TDP cases was similar to normal control cases (Figure 7e and 8e). In FTLD-tau cases, we observed increased TTBK1 and TTBK2 immunoreactivity in both the frontal cortex and hippocampus relative to normal controls (Figure 7c and 7f; Figure 8c and 8f). The increase in TTBK1/2 immunostaining in the hippocampus of FTLD-tau cases is strikingly robust, especially in CA3 pyramidal neurons, and is more pronounced relative to the increase observed in the frontal cortex of FTLD-TDP.

We previously showed that TTBK1 and TTBK2 co-localize with phosphorylated TDP-43 in cytoplasmic inclusions in FTLD-TDP cases. To determine whether TTBK1/2 co-localize with phosphorylated tau in FTLD-tau cases, we performed double label immunofluorescence on hippocampal sections (Figure 8e and 8f). We observed TTBK1/2 immunofluorescence throughout the cytoplasm of hippocampal pyramidal neurons that overlapped with AT180 immunofluorescence in neurons positive for these pathological tau deposits. In general, the co-localization of TTBK1 with this phosphorylated tau species was stronger than that of TTBK2. Taken together with our findings in human brain tissue, this suggests that elevated levels of TTBK1 and TTBK2 proteins are present in patients with both FTLD-tau and FTLD-TDP and co-localize with phosphorylated protein.

Figure 6: FTLD cases exhibit increased levels of TTBK fragments

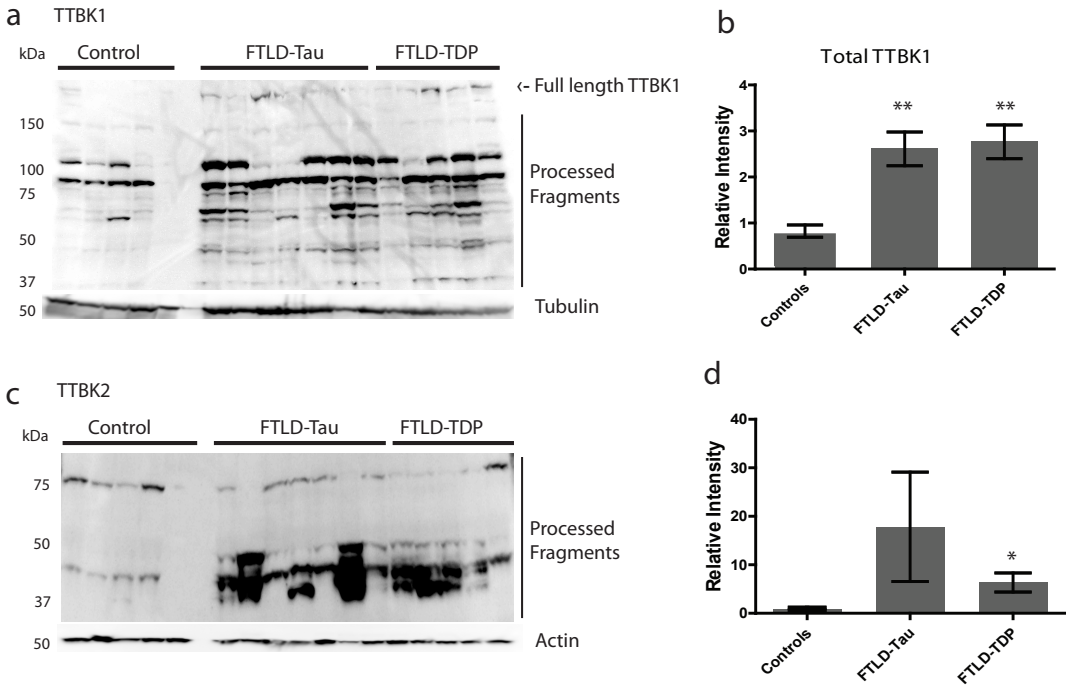


Figure 6 Legend: **a** Both FTLD-tau and FTLD-TDP patients exhibit significantly increased full-length TTBK1 and processed TTBK1 expression as compared to age-matched controls. **b** Bar graphs represent quantification of all TTBK1 bands present plotted in relative intensity. Significance was determined using a one-way ANOVA. $P < 0.001$ (**). **c** Both FTLD-tau and FTLD-TDP patients exhibit significantly increased processed TTBK2 kinase domain expression as compared to age-matched controls. **d** Bar graphs represent quantification of all TTBK2 bands present plotted in relative intensity. Significance was determined using a one-way ANOVA.

Figure 7: FTLD cases exhibit increased TTBK1 immunostaining

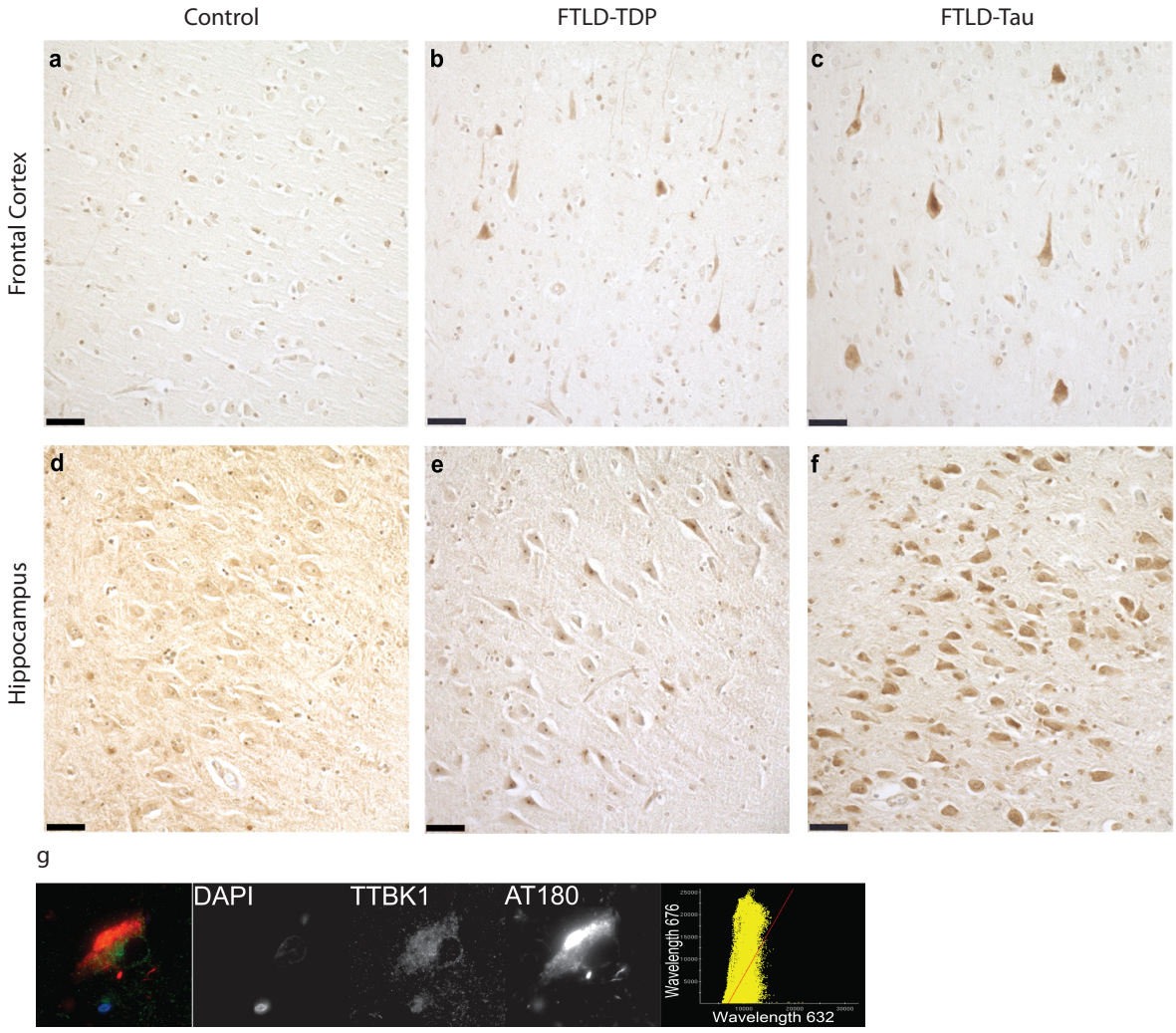


Figure 7 Legend: FTLD cases exhibit increased TTBK1 immunostaining. Representative images demonstrating upregulation of TTBK1 immunoreactivity in FTLD brain tissue ($n = 5$ cases of FTLD-TDP represented in panels b,e; $n = 7$ cases of FTLD-tau represented in panels (**c**, **f**)) compared to that of normal control subjects (**a**, **d**). In the hippocampus (**d-f**), the increase in TTBK1 is highly robust in FTLD-tau cases relative to both FTLD-TDP cases and controls. In the frontal cortex (a-c) there is enhanced TTBK1 immunoreactivity relative to normal controls, but the difference between the FTLD subtypes was less apparent. Scale bar = 50 μm. **g** TTBK1 is expressed throughout the cytoplasm, and overlaps with phosphorylated tau (AT180) in human cells. Shown is an image from a representative case ($n = 3$). Pearson coefficient of correlation for colocalization = 0.69 ± 0.18 for $n = 46$ neurons analyzed.

Figure 8: FTLD cases exhibit increased TTBK2 immunostaining

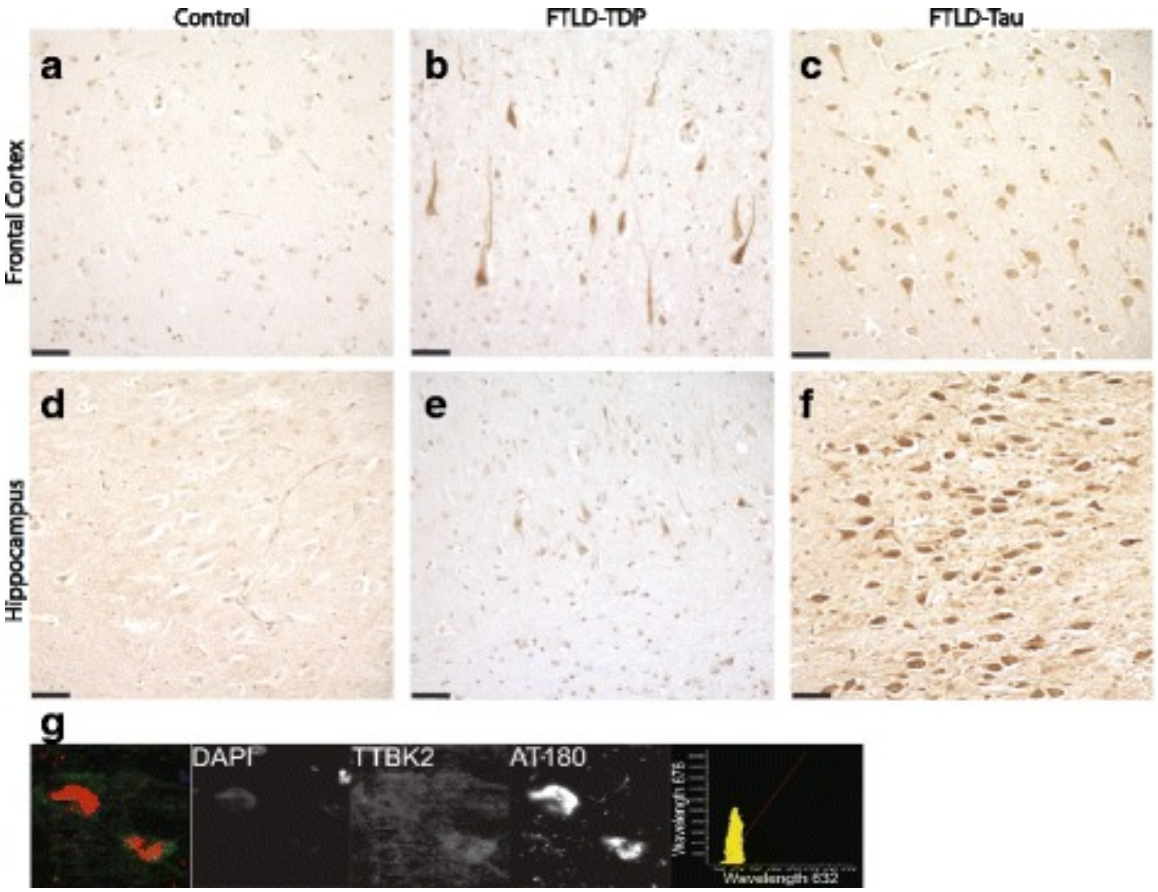


Figure 8 Legend: Representative images demonstrating upregulation of TTBK2 immunoreactivity in FTLD brain tissue ($n = 5$ cases of FTLD-TDP represented in panels b,e; 7 cases of FTLD-tau represented in panels (**c**, **f**) compared to that of normal control subjects (**a**, **d**). In the hippocampus (**d-f**), the increase in TTBK2 is highly robust in FTLD-tau cases relative to both FTLD-TDP cases and controls. In the frontal cortex (**a-c**) there is enhanced TTBK2 immunoreactivity relative to normal controls, but the difference between the FTLD subtypes was less apparent. Scale bar = 50 μm. **g** TTBK2 is expressed throughout the cytoplasm, and overlaps with phosphorylated tau in human cells. Shown is a representative case ($n = 3$). Pearson coefficient of correlation for colocalization = 0.57 ± 0.18 for $n = 32$ neurons analyzed

