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P53 Aggregates and Interacts with Tau in Alzheimer's Disease

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List of Abbreviations

α-synuclein: Alpha synuclein

AD: Alzheimer’s disease

AFL-p53O: Alexa-fluorescence labeled p53 oligomers

AFL-tauO: Alexa-fluorescence labeled tau oligomers

AFM: Atomic Force Microscopy

APP: Amyloid Precursor Protein

Aβ: Amyloid Beta

FAD: Familial Alzheimer’s disease

FPLC: Fast protein liquid chromatography

Htau: Human tau

LOF : Loss of function

MCI : Mild cognitive impairment

NFTs: Neurofibrillary tangles

p53O: p53 oligomers

PCC: Pearson’s Correlation Coefficient

PHF: Paired helical filaments

PTMs: Post-translational modifications

ROI: Region of Interest

SEC: Size Exclusion Chromatography

tauO: tau oligomers

WT: Wild-type

Acknowledgements

Thank you to those that made this International program possible and allowed for the unique opportunity to learn and be a part of something bigger than I ever imagined. Research and the pursuit of knowledge should be without borders. Thoughts and ideas from the minds of a diverse background allows for unique approaches to complex problems.

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For you, my father, “I will press on regardless.”

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by the gradual and progressive death of neurons. This ultimately leads to nervous system dysfunction in the form of memory impairment, which causes severe disability and reduced quality of life, resulting in reduced life expectancy. AD is the most prevalent neurodegenerative disease and the 7th leading cause of death worldwide. Despite the large body of data being generated regarding the pathology of AD, both quality of life and curative treatments remain elusive.

The gradual cell death seen in AD has been primarily attributed to protein aggregates from the proteins tau and amyloid beta (Aβ), the aggregation of which form the hallmark neurofibrillary tangles and plaques seen in AD brains. However, recent studies suggest that other essential proteins form aggregates that interact and contribute to toxicity. The transcription factor p53 shares many characteristics with other aggregation-prone proteins involved in neurodegenerative diseases, suggesting the involvement of p53 in AD pathogenesis. Neuronal death, cell cycle re-entry, and dysregulation of DNA damage response are all associated with AD pathogenesis and are tightly controlled by p53. Previous research has shown that p53 is an intrinsically disordered protein that is prone to aggregation and elevated levels of p53 are found in the brain tissue of AD patients. Therefore, I hypothesize that p53 plays a role in the pathogenesis of AD.

The aggregation and oligomerization of p53 and potential mechanisms of toxicity were characterized. p53 was found to not only form oligomers, but also interact with tau *in vivo* and *in vitro*. The successful completion of this project has provided new insights into mechanisms of pathogenesis in AD and may provide novel cellular targets for therapeutics.

*Note: The background covering aggregation and seeding is in large part, taken from the published review Farmer K, Gerson JE, & Kayed, R. Oligomer Formation and Cross-Seeding: The New Frontier. Isr J Chem. 2017.

Introduction

Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder that causes the gradual and progressive death of neurons. This ultimately leads to nervous system dysfunction, primarily in the form of memory impairment, which causes severe disability and reduced quality of life, resulting in reduced life expectancy. AD is the most prevalent neurodegenerative disease- currently affecting 50 million worldwide with that number expected to grow as high as 152 million in the next 30 years, as the populace ages and life expectancy increases [1]. It is the 7th leading cause of death worldwide [1] and remains the only disease in the top ten causes of death with no early diagnostics, no preventatives, nor effective treatments. The current global cost of dementia is estimated at US $1 trillion per year and is expected to reach US $2 trillion in 10 years [1].

A majority of AD cases (>99%) are termed "sporadic" or late-onset due to the complex combination of causes that do not include a family link. The primary risk factor for sporadic AD is age with a majority of cases at 65 years or older. However, genetic familial forms of AD (FAD) do exist, accounting for less than 1% of all cases. These genetic forms, also falling under the term "early-onset AD," stem from mutations in genes related to amyloid beta precursor protein (APP) and its proteolytic cleavage by the Presenilin genes, PSEN1 and PSEN2. Together, presenilins cleave APP to ultimately form the peptide amyloid-β that makes up the plaques found in AD brains [2,3]. However, most research aims to understand the sporadic or late onset forms of AD as the mechanism of disease remains unknown.