Discussion

Previous work has shown that TTBK1 and TTBK2 phosphorylate both tau [59, 61] and TDP-43 [98]. However, the full extent to which tau and TDP-43 are phosphorylated by TTBK1 and TTBK2 *in vivo* have yet to be described. It remains unknown whether changes in TTBK1 and TTBK2 activity, abundance, and proteolytic processing influence tau- and TDP-43-proteinopathies. To address these gaps in knowledge, we generated transgenic *C. elegans* expressing active human TTBK1 and TTBK2 kinase domains, and evaluated their effects on tau and TDP-43 transgenic models of FTLD. We also characterized TTBK1 and TTBK2 expression patterns and levels in both FTLD-tau and FTLD-TDP subtypes.

We found that hTTBK1-cat expression dramatically increased total and phosphorylated protein levels of human tau and TDP-43 in transgenic *C. elegans* resulting in exacerbated behavioral phenotypes and neurodegeneration. Interestingly, while hTTBK2-cat expression drove accumulation of total and phosphorylated tau and behavioral defects in tau transgenic animals, hTTBK2-cat was relatively neutral in TDP-43 transgenic animals. This suggests an *in vivo* selectivity of TTBK1 and TTBK2 towards their phosphorylation targets that differs from their *in vitro* ability to phosphorylate purified TDP-43. Furthermore, these data could also reflect regional or neuronal subtype selectivity by TTBK2.

Although increases in TTBK1 expression have been previously shown in human AD cases [133], there have been no studies examining changes in TTBK1 or TTBK2 abundance in FTLD. In this study we show that both TTBK1 and TTBK2 protein levels increase as compared to age-matched controls in FTLD-TDP and FTLD-tau cases. These findings were seen in both immunoblot analyses and immunohistochemistry studies. These results suggest that changes in

TTBK1 and TTBK2 abundance or processing may influence their kinase activities towards tau and TDP-43 in FTLD-tau and FTLD-TDP.

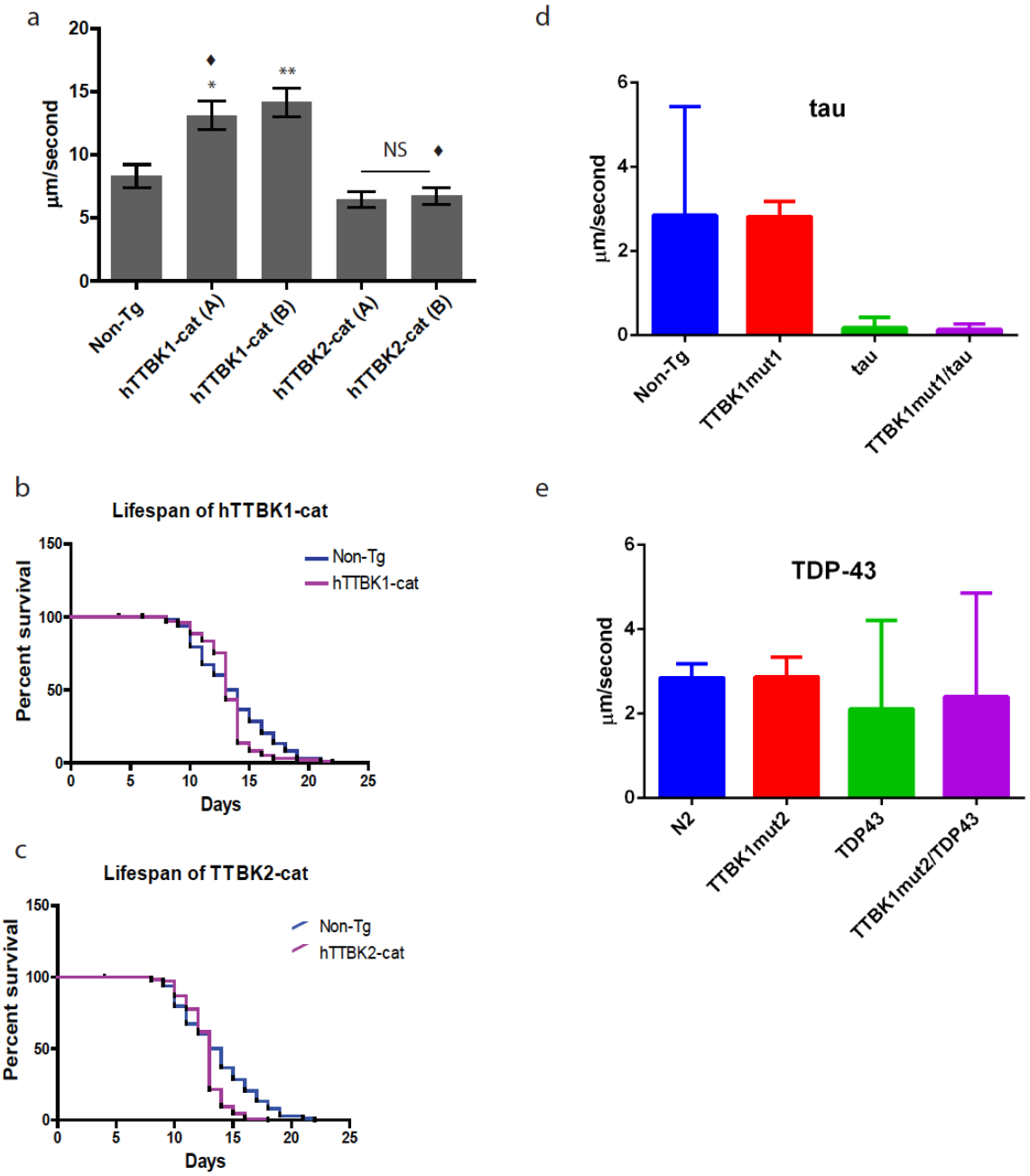
Importantly, this study suggests distinct target selectivity between TTBK1 and TTBK2 kinase activity despite high homology between the kinase domains (88% identity and 96% similarity) [100]. The differential regulation of TTBK1 versus TTBK2 activity is poorly understood. However, here we show that TTBK2 kinase domain has a greater influence on promoting tau-induced neurodegeneration than TTBK1, but it does not appear to affect TDP-43 *in vivo*. Likewise, TTBK1 is able to phosphorylate both TDP-43 and tau *in vivo*, but appears to have a relatively modest effect on tau compared to TTBK2. Investigations into the regulation of TTBK1 and TTBK2 kinase activity and substrate specificity are important next steps in determining the roles of TTBK1 and TTBK2 in FTLD.

Conclusions

The identification of TTBK1 and TTBK2 as both tau and TDP-43 kinases indicates a possible shared mechanism for the initiation of TDP-43 proteinopathy and tauopathy in FTLD. This is supported by the pathological presence of either phosphorylated TDP-43 or tau in the majority of FTLD cases, and the elevated protein expression of TTBK1 and TTBK2 in both FTLD-tau and FTLD-TDP. Therefore, the development of drugs to selectively inhibit TTBK1 and TTBK2 may be a common therapeutic strategy for both FTLD-tau and FTLD-TDP. In general, kinases have become among one of the most important classes of drug targets [137]. TTBK1 in particular makes an attractive drug target due to its restricted expression in neurons [100], unlike other tau kinases such as GSK3 and CDK5 that are ubiquitously expressed [138, 139]. Furthermore, TTBK1 is one of the first kinases to be identified as both a tau and TDP-43

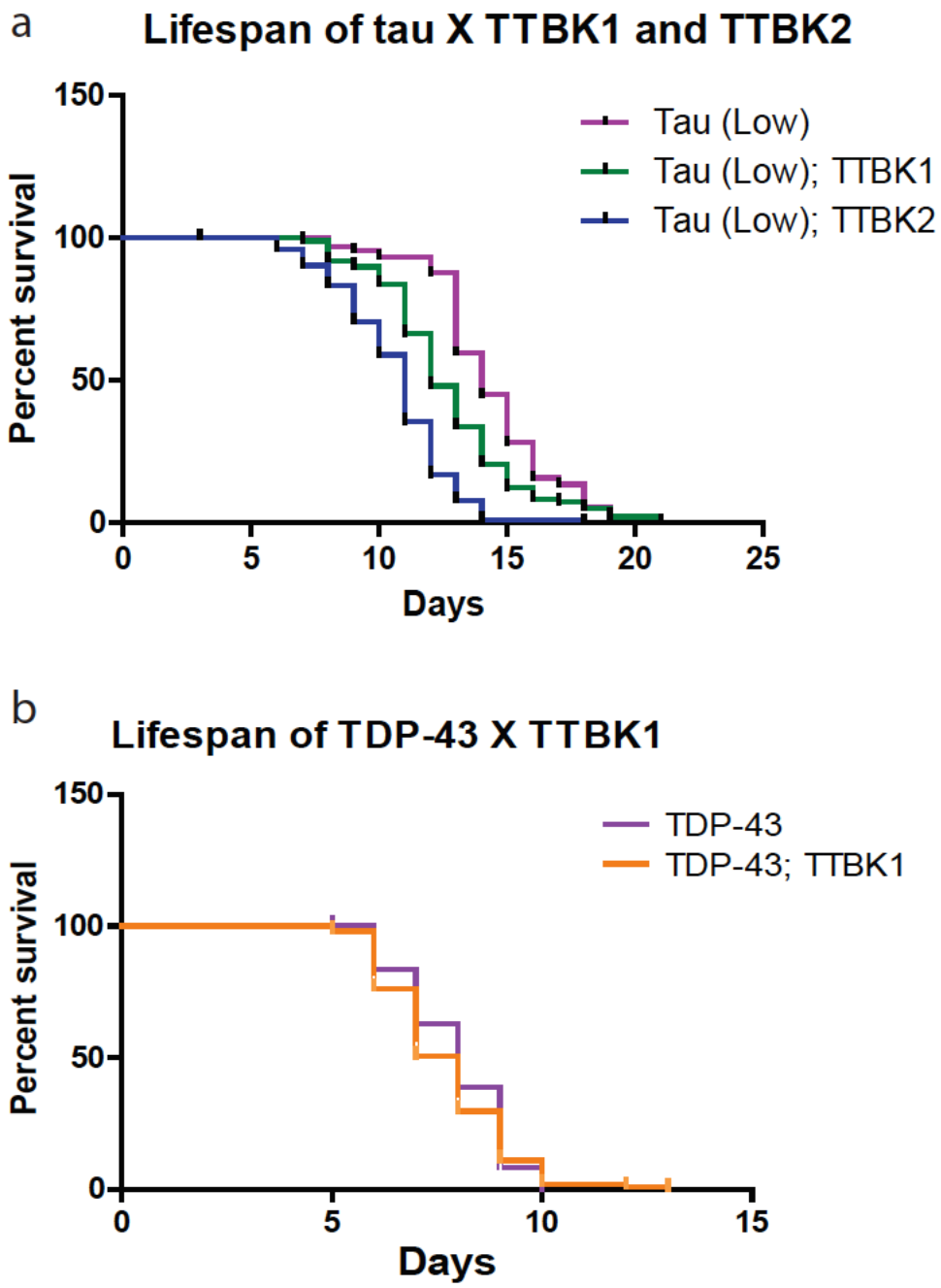
kinase and our results suggest that TTBK1 kinase activity is able to induce neurodegenerative phenotypes in both tau and TDP-43 backgrounds. TTBK1 selective kinase inhibitors therefore represent a potential means to treat both FTLD-tau and FTLD-TDP.