The gradual cell death in AD has been primarily attributed to protein aggregates from the proteins amyloid beta (Aβ) and tau, the aggregation of which, forms the hallmark extracellular plaques and intracellular neurofibrillary tangles, respectively, seen in AD brains. In fact, the only way to confirm a diagnosis of AD today is post-mortem examination where histology from brain

tissue will show the presence of the two major plaque and tangle hallmarks in addition to gross morphological changes including severe cortical shrinkage, hippocampal atrophy, and enlarged ventricles. Alzheimer's disease classification based on pathology, including spread and severity, have been characterized into six stages from the work of Braak & Braak [4-6] and are widely used although other characterizations have been produced. In Braak stage 1, the earliest stage, there is evidence of pathology in the lower brainstem, transentorhinal cortex, and olfactory system, by mid-stage the hippocampus and temporal cortex are effected, and by the last stage or Braak stage 6, the disease is at its most severe and can be found throughout the neocortex-including the motor and sensory regions [3]. Although the exact mechanism for how the disease spreads is unknown, there is evidence to believe that pathology spreads by cell-to-cell transmission [7-11]. The neuropathology of AD is complex with many contributing factors and a compounding factor of age and co-morbidities to consider. However, recent studies suggest that other essential proteins found to aggregate play a vital role in disease pathogenesis. Protein-protein interactions with different aggregates can increase toxicity and spread the disease to vulnerable brain regions [12-15]. With this in mind, we investigated a new protein aggregate in the pathogenesis of AD, with special focus on protein-protein interactions.

Amyloid-Beta

Amyloid-β (Aβ) is a small protein fragment derived from the proteolytic cleavage of amyloid precursor protein (APP). APP is a transmembrane glycoprotein with undetermined function. APP-null mice show decreased locomotor activity and impaired learning and memory [16,17] It is located on chromosome 21 and is expressed in many tissues of the body. Aβ function is also not well understood but has been shown to act as a transcription factor [18] and be involved in synaptic function, learning, and memory. Structurally, Aβ is considered an intrinsically disordered protein and is prone to aggregation [19]. Aggregated Aβ makes up the characteristic fibrillary plaques

that occur in AD brains [2,20]. Aβ has also been found to interact with tau and tau aggregates in AD and this interaction contributes to disease pathology.

Tau

The MAPT gene, located on chromosome 17 in humans, encodes the microtubule associated protein tau. Tau is expressed in neurons, particularly axons, of the central nervous system [21]. Tau is largely soluble and is known for stabilizing the microtubules, making it important for neuron flexibility and intracellular support and trafficking. Tau is found in many cellular compartments including the cytoskeleton, plasma membrane, cytosol, and nucleus [22-24].

Tau can be alternatively spliced to produce six isoforms, which are distinguished by their containing three (3R) or four (4R) repeat binding domains [25]. Multiple post-translational modifications help regulate tau, including acetylation and phosphorylation [26]. However, hyperphosphorylated tau has been found to occur in some diseases, causing microtubule disassembly and therefore a disruption in cell structure, transport, and signaling [27,28]. Tau is also an intrinsically disordered protein and has been shown to form aggregates [19,29]. These aggregates are under heavy investigation in AD as the neurofibrillary tangles (NFTs) found in AD are made up of hyperphosphorylated tau [30-32]. NFT amount and distribution highly correlate with duration and severity of dementia [33-36]. Tau is also involved in a larger set of diseases, called tauopathies and include diseases such as Alzheimer's disease, Pick's disease, frontotemporal dementia, cortico-basal degeneration and progressive supranuclear palsy [37].