Supplemental Figure 1: Characterization of hTTBK-cat C. elegans lines



Supplemental Figure 1 Legend: (a) Synchronized TTBK-cat transgenic L4 larvae behavior was measured by radial velocity relative to non-transgenic (non-Tg) animals. Animals were measured for the linear distance traveled from a central reference point over one hour, N>75 for each genotype. Significance was determined using an unpaired T-test. P=0.04 for Non-TG vs. hTTBK1-cat and P= 0.007 for Non-Tg vs. hTTBK1-cat. Lines that were selected for further analysis are indicated by black diamonds. (b) Lifespan analysis for hTTBK1-cat. (c) Lifespan analysis for hTTBK2-cat. (d) Extrachromasomal array transgenic TTBK1 kinase dead mutants crossed with Tau transgenic animals. Note that TTBK1 kinase dead mutants have normal locomotion relative to non-tg and do not significantly change tau mediated locomotion defects. (e) Kinase Dead TTBK1 transgenes crossed with TDP-43 transgenic lines. Note that TTBK1 kinase dead mutants do not significantly change TDP-43 mediated locomotion defects.

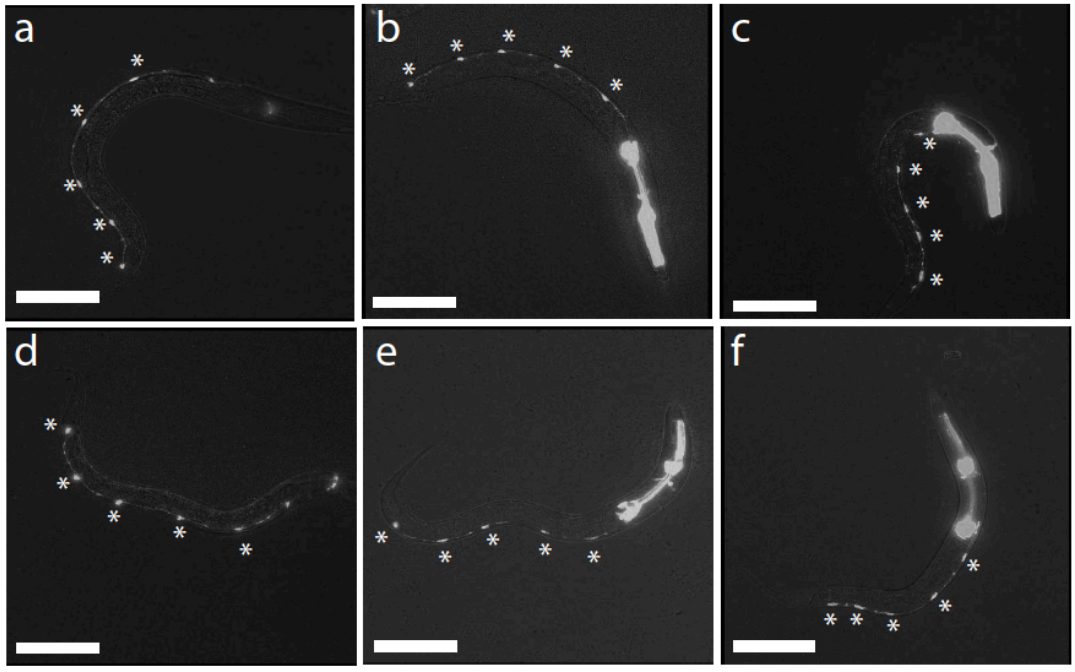
Supplemental Figure 2: Lifespan Analyses

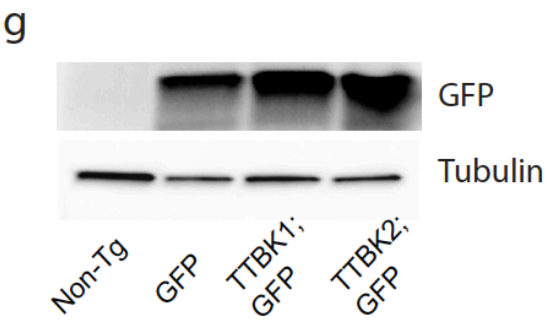


Supplemental Figure 2:

Lifespans of each transgenic line was assessed at 25°C. (a) Lifespan analysis of tau versus tau;TTBK1 or tau;TTBK2. Tau median lifespan = 14 days, tau; TTBK1 median lifespan = 13 days, tau; TTBK2 median lifespan = 12 days. Significance was analyzed using a Chi-squared analysis with a Mantel-Cox test. $P=0.04$ for tau vs. tau; TTBK1 and $P<0.001$ for tau vs. tau; TTBK2. (b) Lifespan analysis of TDP-43 versus TDP-43; TTBK1. TDP-43 median lifespan = 8 days, TDP-43; TTBK1median lifespan = 7.5 days. No significance.

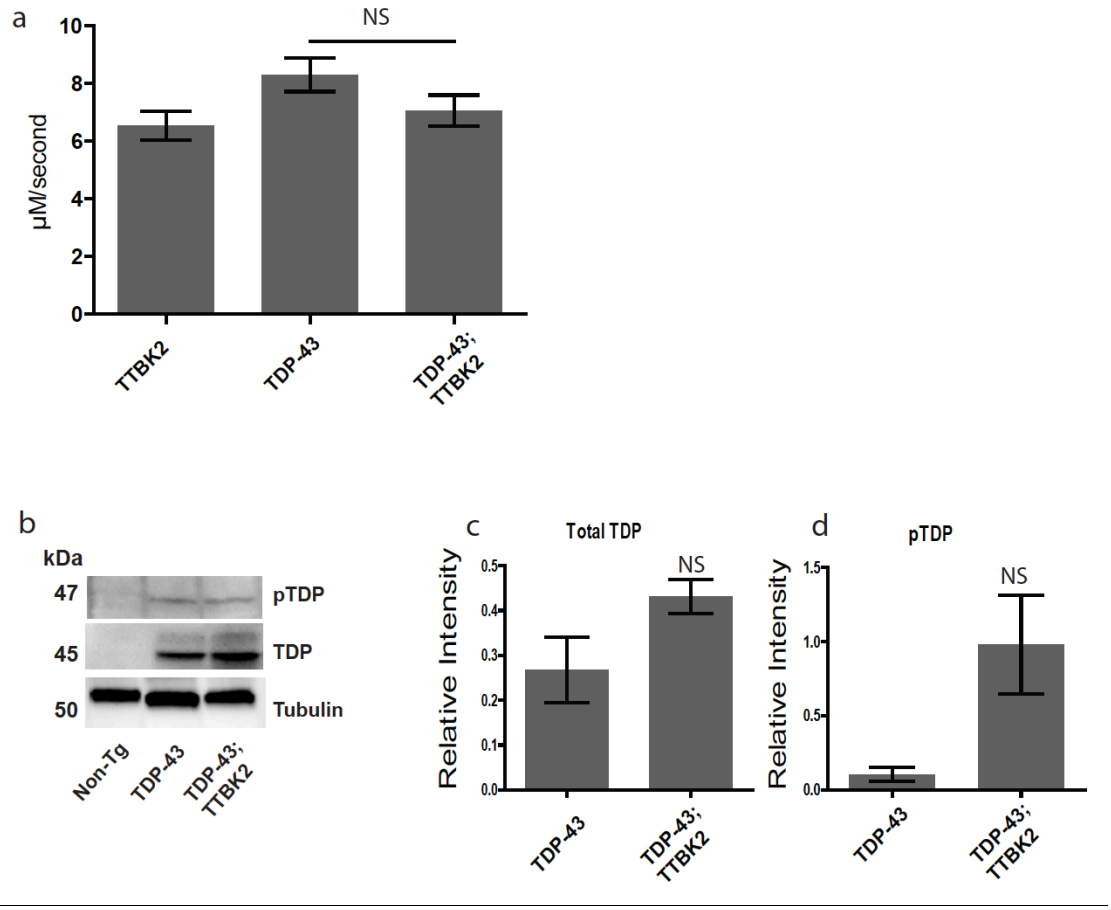
Supplemental Figure 3: L1 transgenic C. elegans are developmentally normal and GFP levels are unaffected by hTTBK1 or hTTBK2 expression





Supplemental Figure 3 Legend: GFP-labeled D-type GABAergic motor neurons were observed in L1 larval transgenic animals *in vivo* living worms. (a) TTBK1 (b) tau (high) (c) tau (high); TTBK1 (d) TTBK2 (e) tau (low) (f) tau (low); TTBK2. Asterisks indicate live neurons. Scale bar=50um. (g) GFP expression levels are not influenced by the presence of hTTBK1-cat or hTTBK2-cat.

Supplemental Figure 4: TTBK2 does not affect TDP-43 phenotypes



Supplemental Figure 4 Legend: (a) Staged hTTBK2-cat; TDP-43 transgenic L4 larvae do not exhibit significantly decreased radial velocity relative to TDP-43 transgenic animals. Animals were measured for the linear distance traveled from a central reference point over one hour, N>100 for each genotype. Significance was determined using an unpaired T-test. P=0.13 versus TDP-43. (b) hTTBK2-cat; TDP-43 transgenic animals have slightly increased but not statistically significant total TDP-43 and pTDP-43 relative to TDP-43 animals. Bar graphs represent six independent replicate immunoblots of (c) total TDP-43 and (d) pTDP. Graphs are plotted in relative intensity. Significance was determined using an unpaired T-test. P=0.059 for total TDP and p=0.13 for pTDP.

4 TTBK1/2 processing is altered in tauopathy-related diseases

Introduction

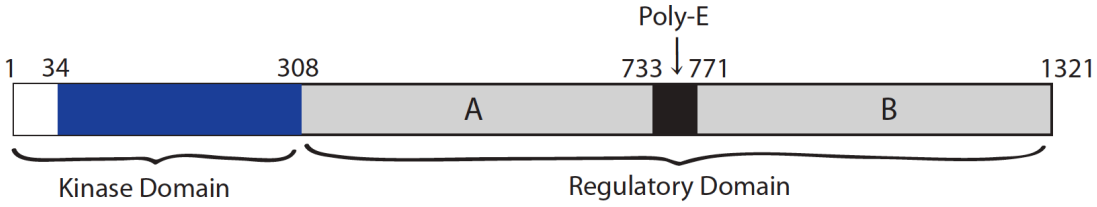
Aberrant protein deposits characterize many neurodegenerative diseases. In a number of neurodegenerative disorders, these deposits contain phosphorylated TDP-43 or tau. Though highly debated, evidence suggests that the phosphorylated species of TDP-43 and tau may be the key toxic factor for initiating disease course [17, 74, 93, 126]. Therefore, understanding which kinases are able to phosphorylate TDP-43 and tau, may lead to potential therapeutic interventions. The Tau Tubulin Kinases 1 and 2 (TTBK1/2) are two recently characterized kinases that are able to phosphorylate both tau and TDP-43[59, 97, 100]. Furthermore, recent work has shown that TTBK1 is upregulated in Alzheimer's disease and TTBK1/2 are upregulated in Frontotemporal dementia [59, 62].