Aggregates Contribution to AD Pathology

During protein translation, it is critical for nascent proteins to fold into the correct conformation to ensure stability and therefore correct function. However, protein misfolding can occur with some proteins being predisposed to misfolding due to an intrinsic disorder within their structure. In an environment without appropriate chaperones, cofactors, or functioning degradation systems, these misfolded proteins can accumulate, aggregate, and begin to interfere with normal cellular processes. Some misfolded proteins can form insoluble fibrillar aggregate deposits called fibrils, that represent a low-energy end result from a complex process.

To start this process, a single protein begins as a monomer and due to misfolding, will begin to form a low molecular weight, transient intermediary species called an oligomer. Oligomers typically begin as dimers/trimers and gradually form into soluble high molecular weight aggregates with spherical morphology. Oligomers are characterized as small (3-50 nm), with a partially folded β-sheet quaternary structure and exposed hydrophobic patches [38-43]. Although the majority of oligomers exhibit similar structural features including β-sheets, in some instances, α-helical oligomers can form as well. These oligomers, notably, include α-synuclein, amyloid-β (Aβ) and islet amyloid polypeptide (IAPP) that have all been previously found in disease. Once formed, oligomers will then arrange, like beads on a string, into late stage intermediate structures, called protofibrils, which are characterized by a curvilinear fiber morphology (size <10 nm in diameter and up to 400 nm in length). Protofibrils will continue aggregating before ultimately forming stable, straight and highly regular insoluble fibrils of varying high molecular masses [38,44-47]. Fibrils are characteristic of fibrillar amyloid deposits found in amyloidosis or amyloid diseases.

Soluble monomeric proteins are currently known to form oligomers by either primary or secondary self-assembly. Primary self-assembly occurs independently of preexisting aggregates, whereby aggregation is coupled to growth through monomer addition and strongly depends on the level of soluble monomer. Secondary self-assembly, on the other hand, involves preexisting

aggregates, such as fibrils, that fragment and release monomers and oligomers that can then self-assemble of their own accord [48,49]. Secondary self-assembly by filament breakage has emerged as a major pathway for the proliferation of both amyloid fibrils and prion aggregates by *in vitro* and *in vivo* methodologies [48].

Many proteins are known to undergo similar misfolding pathology, more generally known as proteopathies or protein misfolding/conformational disorders. Many neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and others, are considered proteopathies with either one or numerous different proteins involved in each disease. In fact, these fibrils make up the dense-core plaques and neurofibrillary tangles (NFTs) characteristic in AD [4,5].

For many decades, the end stage fibrils were largely believed to be the cause of AD and many other proteopathies due to their presence in postmortem examination of patient disease brains [50]. However, members of the neurodegenerative field now widely accept that the small, soluble species of oligomers are the most toxic species in neurodegenerative diseases [51-56]. This has been in large part due to the following observations: 1) amyloid deposits correlate poorly with progression of disease in AD patients [54,55]; 2) Aβ oligomers are increased in AD brains and CSF in comparison to controls and correlate with loss of synaptic markers and cognitive impairment in humans [57-64]; 3) in human cases with equal amyloid plaque load, those with cognitive deficits correlated with higher levels of oligomers [65]; 4) APP transgenic mouse models show an age-related increase in Aβ oligomers that correlate with synaptic loss and cognitive dysfunction in the absence of plaques [62,66-70]; 5) lack of correlation between NFTs and synaptic dysfunction [71]; 6) numerous tau mouse models show the appearance of degenerating neurons and synaptic loss prior to the appearance of NFTs [72,73]; 7) the reduction of tau overexpression in a mutant tau mouse model reduced neuronal loss despite the continued formation of NFTs [74]; 8) the removal of dense-core plaques in clinical trials failed to reverse the damage or prevent the cognitive decline associated with AD [75,76] ; 9) little to no

cytotoxicity in cell-culture models associated with fibrillar amyloid deposits of a number of proteins, including tau and Aβ associated with AD, huntingtin protein associated with HD, and α-synuclein associated with PD; 10) α-synuclein Lewy body (LB)-associated disorders [77] and an HD cell-culture model [78] demonstrated that neurons with amyloid deposits were healthier than those without them; and 11) mouse models overexpressing Aβ, tau, huntingtin or a-synuclein exhibit pathology prior to, or independently from, the appearance of amyloid deposits.

Recent evidence suggests that fibrillar amyloid deposits may represent the physiologic sequestration of the spread of the disease and may actually play a protective role [79]. Although there is evidence that fibrils are toxic to some degree, many believe this to be the result of secondary self-assembly by means of fibril breakage into monomers that will aggregate into more toxic intermediates [80,81]. Thus, the relatively inert properties of fibrils suggest that the soluble intermediate aggregates, specifically oligomers, represent the more toxic species.

Numerous studies have suggested that toxicity is associated with the aggregation phase of the peptide, which would implicate oligomers, rather than fibrils, as the toxic species [38,82-86]. Aβ and tau oligomers are more strongly correlated with disease severity in AD than Aβ fibrillary deposits and Aβ oligomers have been shown to produce cognitive deficits in the absence of plaques [58,61,67]. Tau oligomers have been identified in AD, progressive supranuclear palsy (PSP), PD, dementia with Lewy bodies (DLB), and HD [87-90]. Tau oligomers have been found in the early stages of AD, prior to the onset of clinical symptoms, and also have been found in AD brain samples at concentrations four-fold higher than healthy control samples [89,91]. In both AD and PSP, tau oligomer levels were elevated and correlated with the onset of clinical symptoms [89,92]. In addition, when tau oligomers were intracranially injected into wild-type mice, they induced cognitive deficits, synaptic loss, and mitochondrial dysfunction that were absent with tau monomer and tau fibril injection [39,84,93]. It has also been demonstrated in a transgenic animal model that intracerebral injection of endogenous or synthetic Aβ seeds induces widespread deposition of Aβ, even to areas

distant from the site of injection, implicating oligomer propagation as a mechanism of toxicity [94]. This is consistent with the finding that oligomers have been found in both the intracellular and extracellular space and are capable of moving in between these spaces [95-97].

In the case of AD, the proteins Aβ and tau are the major contributors of disease pathogenesis, but there is evidence that soluble amyloid oligomers are generally toxic and can arise from almost any misfolded protein. Numerous proteins have been found to gain pathogenic/ pathological functions upon aggregation and proteins not directly involved in proteopathies can become toxic upon forced aggregation. Moreover, it has been demonstrated that proteins both related and unrelated to disease are equally likely to form toxic amyloid oligomers that are also more toxic than their respective fibrils [98]. This evidence would allow for the conclusion that soluble amyloid oligomers are generally toxic, follow a common mechanism of toxicity between diseases, and can arise from almost any misfolded protein, regardless of specific amino-acid sequence. This would indicate that proteopathies may be caused by different proteins in various concentrations relative to one other and could explain why targeting just one protein oligomer may not fully halt the spread of disease.

Some common properties of oligomers have been established, though oligomers remain difficult to characterize due to their highly disordered and transient nature, and thus, the variability of aggregate species. Oligomers may vary in their formation (primary versus secondary self-assembly; relationship to fibril formation), size (from dimers to high-order multimers), structure (from random coil to a similar degree of β-sheet content to that observed in the fibrillary species), interrelationships with other proteins and aggregates, toxicity, and how they affect the function and viability of neurons [99,100].

Liu et al. categorized Aβ oligomers into two types (Type 1 and Type 2) based on their temporal, spatial, and structural relationship to amyloid fibrils through the use of conformational antibodies, A11 and OC, that classify oligomers based on underlying structural conformation.[3a]