Although the kinase activity of TTBK1/2 has been well studied, the mechanisms by which TTBK1 and TTBK2 are regulated are elusive. The kinase domain only comprises the first 297 amino acids of TTBK1 and 331 amino acids of TTBK2, accounting for only 22-26% of the whole protein. The kinase domain is highly conserved across species including *C. elegans, D. Melanogaster,* and all mammals [100]. The regulatory domains of both TTBK1 and TTBK2 in humans are distinct and show very little similarity to other known proteins. Furthermore, between TTBK1 and TTBK2 there is only one region of homology that has been identified in the C-terminus of the regulatory region, spanning amino acids 1053–1117 in TTBK1 and 942–1006 in TTBK2 with 43% identity and 58% similarity, and its role remains unclear [100]. This suggests that TTBK1 and TTBK2 are likely regulated in unique pathways and the functional overlap they share is specific to kinase activity. Of particular interest is the regulatory domain of TTBK1, which contains a stretch of 39 glutamate residues interrupted by three aspartate residues

towards the end of the repeat region. No other protein contains a polyglutamate region this extensive, and the role it plays in TTBK1 regulation and signaling in unknown. In the initial characterization of TTBK1, there was evidence for processing of the protein into fragments that ranged in size from 25-100kda [59]. Although cleavage of TTBK1 occurs both in normal and diseased tissues, there is no clear understanding of which truncated protein species are disease relevant. Furthermore, the roles that these processed TTBK fragments play in normal protein function are unknown. It is possible that protein processing is an indicator of abnormal lysosomal processing, as suggested by Ikezu, et al. [100]. A clear understanding of which species are upregulated or downregulated during disease progression as compared to age-matched controls will reveal how the function of the TTBKs and their regulation changes during the course of disease progression.

To better understand whether TTBK1 is processed differentially in disease, we performed western blot protein analyses of several tauopathies and related diseases. Specifically, we targeted various TTBK1 epitopes across the regulatory domain, designated as domain A, polyE, and domain B (Fig. 1). We observed that some regions of the regulatory domain are in fact upregulated in certain diseases, but not all. We also observed that within diseases, there is variability in expression levels on a case-by-case basis. This suggests that the regulation of TTBK1 in disease is variable and complex and that further studies are necessary to better elucidate how TTBK1 processing changes in disease.

Figure 1: TTBK1 Protein Model



Methods

C. elegans *strains*

The full-length TTBK1 *C. elegans* strain was constructed by introducing human TTBK1 cDNA, driven by the pan neuronal *rgef-1* promoter (*Prgef-1*::hTTBK1) into the *C. elegans* genome. *Prgef-1*::hTTBK1 microinjected into N2 at a concentration of 20ng/µl with an *elt-2*::mCherry coinjection marker at a concentration of 25ng/µl. For all transgenic strains, extrachromosomal arrays were then integrated by exposing animals to a dose of 4000 Rad Gamma rays and subsequently outcrossed back into the N2 background at least twice.

Radial locomotion assay

Behavior was assessed by placing 15-20 age-matched (L4) *C. elegans* at the center of a 150mm NGM plate supplemented with 5x peptone (5xPEP), with a uniform OP50 bacterial lawn. After one hour of free movement at room temperature, the radial distance traveled from the origin by each animal was measured. Distance from the origin traveled per unit of time was expressed in micrometers per second to give a radial velocity. The assay was performed in triplicate by an observer blinded to genotype and statistical analyses were performed using GraphPad Prism software.

Immunoblotting

Human post-mortem brain lysates and *M.Musculus* brain lysates were prepared by homogenization of tissue in lysis buffer (50mM HEPES pH 7.5, 1mM EDTA, 150mM NaCl, 10% Glycerol, 0.1% Triton X-100, 1mM PMSF, 1 protease inhibitor pellet (Roche cOmplete Mini)) followed with a 10 second sonication at 50% amplitude using a Branson Sonifier with

micro tip. Total protein lysate was loaded in 5XSDS buffer (5% SDS, 200mM DTT, 50mM Tris pH 6.8, 5mM EDTA, 50% sucrose, 0.05% Bromophenol Blue), resolved on a precast 4-15% gradient SDS-PAGE gel (BioRad) at 200V, and transferred to PVDF membrane (Bio-Rad Immun-blot PVDF membrane) at 100V for 32m. TTBK1 domain A was detected with the commercially available polyclonal antibody directed at a C-terminal region of the TTBK1 regulatory domain (Sigma, SAB3500002, 1:2000). TTBK1 domain B was detected with the commercially available polyclonal antibody directed at amino acids 1203-1218 (DLRPKQPPGRGLGPGR) (ProSci, Cat. No. 5013, 1:2000). The polyglutamate region of TTBK1 was detected with a polyclonal antibody (1:5000) generously provided to us by the Gorovsky lab [140].

Mixed-stage populations of *C. elegans* were grown on 150mm 5XPEP plates, washed with M9 buffer, and frozen with liquid nitrogen. Protein lysates were prepared by sonication of frozen *C. elegans* pellets in lysis buffer (10 mM Tris-HCL pH 7.5, 5 mM EDTA, 10% sucrose) at 70% amplitude for 10 seconds, repeated three times. The lysate was loaded and resolved on precast 4-15% gradient SDS-PAGE gels (BioRad) and transferred to PVDF membrane (Bio-Rad Immun-blot PVDF membrane) at 100 volts for 32m. TTBK1 domain B was detected with the commercially available polyclonal antibody directed at amino acids 1203-1218 (DLRPKQPPGRGLGPGR) (ProSci, Cat. No. 5013, 1:2000). Load controls were detected by probing for β-Tubulin as previously described [125].

Post-mortem human tissue

We obtained de-identified samples of *postmortem* tissue from the University of Washington Alzheimer's Disease Research Center (ADRC) Neuropathology Core (PI, Dr. C.

Dirk Keene) after receiving human subjects approval (University of Washington human subjects division approval: HSD# 06-0492-E/A 01). Cases were selected on the basis of having an autopsy-confirmed diagnosis of FTLD-tau, FTLD-TDP, PSP, CBD, or Pick's disease. Control samples were from neurologically healthy control participants, who were of a similar age and were confirmed to be negative for neuropathologic changes using routine and immunohistochemical assays. Frontal cortex (prefrontal middle frontal gyrus) and hippocampus (at the level of the lateral geniculate nucleus) samples were dissected at the time of autopsy in coronally sliced brains fixed approximately 3 weeks in 10% neutral buffered formalin according to routine protocols. Samples were processed and embedded in paraffin according to standard protocols.

Results

TTBK1 processing is variable across the tau-related disease spectrum.

Tauopathies are a group of diseases characterized by abnormal tau accumulations. Phosphorylated tau is a common feature of a majority of tauopathies [141] and TTBK overexpression has been implicated in several tauopathies including Alzheimer's disease [59] and FTLD [62]. To understand whether TTBK1 processing differed across diverse tau-related pathologies, we performed a western blot panel targeting two epitopes of the TTBK1 regulatory domain against post mortem tissue from FTLD-17 (mutant tau), FTLD-tau, FTLD-TDP, CBD, Pick's, C9orf72, and PSP confirmed cases (**Fig. 2a and b**). We observed that TTBK1 is overexpressed in all diseases, with the exception of the C9orf72 mutation case, as compared to the age-matched controls.

When we probed for domain A of TTBK1 (**Fig. 2a**), we observed consistent fragmentation patterns across most FTLD, CBD, and PSP cases. Surprisingly, the Pick's case displayed only a triplet of bands between 40-50kDa, which only slight evidence of full-length TTBK1. This suggests that processing in Pick's disease may vary from the other disease types. It was also apparent that processed fragments of TTBK1 were more highly expressed than full-length TTBK1 in disease cases, whereas in controls, processed fragments cannot be detected. This provides evidence that the processed species of TTBK1 may be more disease relevant than full length TTBK1.

In contrast, when we probed for domain B of TTBK1 (**Fig. 2b**), processed fragments in the control group became more apparent. In agreement with the results from the domain A blot, we observed no evidence of overexpression in the C9orf72 mutant case, and observed large overexpression of fragments ranging in size from 40-50kDa in the Pick's disease case. This suggests that the fragments that migrate at 40-50kDa likely contain some of both domain A and domain B regions of the regulatory domain, and further suggests that this region of the protein may be disease relevant. Additionally, we observed significant overexpression of TTBK1 in two of the FTLD cases, signifying that pathology is variable across disease, and likely occurs on a per cases basis.

To better understand what is happening with TTBK1 processing with each disease, we expanded the number of cases and probed for each regulatory domain region.

Figure 2: Panel of TTBK1 Processing across Tauopathy-related Disorders

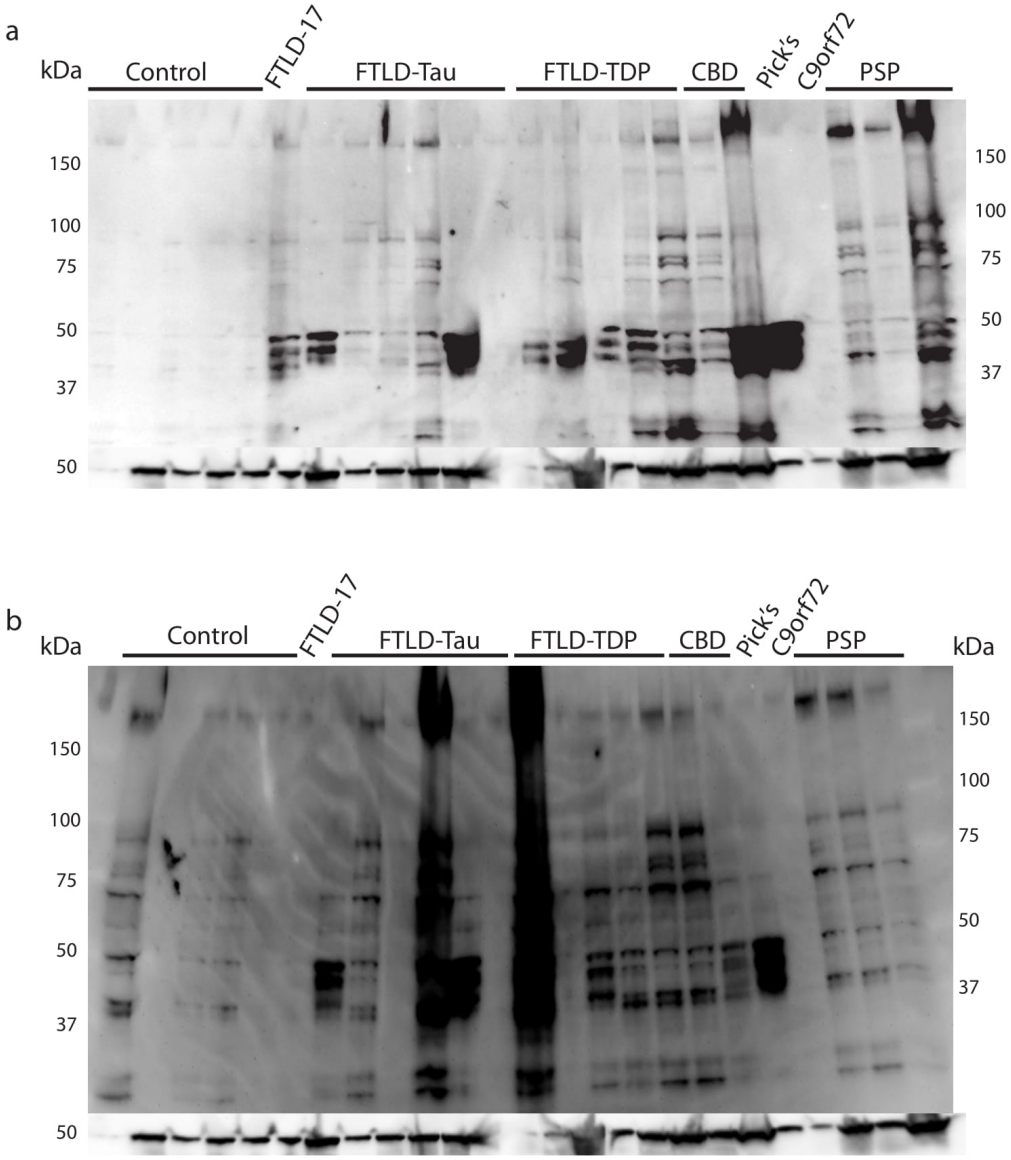


Figure 2 Legend: **a** Patients diagnosed with FTLD, CBD, Pick's, and PSP exhibit increased processed TTBK1 expression when probed for domain A as compared to age-matched controls. **b** Patients diagnosed with FTLD, CBD, Pick's, and PSP exhibit increased processed TTBK1 expression when probed for domain B as compared to age-matched controls.