Type 2 Aβ oligomers are more closely related to amyloid fibrils in that they share a common quaternary structure of in-register parallel β-sheet conformation recognized by the OC antibody, found only after fibrillar plaque formation and within the vicinity of the plaques. This subtype represents the majority of oligomers generated *in vivo* and does not impair cognition. On the other hand, Type 1 Aβ oligomers can be differentiated from amyloid fibrils in distinct ways. Type 1 Aβ oligomers have an out-of-register antiparallel β-sheet structure recognized by the A11 antibody, represent a small proportion of oligomers *in vivo*, are present both before and after dense core plaque formation, are found in various parts of the brain, and are associated with cognitive impairment. Based on their features, Type 1 oligomers appear to be formed *de novo*, while Type 2 oligomers appear to form following fibril breakage. It is therefore hypothesized that Type 1 Aβ oligomers represent the more toxic subspecies and are also likely the cause of disease progression [49]. Similar characterization for α-synuclein classified oligomers into two distinct species (Type A and Type B), which differ in their kinetics of formation, degree of compactness, and susceptibility to degradation processes. Using rat primary neurons, Chen et al. showed that although both types had a slow rate of conversion, Type A oligomers were the first to aggregate, and were essentially benign, while Type B oligomers induced aberrant production of reactive oxygen species (ROS) [99].

Characteristics of Toxic Oligomers

At the start of the aggregation process, monomeric protein sequence and structure have a direct effect on misfolding and aggregation, and thereby affect toxicity. Aβ has two isoforms, Aβ-40 and Aβ-42, which differ by only two amino acids, and yet, are known to vary in their toxicity, with Aβ-42 being the more damaging of the two. Tau protein has six isoforms that may alter the toxicity of aggregates, depending on the number of microtubule binding repeats (either 3R or 4R) present. Structural flexibility and exposure of hydrophobic residues of the peptide have also been shown to affect the toxicity of amyloid species [101,102]. Additionally, posttranslational modifications,

amino-acid side chains [103], and the formation of disulfide bridges have been shown to increase a protein's ability to self-aggregate into oligomers [91,104,105].

As a protein conformationally changes into an oligomer, the contribution of the peptide sequence to the toxicity becomes minimal, while quaternary structure becomes vastly more important. Thus, all proteins are potentially toxic if aggregated, regardless of sequence [106,107]. Pastor et al. demonstrated that oligomer toxicity derives from a specific quaternary structure, but is completely independent of the peptide sequence, polypeptide length, and peptide chirality of the aggregated polypeptide [103]. However, the size of the aggregate becomes highly important for toxicity. It appears likely that an inverse relationship exists between oligomeric assembly size and toxicity, with larger aggregates exerting less toxicity than smaller aggregates [83]. Thus, proteins that are able to aggregate rapidly into fibrils are less toxic than those that remain kinetically trapped at the toxic intermediary oligomer stage for extended periods of time before eventually converting to relatively inert amyloid fibrils. Chen et al. used various complementary biophysical techniques to characterize a set of α-synuclein oligomer subgroups that were found to possess a cylindrical architecture similar to amyloid fibrils. These subgroups were more likely to become kinetically trapped during protein self-assembly. This group hypothesized that the multiplicity of the pathways involved in protein misfolding and the rate of structural conversion are likely to dictate the kinetic stability and pathological potential of different oligomer species [99]. They described two subgroups: one subgroup similar to fibrils, with parallel β-sheet structure and a higher susceptibility to elongation, that most likely was more involved in the transmission and spread of disease, while the other oligomer subgroup had an antiparallel β-sheet structure, likely accumulated within cells, and had a highly hydrophobic nature, which, combined with a resistance to degradation, likely made them the more toxic species [99].

These studies support the hypothesis for the formation of different oligomeric strains or structures that differ in their secondary structure, rates of elongation, toxicity, and disease

pathophysiology. As many of these proteins are natively unstructured, they can take on an indefinite number of folding states, which belies the idea that there are many different strains that can vary in toxicity and ability to spread. Recent studies have demonstrated the contribution of additional aggregated proteins not typically associated with AD to toxicity, including α-synuclein [108]. Our lab has demonstrated that in addition to individual oligomeric assemblies, different proteins can co-aggregate to form hybrid oligomers of tau and α-synuclein, as well as TAR DNA binding protein 43 (TDP-43), with Aβ, α-synuclein, and cellular prion protein (PrPc) in AD patients [38,88], which may contribute to aggregate and disease diversity. Conversely, it has been reported that oligomers of different proteins have a common sequence-independent conformation, which may suggest that a similar mechanism of toxicity would exist for all proteopathies [12,98,101,109,110].