FTLD-tau and FTLD-TDP show consistent overexpression and processing patterns of both TTBK1 domain A and domain B regulatory regions.

To observe whether TTBK1 fragment overexpression is consistent across FTLD-Tau and FTLD-TDP cases, we ran seven confirmed FTLD-tau and five confirmed FTLD-TDP cases alongside two age-matched controls. We specifically performed western blot analysis targeting two regions of the TTBK1 regulatory domain, termed domain A and domain B (see **Fig. 1**) in order to better understand which regions of the regulatory domain are present in each processed species. When domain A was probed, processing patterns were apparent in both controls and diseased specimens. For controls, the most prominent bands were featured at roughly 200kDa, 4 bands around 75kDa, and a series of smaller fragments ranging in size from 40-50kDa (**Fig. 3a**). In contrast, the FTLD disease cases showed overexpression patterns of a fragment resting at around 150kDa and overexpression of a triplet band in the range of 40-50kDa. Several FTLD-tau and most FTLD-TDP cases also had a reduction in expression of the bands around 75kDa. There were no clear differences between the FTLD-tau and FTLD-TDP samples. Similar to what was observed with domain A, extensive overexpression of TTBK1 fragments ranging in size from 40-50kDa were apparent when probing for domain B (**Fig. 3b**) Banding followed a similar pattern across all disease cases, suggesting that small upregulated fragments contain both TTBK1 domains A and B.

These data indicate that TTBK1 processing mechanisms are altered during disease progression. The reduction in full-length TTBK1 and increase in fragments suggests that TTBK1 is getting processed or possibly degraded at a faster rate in disease. The presence of a significantly overexpressed triplet band in the FTLD samples also suggests that the small processed fragments of TTBK1 may be the disease-relevant species.

Figure 3: TTBK1 Processing in FTLD-tau and FTLD-TDP

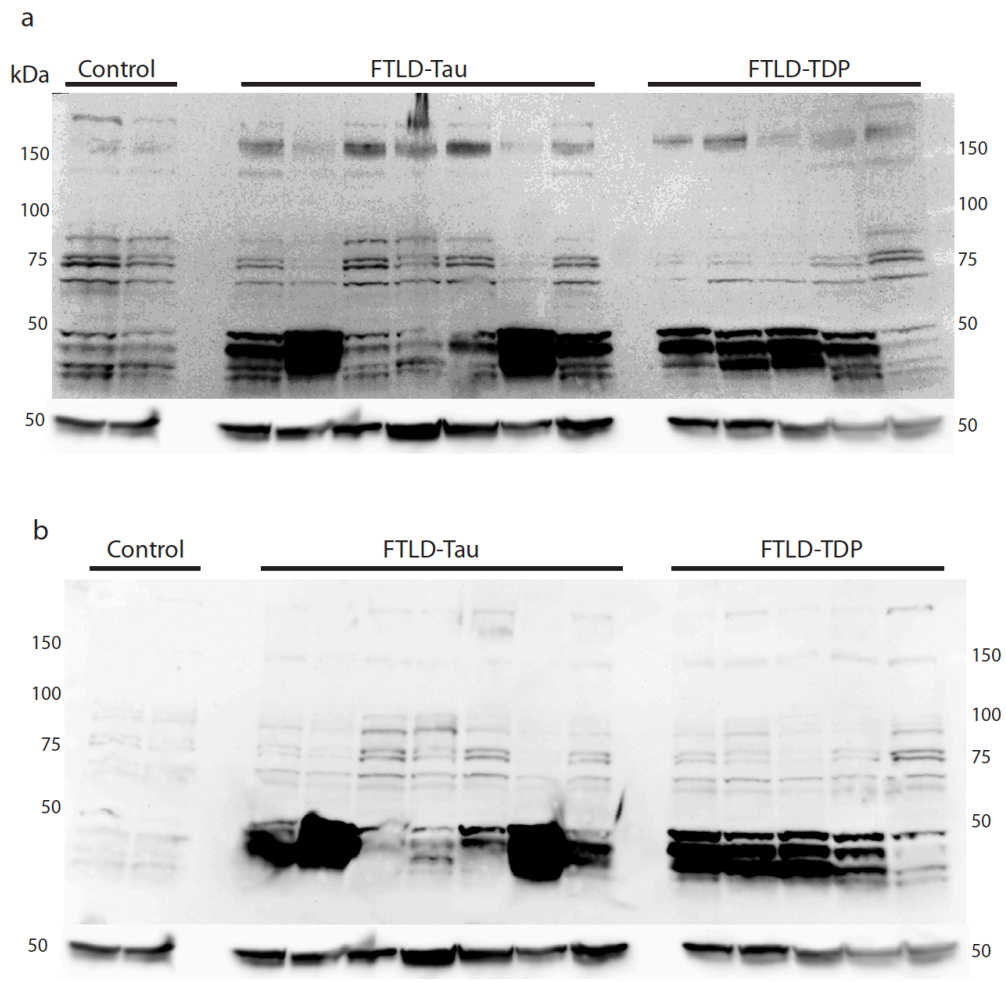


Figure 3 Legend: **a** Patients diagnosed with FTLD-tau and FTLD-TDP exhibit increased processed TTBK1 expression when probed for domain A as compared to age-matched controls. **b** Patients diagnosed with FTLD-tau and FTLD-TDP exhibit increased processed TTBK1 expression when probed for domain B as compared to age-matched controls.

TTBK1 is upregulated in PSP, CBD, and Pick's disease cases.

Because of the apparent expression increase in FTLD cases, we decided to look at whether TTBK1 processing was also changed in related tauopathies: PSP, CBD, and Pick's disease. To date, there has been no work done looking at TTBK1 expression levels in any of these diseases. We performed western blot analyses for the different regulatory domain regions of five confirmed PSP cases, five confirmed CBD cases, and seven Pick's disease cases in comparison to age-matched controls. We observed a significant increase in TTBK1 expression when probed against TTBK1 domain A or domain B, but not when probing for polyglutamate (**Fig. 4a**). Surprisingly, one PSP case had marked overexpression of both domain A and domain B fragments. Further work is ongoing to better understand the identity of the unknown TTBK1 species. To get a better idea of how the other cases compared to controls without the risk of oversaturation, we removed the outlying PSP case and reprobed for domain A and domain B (**Fig. 4b**). From this we were able to show that TTBK1 fragments containing regions of domain A and domain B are upregulated in disease. Again, the greatest upregulation was observed in fragments ranging is size from 40-50kDa. These findings suggest that there is a common mechanism of TTBK1 processing that is disrupted during tauopathy progression. The Pick's disease cases showed a similar triplet-banding pattern (**Fig. 5a**) when probed for both domain A and domain B of TTBK1. Not all cases were upregulated to the same extent. This could be the result of variable post-mortem intervals or severity of disease course.

Figure 4: TTBK1 Processing in CBD and PSP

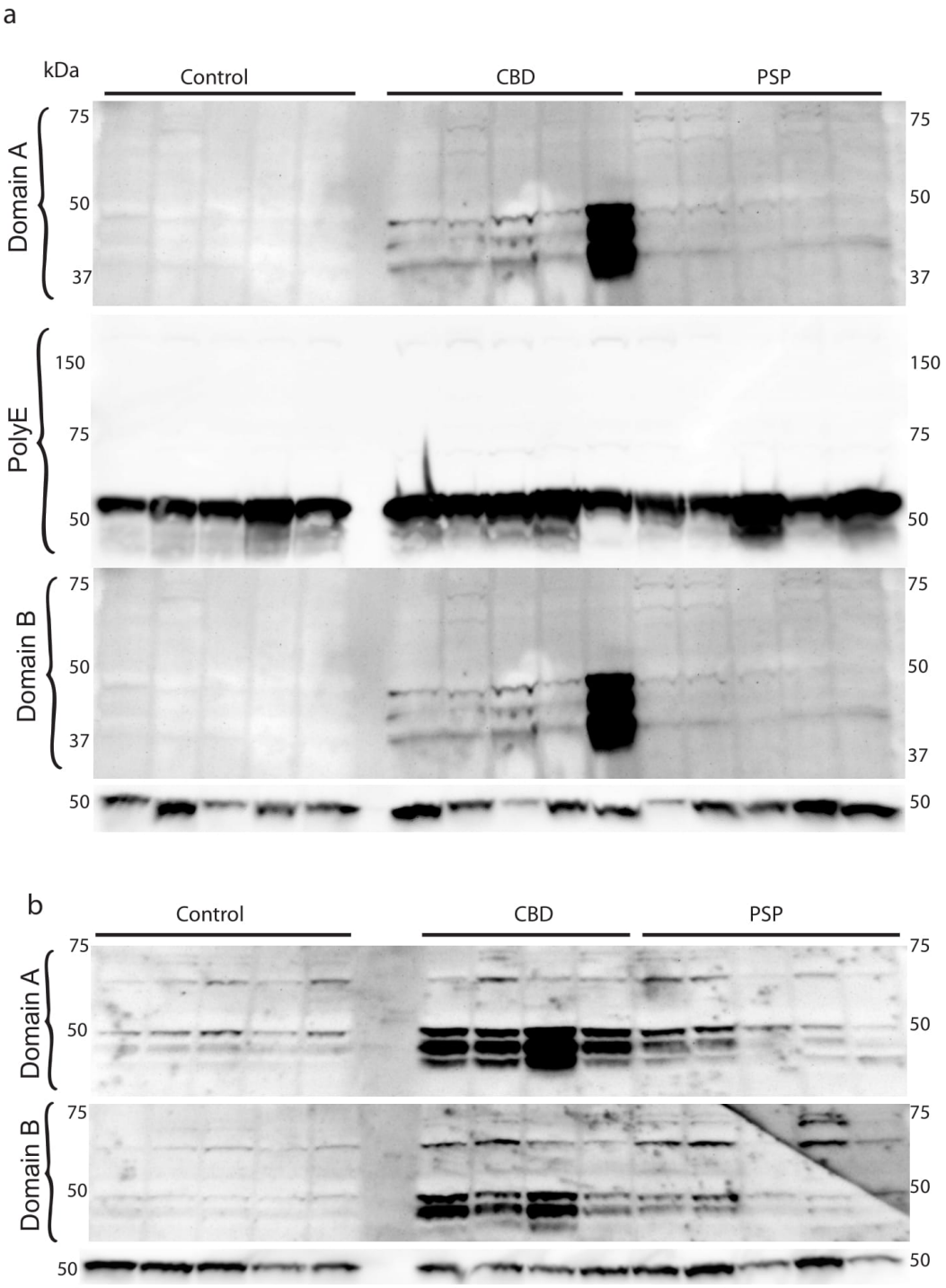


Figure 4 Legend: **a** Patients diagnosed with CBD, but not PSP, exhibit increased processed TTBK1 expression when probed for domain A or domain B as compared to age-matched controls. PolyE expression patterns are unaltered. **b** Patients diagnosed with CBD, but not PSP, exhibit significantly increased processed TTBK1 expression when probed for domain A and domain B as compared to age-matched controls.

Figure 5: TTBK1 Processing in Pick's Disease

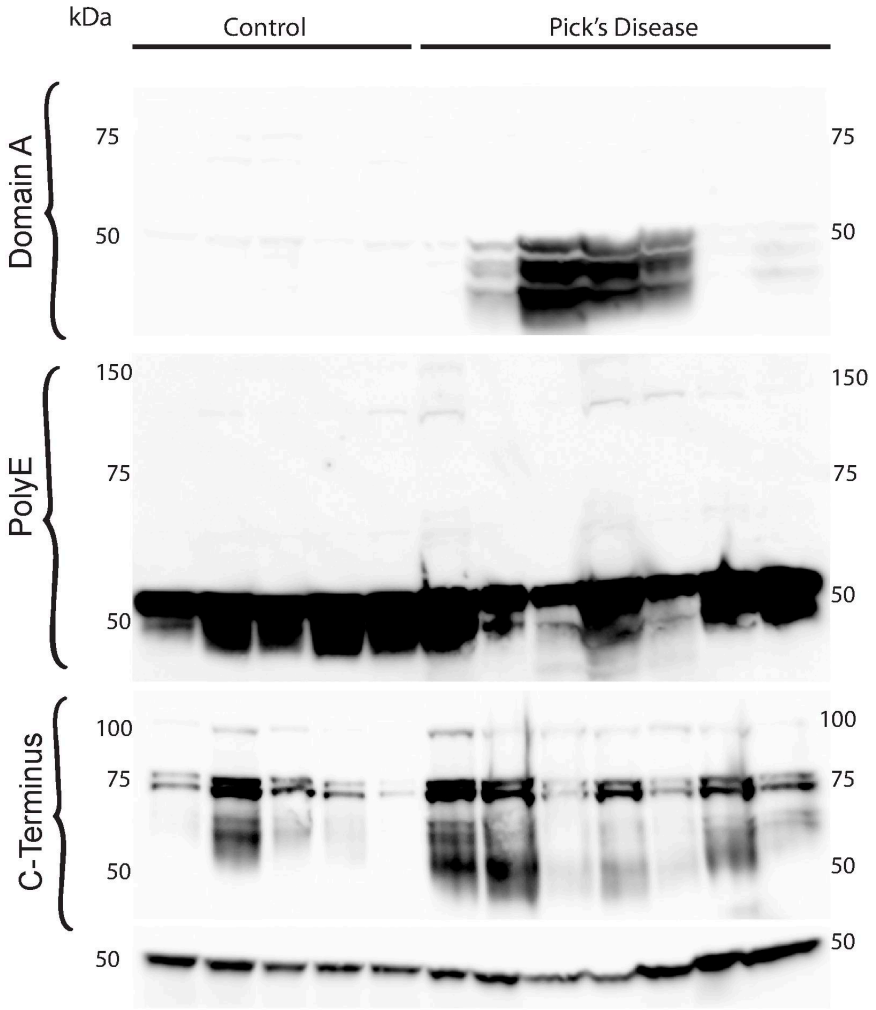


Figure 5 Legend: **a** Some, but not all patients diagnosed with Pick's disease exhibit increased processed TTBK1 expression when probed for domain A, but not domain B or PolyE, as compared to age-matched controls.

TTBK1 processing does not change in ALS tissue.

Our previous work demonstrates that TTBK1 is a TDP-43 kinase and co-localizes with phosphorylated TDP-43 in ALS tissue [97]. However, we recently demonstrated that TTBK1 active kinase does not phosphorylate TDP-43 to the same extent as tau *in vivo* [62]. To date, no studies have been performed to assess TTBK1/2 levels in ALS tissue. To address whether TTBK1 is overexpressed and also exhibits changes in its processing patterns in ALS tissue with confirmed phosphorylated TDP-43, we performed the same western blot analyses, targeting each regulatory domain region on five confirmed ALS cases alongside five age-matched controls. As our previous data would suggest, we did not observe a significant increase in any TTBK1 processed fragments as compared to age-matched controls (data not shown). These findings suggest that aberrant TTBK1 processing plays a role in tauopathy, but not TDP-43 pathology.

TTBK1 processing patterns are not consistent across species.

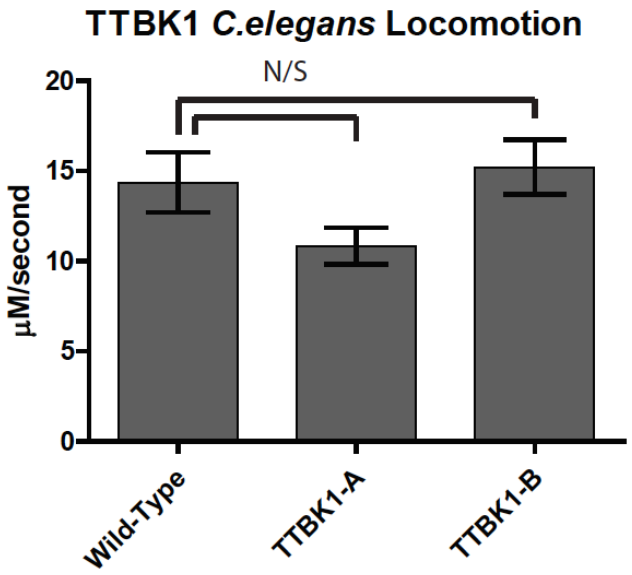
In order to further explore the role that TTBK1 processing plays in disease, we chose to examine transgenic human TTBK1 protein expression in two non-human models: *C. elegans* and *M. musculus.* We constructed transgenic (Tg) *C. elegans* lines expressing full-length human TTBK1 (hTTBK1) under the pan-neuronal promoter *rgef-1* by micro-injecting a plasmid transgene (P*rgef-1*::hTTBK1). The resultant extrachromosomal transgenic strains were exposed to gamma radiation to create stable genomically integrated transgenic lines. Two lines of hTTBK1 were then characterized for behavior using a radial locomotion assay. We observed that both hTTBK1 lines were behaviorally normal (**Fig. 6a**).

To analyze whether TTBK1 processing patterns were consistent across species, we performed western blot analysis of each sample run side by side on the same gel. We chose to target TTBK1 domain B, since it produced the most striking results in our disease cases in

comparison to controls. We observed that processing patterns are not consistent across species (**Fig 6b**). The full-length *C. elegans* lines only displayed two bands, one at roughly 230kDa and another at roughly 140kDa. In contrast, the mouse lines displayed prominent overexpression of both full-length TTBK1 at 230kDa and several bands of processed TTBK1 at roughly 90kDa, with a slight fragment band located at around 70kDa. This expression pattern was previously displayed in the original characterization of TTBK1 overexpression mouse lines and Alzheimer's disease cases [104]. In contrast, the two human tauopathy cases (including the CBD case with significant domain B overexpression) displayed extra bands in the 40-55kDa range, that were not shown in the transgenic models. However, these cases did have a consistent band at 90kDa, which match the mouse overexpression model. This data implies that the processing patterns of TTBK1 are either species-specific or only displayed in pathological conditions. This would further suggest that the proteases and machinery necessary for processing TTBK1 disease-relevant fragments lack a *C. elegans* or *M. musculus* functional homolog or signaling mechanism. Alternatively, this could suggest that the fragments are only activated or expressed during disease conditions, which are not fully mimicked in our transgenic models.

Figure 6: TTBK1 processing is variable across species

a



b

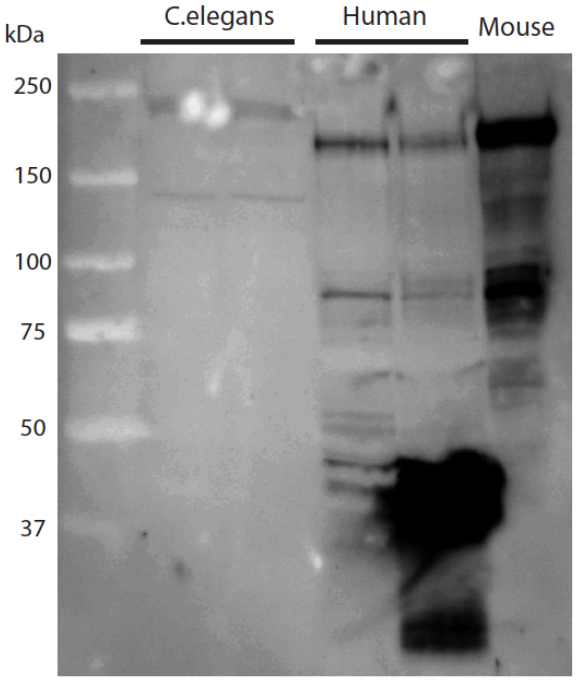


Figure 6 Legend: (**a)** Two lines (A and B) of staged hTTBK1 full-length transgenic L4 larvae do not exhibit behavioral abnormalities relative to non-transgenic animals. Animals were measured for the linear distance traveled from a central reference point over one hour, N>50 for each genotype. Significance was determined using an unpaired T-test. P=0.13 versus TDP-43. **(b)** When probed for domain B of TTBK1, *C. elegans*, Human PSP tissue, and Mouse tissue display differential patterns of TTBK1 processing.

Discussion

Previous work on the initial characterization of TTBK1 showed processing in both human control and AD cases [59]. There is also evidence that TTBK1 is upregulated in disease [59, 62]. However, it is not understood how this processing event is altered in disease and whether TTBK1 overexpression is a consistent feature of other tauopathies, including PSP, CBD, and Pick's disease. To address these gaps in knowledge, we performed a series of protein expression analyses targeting different regions of TTBK1, to better understand where processing occurs in the protein and to provide evidence for overexpression across diseases.

Our results give evidence for the accumulation of short cleaved protein fragments and decreased expression of full-length protein in four types of tauopathy: FTLD, PSP, CBD, and Pick's disease. Specifically, we demonstrate a consistent processing pattern where fragments ranging in size from 40-50kDa are significantly upregulated in most disease cases. The role that these fragments play in pathology remains unknown; however, I hypothesize that this aberrant processing of TTBK1 results in an imbalance of full-length protein to fragment ratios, favoring increased accumulation of TTBK1 fragments. This abnormal processing likely alters signaling pathways and results in further upregulation of TTBK1.

We hypothesize that the changes we observed in TTBK1 processing may lead to increased TTBK1 activity, which would result in more tau phosphorylation. Our data also suggest that the aberrant overexpression of TTBK1 fragments in disease cases are performed by human specific proteases. To further explore which proteases may be involved, a protease-targeted screen could be performed.

Conclusion

Ultimately, the identification of disease-relevant cleavage products and proteases that drive disease progression could lead to the development of targeted modulators of specific species or cleavage sites, which may serve as a novel therapeutic approach to ALS and FTLD treatment. It also provides insight into how TTBK1 may be self-regulated, and provides motivation for the further study of TTBK1 regulatory mechanisms.

5 A Screen for TTBK1 and TTBK2 Interacting Proteins

Introduction

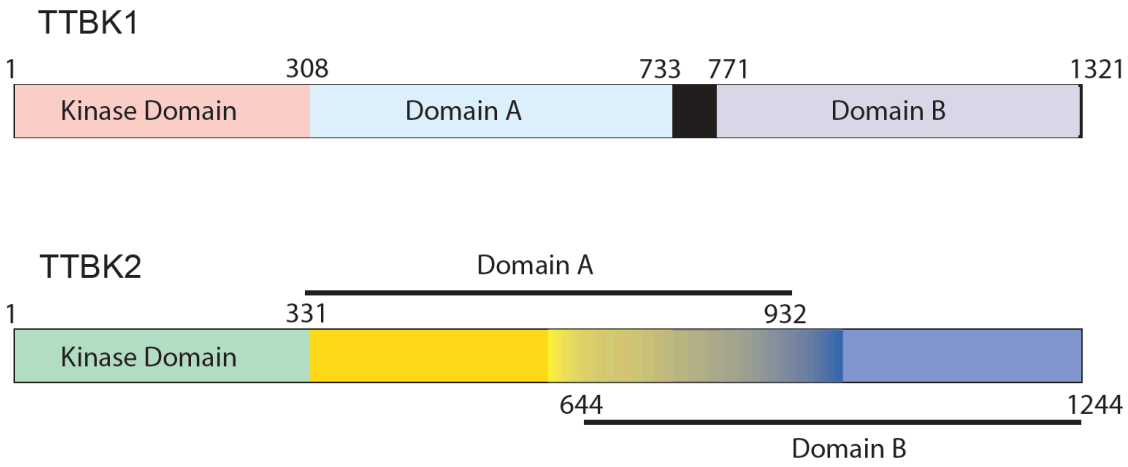
Tau tubulin kinases 1 and 2 (TTBK1 and TTBK2) have been implicated in a number of neurodegenerative diseases including Alzheimer's disease, Frontotemporal Dementia, and other tauopathies [59, 62, 107, 110]. They are a pair of kinases that are able to phosphorylate both TDP-43 and tau and are upregulated in the presence of pathological lesions [62, 97]. Although both are expressed in brain tissue [60, 100], their function in neurons and the central nervous system remains unknown. Additionally, the regulatory mechanisms of TTBK1/2 are not yet understood.

Several binding partners of TTBK2 have been identified, demonstrating the role that TTBK2 plays in initiating ciliogenesis. These binding partners include CEP164 [112, 114], EB1 [112, 113], and EB3 [113]. During cilium formation, TTBK2 forms a complex with and phosphorylates CEP164 to trigger the assembly of the primary cilium [114]. Specifically, it binds to CEP164 in the proline rich C-terminal region [112]. TTBK2 also binds to EB1 in its C-terminal domain, which likely results in its migration to the plus-ends of microtubules during ciliogenesis. TTBK2 was also found to phosphorylate Cep97 during centriole recruitment [112]. EB3, another plus-end microtubule tracking protein, was further discovered to bind TTBK2, but only when TTBK2 kinase domain is not regulated by its own C-terminus. The same study demonstrated that phosphorylation of KIF2a, a kinesin depolymerase, by TTBK2 inhibits KIF2a activity [113]. These interactions all emphasize the regulatory role that TTBK2 plays in primary cilium formation. Protein binding partners of TTBK1, on the other hand, have not been identified.

A better understanding of what proteins interact with TTBK1/2 will allow us to better elucidate the potential mechanisms for TTBK1/2 regulation and provide further evidence for additional roles that TTBK1/2 may play in the nervous system. Moreover it will give us insight into non-developmental functions of TTBK1/2 and other cellular processes that may be affected during disease by aberrant TTBK activity. Identification of novel protein interactions could also provide a basis for the development of targeted therapeutics.

To identify proteins that interact with TTBK1/2 we performed a yeast two-hybrid screen targeting various domains of both TTBK1 and TTBK2 (Fig. 1). Specifically, we expressed individual domains of either TTBK1 or TTBK2 as bait in yeast and screened a human fetal brain cDNA library for binding partners. Positive interactions were confirmed using a LacZ/X-gal filter assay and curated into a list. We were able to identify a total of 30 unique hits, two of which were previously characterized, which demonstrates the efficacy of the screen. For both TTBK1/2 the majority of protein binding partners were involved in cellular growth, cytoskeleton regulation, or autophagosome maturation. This suggests that the TTBKs play a diverse set of roles centered on microtubule structure and mitotic regulation and provides evidence for a novel role for TTBK1/2 in the maturation of autophagosomes.

Figure 1: TTBK1 and TTBK2 Yeast Two-Hybrid Domains



Methods

Yeast two-hybrid screen

Bait plasmids for each TTBK1 and TTBK2 region were constructed in the pLexA-N vector. The following constructs were inserted under control of the LexA promoter: TTBK1 kinase domain (AAs 1-308), TTBK1 domain A (AAs 297-733), TTBK1 domain B (AAs 775-1321), TTBK2 kinase domain (AAs 1-331), TTBK2 domain A (AAs 331-932), and TTBK2 domain B (AAs 644-1244). Cultures of TTTBK1/2 bait plasmid yeast were grown overnight at 30°C, brought to log phase the next day, and transformed with the Clontech Matchmaker Human Fetal Brain Library (1.3 mg/mL), a cDNA library in the pACT2 vector, resulting in greater than 1X10^6 transformants. Transformants were plated on 150mm plates of SD minimal media lacking leucine, tryptophan, and histidine and containing 5-100mM 3-Amino-1,2,4-Triazole (3-AT) and grown at 30°C. Colonies were picked and grown in SD broth lacking tryptophan and leucine. The candidate prey plasmid from the matchmaker library was isolated, transformed and amplified in *E. coli*, re-isolated, and identified by sequencing from primers within the pACT2 backbone.

Filter Assay for Selectivity

The prey plasmid for positive, unique interacting proteins was transformed back into L40 yeast along with either the TTBK1/2 bait or a MS2 plasmid. Four biological replicates of each transformation were struck onto SD minimal media lacking both tryptophan and leucine or SD minimal media lacking only tryptophan for controls and grown at 30°C overnight. The colonies were then transferred onto nitrocellulose paper (GE Healthcare, Whatman Protran BA 85). The nitrocellulose was immersed in liquid nitrogen for one minute and allowed to thaw for two

minutes. The nitrocellulose was transferred to three layers of blotting paper that were saturated in Z buffer ($Na_2HPO_4 \cdot 7H_2O$, $Na_2HPO_4 \cdot H_2O$, KCl, $MgSO_4 \cdot 7H_2O$, adjusted to pH 7.0) with X-GAL (Cat No. 8060-1) dissolved in DMF at 20mg/ml, and sealed with parafilm within a petri dish. The plates were incubated at room temperature until the controls began to display a blue hue. Selectivity was determined based on which interacting transformants displayed B-gal activity prior to the controls, and did not show B-gal activity with the MS2 plasmid.

Results

To identify potential regulators of TTBK1/2 protein activity, I performed yeast two-hybrid screens for proteins that interact with TTBK1 or TTBK2. To gain a better understanding of where each protein interaction was taking place, we cloned specific regions of both TTBK1 and TTBK2 into a bait vector. The genetic content of each resultant yeast colony was isolated, transformed back into yeast, and tested for selectivity. Positive hits were curated and categorized on the basis of their predicted intracellular function.

From the screen, we isolated six unique protein interactions for TTBK1 and 25 unique protein interactions for TTBK2 (Table 1). We were unable to identify any protein- binding partners for TTBK1 kinase domain. Both TTBK1 domain A and TTBK2 domains A and B pulled down members of the GABARAP family (GABARAP, GABARAPL1, and GABARAP L2). TTBK2 domain B pulled down two known protein interactions, EB1 and EB3, demonstrating the efficacy of our yeast two-hybrid protein interaction assay. Hits were then classified by cellular function (Fig. 2). The largest number of hits were involved in cytoskeleton regulation (5, 14%), cell growth and adhesion (5, 14%), or autophagosome maturation (4, 11%). The remaining proteins had functions in intracellular trafficking, transcriptional regulation,

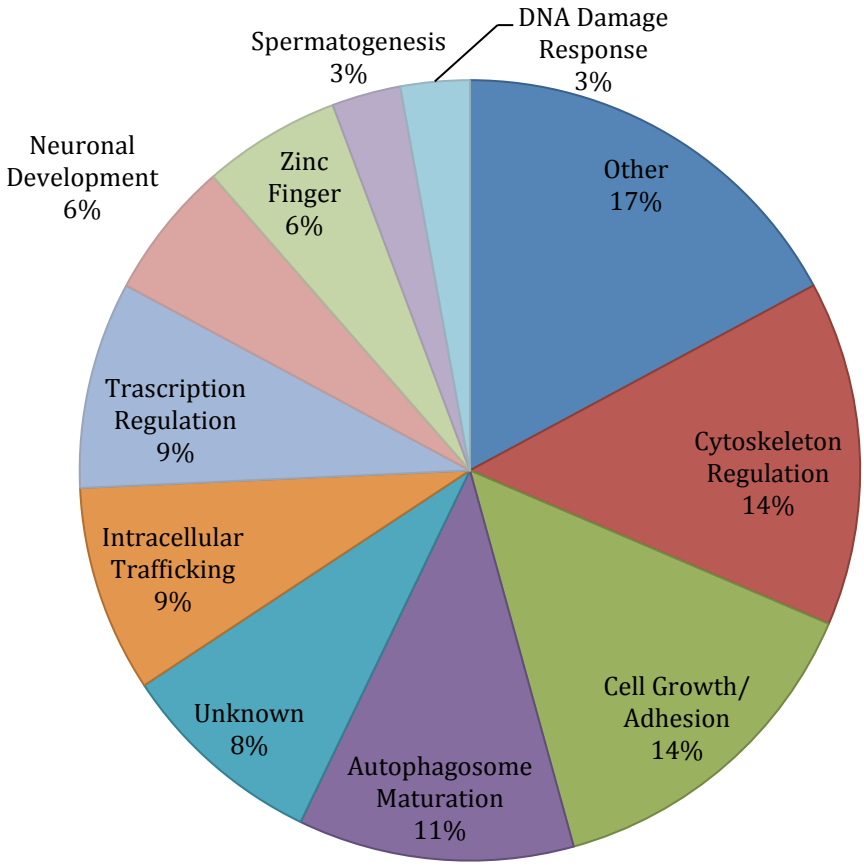
neuronal development, zinc finger activity, spermatogenesis, DNA damage response, and other or unknown functions.

Table 1: Proteins that Interact with TTBK1/2

TTBK1 Bait Region	Interacting Gene	Protein Name	Cellular Function
Kinase Domain	None Identified		
Domain A	GABARAP	Gamma-aminobutyric Acid Receptor –Associated Protein	GABA receptor transport, autophagosome maturation
	GABARAPL2	Gamma-aminobutyric Acid Receptor –Associated Protein	Intra-golgi trafficking, autophagosome maturation
	ITSN2	Intersectin 2	Endocytic membrane trafficking, microtubule assembly, dendrite formation
	PTPRK	Protein-Tyrosine Phosphatase Kappa	Cell contact and adhesion
Domain B	SMS	Spermine Synthase	Spermine to spermidine catalysis
	XRCC6	X-Ray Repair Cross Complementing Protein 6	Helicase, DNA damage repair, innate immune response
TTBK2 Bait Region	**Interacting Gene**	**Protein Name**	**Cellular Function**
Kinase Domain	ANKRD24	Ankyrin Repeat Domain-containing Protein 24	Unknown
	PRPF3	U4/U6 Small Nuclear Ribonucleoprotein PRP3	Pre-mRNA splicing
Domain A	ALKBH5	RNA Demethylase ALKBH5	Demethylation during spermatogenesis
	ASAP1	Arf-GAP with SH3 Domin, ANK Repeat and PH Domain-containing Protein 1	Cilium biogenesis and degradation
	CCDC149	Coiled-coil Domain-containing Protein 149	Possible zinc finger function
	CDH10	Cadherin-10	Ca-dependent cell adhesion
	FLOT1	Flotillin-1	Caveolar membrane scaffolding
	GABARAP	Gamma-aminobutyric Acid Receptor –Associated Protein	GABA receptor transport, autophagosome maturation
	GABARAPL1	Gamma-aminobutyric Acid Receptor –Associated Protein-Like 1	Intracellular receptor trafficking, autophagosome maturation
	GABARAPL2	Gamma-aminobutyric Acid Receptor –Associated Protein-Like 2	Intra-golgi trafficking, autophagosome maturation

	SDR39U1	Emiperase Family Protein SDR39U1	Oxidoreductase
	SLN	Sarcolipin	Modulator or sarcoplasmic reticulum activity and Calcium re-uptake
	SOX2	Transcription Factor SOX2	Transcription factor for embryonic development
	TMEM53	Transmembrane Protein 53	Unknown
	ZDHHC15	Palmitoyltransferase ZDHHC15	Palmitoyltransferase
Domain B	FBLN1	Fibulin-1	Cell adhesion and migration, basement membrane development
	GABARAPL2	Gamma-aminobutyric Acid Receptor –Associated Protein-Like 2	Intra-golgi trafficking, autophagosome maturation
	GLOD4	Glyoxalase Domain Containing Protein 4	Enzymatic activity, potential cadherin binding
	GSPT1	Eukaryotic Peptide Chain Release Factor GTP-Binding Subunit	Translation termination, regulation of cell growth
	KAT5	Histone Acetyltransferase Kat5	Transcriptional Regulation
	MAPRE1	Mictrotubule-associated Protein RP/EB Family Member 1	Regulation of microtubule dynamics, regulator of autophagosome transport
	MAPRE3	Mictrotubule-associated Protein RP/EB Family Member 3	Regulation of microtubule dynamics, microtubule tethering to golgi
	MORC3	MORC Family CW-type Zinc Finger Protein 3	Zinc finger, RNA binding
	PAK3	Serine/Threonine Protein Kinase PAK3	Cytoskeleton regulation signaling, cell migration signaling, dendrite spine morphogenesis and synapse formation
	PHLDB1	Pleckstrin Homology Domain Family B Member 1	Basement membrane Assembly, regulation of microtubule cytoskeleton organization
	UNC45A	Protein Unc-45 Homolog A	HSP-90 co-chaperone

Figure 2: Roles of TTBK1/2 Interacting Proteins



Discussion

The role TTBK2 plays in the initiation of ciliogenesis has been heavily characterized, and many protein-binding partners that are involved in this process have been identified, including CEP164, EB1, and EB3. Yet, there is no evidence for proteins that interact with TTBK1. Furthermore, to date there is very little evidence for what proteins are involved in TTBK1/2 protein regulation. We hypothesize that TTBK1/2 are regulated through protein interactions. We set out to identify proteins that bind to TTBK1 and TTBK2 by performing a yeast two-hybrid screen using regions of TTBK1 and TTBK2 as bait. From this screen we were able to identify 29 unique protein interactions.

Upon classification, most interacting proteins functionally divided into five main categories: cytoskeleton regulation, cell growth and adhesion, autophagosome maturation, intracellular trafficking, and transcription regulation. Interestingly we were able to identify two previously demonstrated protein interactions, EB1 and EB3, which give support for the efficacy of our strategy. We observed several additional proteins that bound to TTBK2 that also play roles in microtubule structure and regulation, including ASAP1, PAK3, and PHLDB1, which give further support for the involvement of TTBK2 in the cytoskeleton regulation processes during cilia development.

Despite our efforts, we only identified a small number of protein binding partners to TTBK1 that have a variety of cellular functions. These binding partners could provide some insight into the role that TTBK1 plays. Of utmost interest is the interaction with ITSN2, a protein that is involved in microtubule assembly and dendrite formation. This points to a potential role that TTBK1 may play in neurogenesis. I hypothesize that TTBK1 kinase activity is essential for the proper guidance of dendritic connections, facilitated by its binding to ITSN2.

We also identified some similarity between the binding partners of TTBK1 and TTBK2 by isolating GABARAP and GABARAPL2. GABARAP bound to both TTBK1 domain A and TTBK2 domain B. GABARAPL2 bound to both TTBK1 domain A as well as both TTBK1 domains A and B. This suggests that GABARAPL2 binds between amino acids 644 and 932 of TTBK2. The GABARAP proteins are members of the LC3/GABARAP family and are responsible for autophagosome development. The identification of the GABARAP family as binding partners for both TTBK1 and TTBK2 provide insight that both TTBK1 and TTBK2 may function in the regulation of autophagosome formation. Alternatively, the GABARAP family could be involved in regulating TTBK1/2 activity. This could also indicate a common mechanism of neuronal function and regulation between the two kinases and give us insight into potential common mechanisms of misregulation in disease.

Further analysis of the model of protein interaction is critical for a more complete understanding of how TTBK1/2 are regulated. To follow-up on these data interactions will be verified using an *in vitro* radioactive pull-down assay. To further elucidate whether the interaction is involved in the regulation of the TTBKs, kinase activity will be assessed in the presence of the protein-binding partner with a luminescent kinase activity assay (KinaseGlo). Finally, interactions will be analyzed *in vivo* using *C. elegans.*

Conclusions

The identification of novel protein interactions will help us to better understand the roles that TTBK1/2 play and potentially provide insight into the function these kinases have in neuronal populations. Furthermore, the identification of protein binding partners may shed light on the mechanisms for TTBK1/2 regulation. Because both TTBK1 and TTBK2 have been

identified as key players in a number of diseases, knowledge of their regulatory pathways has the potential to lead to the development of kinase-targeted therapeutics.

6 Discussion and Future Directions

The neurodegenerative disease research field has grown rapidly in recent years as we have identified numerous genes and pathways that are involved in disease initiation and progression. However, to date very few treatments are available that both assuage the symptoms and eliminate the threat of these disorders. Despite the efforts of clinical trials, we have made little progress towards a cure. For these reasons, it is essential for scientists to parse out the basic mechanisms and contributing factors that ignite the cascade of cell death in neurodegenerative disease. Because of the overwhelming abundance of neurodegenerative diseases that present with phosphorylated toxic protein species, I have turned my focus towards investigating the role that kinases play in disease progression.

The work demonstrated in this dissertation has helped to advance the field of neurodegenerative research because it highlights the role that the Tau Tubulin Kinases 1 and 2 (TTBK1/2) play in driving the pathogenesis of frontotemporal lobar degeneration and other tau-related disorders. I illustrate that TTBK1/2 are able to phosphorylate both tau and TDP-43 *in vivo* and are overexpressed in FTLD tissues. I also show that both TTBK1 and TTBK2 preferentially target tau over TDP-43. Additionally, I demonstrate that TTBK1 is overexpressed in a variety of FTLD-related disorders including PSP, CBD, and Pick's disease, but not ALS. Lastly, I establish that processing patterns are variable across different diseases and that small processed species of TTBK1 are upregulated in several tau-related disorders.

The identification of TTBK1/2 as kinases that robustly phosphorylate both tau and TDP-43 provides a novel connection between a number of neurodegenerative diseases including ALS, FTLD-TDP, FTLD-tau, and other tauopathies. Additionally, the connection of TTBK1/2 to both tau and TDP-43 phosphorylation may explain the etiology of both FTLD subtypes. Further

exploration of how TTBK1/2 contribute to disease progression may help to elucidate an underlying mechanism of neurodegeneration in the ALS-FTLD spectrum and across tauopathies. Therefore, a better understanding of how TTBK1/2 are regulated may provide insight into a therapeutic strategy that has the potential to target the entire spectrum of ALS and FTLD and other tauopathies.

As a result of these findings, several questions remain unanswered and even more have surfaced:

Is TTBK1 and TTBK2 activation upstream or downstream of Tau and TDP-43 pathology?

Previous work in our lab showed that TTBK1 and TTBK2, originally characterized as tau kinases, are able to phosphorylate TDP-43 *in vitro*. My work further demonstrates the kinase activity of TTBK1 and TTBK2 *in vivo* in a *C. elegans* transgenic model. However, we still do not understand whether kinase activity is contributing to pathogenesis prior to tau or TDP-43 phosphorylation or as a result of tau/TDP-43 phosphorylation. If acting upstream of tau or TDP-43, then one could postulate that TTBK1/2 overexpression is an early event in disease initiation. On the other hand, if TTBK1/2 act downstream of tau/TDP-43 phosphorylation, then I would hypothesize that TTBK1/2 may be regulated by the presence of phospho-tau and phospho-TDP-43 species, resulting in its upregulation. To answer these questions, we need to discern whether TTBK1/2 overexpression is present in early mammalian models of tau and TDP-43 pathology.

Which proteases are responsible for processing, and how is TTBK1 processing signaled?

When TTBK1 was originally identified and studied in Alzheimer's disease cases, processed protein fragments were apparent but had not been further characterized. When I ran a panel of FTLD-related tau and TDP-43 disorders, I noticed that TTBK1 processing was not consistent across disease sub-types. This observation produced the hypothesis that TTBK1 processing is highly regulated but gets disrupted during disease pathogenesis, or is an artifact of disrupted lysosomal degradation. To parse out the patterns of processing, I looked at the abundance and size of processed TTBK1 species in FTLD-tau, FTLD-TDP43, PSP, CBD, Pick's disease, and ALS. I was able to demonstrate that TTBK1 processing is consistent across many of the tauopathies. Most commonly, I observed the upregulation of a series of fragments ranging in size from 40-50kDa that was detected with both TTBK1 domain A and domain B antibodies, with a marked decrease in full-length TTBK1. This suggests that TTBK1 processing is misregulated in disease, favoring the production of short TTBK1 fragments that consist of regions of the regulatory domain.

I hypothesize that the processed fragments likely paly a role in normal protein regulation, but when neurons experience cellular stress seen in neurodegeneration, the processed fragments can no longer function or are over-activated, and are not properly turned over, thereby increasing their presence in the cell. Over time, the buildup of processed TTBK1 fragments may lead to the disruption of other cellular processes and contribute to other pathogenic processes. The kinase domain of TTBK1 may be more active when it is freed from the regulatory domain. It is possible that an increase in regulatory domain fragments in disease indicates an increase in kinase activity as a result.

To identify which proteases may be involved in this processing, it would be apt to identify which amino acids of TTBK1 are present in the disease-associated fragments. From that information, we could glean the approximate cut sites. Proteases that are able to slice at those sites will them be systematically screened to see whether they are responsible for TTBK1 processing. Identification of proteases that process TTBK1 could lead to the development of therapeutics that target that protease activity, in order to slow down the accumulation of processed TTBK1 species.

What roles do TTBK1/2 play in neurons?

A recent study demonstrated that TTBK1 is able to phosphorylate SV2A, a synaptic vesicle protein that recruits synaptotagmin-1 during endocytosis [105]. This suggests that TTBK1 may play a role in synaptic vesicle release. However, there is no further supporting evidence for this theory. Most work on TTBK2 has focused on its function in ciliogenesis, but has not addressed its specific role in neurons. Data produced by this dissertation suggests that both TTBK1 and TTBK2 may be involved in neuronal development. Through the yeast two-hybrid screen I identified two protein-binding partners of both TTBK1 and TTBK2 that function in dendrite development. ITSN2, which binds to domain A of TTBK1, was primarily characterized for its involvement in endocytosis [142] but also functions in microtubule assembly and dendrite development. This gives support to the idea that TTBK1 may be involved in synaptic vesicle endocytosis. PAK3 is a serine/threonine kinase, which binds to TTBK2 domain B and functions in synapse formation and plasticity [143]. This suggests that TTBK1/2 may be involved in both synaptic formation and may play roles in dendrite formation. To further explore this role, it would be ideal to produce a model of TTBK1/2 knockout in mammals.

Unfortunately, attempts to produce a homozygous TTBK2 knockout model have resulted in embryonic lethality, demonstrating that TTBK2 has a critical function during embryogenesis. TTBK1 KO mice are currently under construction and will be characterized in future studies.

How are TTBK1/2 regulated?

Despite growing interest in the native roles of TTBK1/2, no regulatory mechanisms have been elucidated. Some evidence points towards an autophosphorylation event [100, 112], which could provide a positive or negative feedback loop for kinase activity. TTBK2 is able to interact with a number of cilia assembly proteins, but no work has addressed how its kinase activity is triggered. To gain a better understanding of what may be involved in TTBK1/2 regulation, I performed a yeast two-hybrid interaction screen, looking for protein interactions from fetal brain tissue. From the screen, I was able to identify 29 unique protein interactions, several of which are of protein modulators. Further validation of these protein interactions is under way. It is possible that the interaction of TTBK1/2 with any of these proteins can result in regulation of TTBK1/2 kinase activity. In order to reach an answer on whether or not these protein interactions regulate TTBK1/2 function, a kinase activity assay will need to be performed.

It is also possible that TTBK1 is self-regulated by either the processed fragments that it produces, or signaling by its polyglutamate region. The processing of TTBK1 is still poorly understood and requires more experimentation before any conclusions can be reached. Based on evidence that TTBK2 C-terminus can bind and inhibit its kinase domain [112], I hypothesize that TTBK1 may be regulated in a similar fashion, by the biding of processed regulatory domain fragments to the kinase domain. I also hypothesize that the polyglutamate region of TTKB1 acts as a signaling domain and recruits proteases to TTBK1 to promote processing.

7 Conclusion

In conclusion, this work provides evidence for the importance of TTBK1/2 in initiating neurodegenerative pathways. I provide support for the hypothesis that TTBK1 and TTBK2 kinase activity influences both tau and TDP-43 phosphorylation *in vivo.* Furthermore, I demonstrate that TTBK1 and TTBK2 are upregulated in numerous tau-related disease cases, which offers novel evidence for the role that these kinases play across tauopathies. This dissertation provides a basis for the future study of TTBK1/2 processing and regulation and gives evidence for why we should consider TTBK1 targeted drugs to treat TDP-43 and tau mediated neurodegenerative proteinopathy disorders.

8 References

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