Several mechanisms have been proposed for how oligomers exert their toxicity, including: 1) inflammatory effects on the cell membrane [111]; 2) induction of oxidative stress [112]; 3) sequestering of transition metals [113]; 4) specific and nonspecific interactions with cellular and lipid membranes [109,114,115]; 5) damage to DNA [102,116], 6) endoplasmic reticulum stress [117]; 7) proteasome impairment; 8) mitochondrial dysfunction [118,119]; and 9) autophagy disturbance [120]. Some studies using immunohistochemistry, biochemistry, and electrophysiology have shown that Aβ oligomers are able to exploit certain receptors to exercise their toxic effects as well. For example, studies have shown that extracellular Aβ oligomers can functionally disrupt the N-methyl-D-aspartate (NMDA) receptor, which ultimately causes synaptic dysfunction and neurodegeneration [95,121]. In addition, Aβ oligomers can bind PrPc, which starts a cascade of events that ultimately leads to the disruption of synaptic function in hippocampal neurons [122-124]. Despite these numerous contributions, the exact mechanism of oligomer toxicity has yet to be resolved.

In addition to the underlying mechanism of toxicity behind aggregates, the precise factors underlying their initial formation are also unknown. However, reduced pH, increased temperature, agitation, and increased concentration of aggregates have all been identified as factors that can

accelerate amyloid aggregation *in vitro* [125,126]. Therefore, it may be possible that changes in the environment *in vivo* (due to unknown mechanisms) may be promoting amyloid oligomer formation and extending the amount of time they remain in the most toxic state. Thus, changes due to the toxicity of oligomeric aggregates may perpetuate a toxic cycle in which aggregates induce environmental changes that promote the formation of further aggregates. One major measurement of toxicity of amyloid oligomers is the extent to which they disrupt lipid membranes. Numerous studies have shown that toxic amyloid oligomers target the lipids of the cell membrane from both the extracellular and intracellular space; lipid disruption seems to be a general feature of all amyloid oligomers [81,99]. By interfering with the cell membrane, toxic oligomers have been found to form channels that increase intracellular calcium levels, disrupt homeostasis of other ions, perturb membrane fluidity, produce toxic levels of hydrogen peroxide, thin the membrane, and induce cell apoptosis [102,127]. Indeed, by disrupting the cell membrane, these toxic oligomers may be promoting an environment suited to an aggregation state, as well as ensuring their propagation to other cells. However, different cell types have unique membrane compositions and oligomers may only be able to affect certain membrane subtypes, which may partially explain the vulnerability of certain cell types in disease. For example, α-synuclein oligomers have been observed to be more efficient at permeabilizing lipid vesicles when the vesicles are primarily composed of negatively charged phospholipids [99].

Seeding of Oligomers

Amyloid aggregates are proposed to form either via the oligomer-nucleated conformational induction model or template-assisted growth, in which monomer is directly incorporated into amyloid fibrils. The oligomer-nucleated conformational induction model is divided into two phases: the nucleation/lag phase and the polymerization/elongation phase. The nucleation or lag phase requires overcoming a high energy barrier and involves the production of various intermediates,

including oligomeric, misfolded protein "seeds." During the polymerization/elongation phase, the oligomeric seeds cause a rapid and exponential recruitment of the native monomeric protein. Ultimately, the recruited monomers and seeds, together, produce a spectrum of misfolded structures from small soluble oligomers to large insoluble fibrils. *In vitro* studies have shown that the addition of preformed seeds can accelerate or bypass the lag phase, leading to faster aggregation [128,129]. The mechanism behind this is hypothesized to involve the oligomeric seeds providing the nucleation template for other normally folded monomeric proteins to aggregate. These seeds can be homologous/homotypic, meaning the same monomeric and amyloidogenic protein, or can be heterologous/heterotypic (cross-seed), meaning the seed and affected monomer are not the same protein[12]. Seeding is likely the basis by which misfolded proteins propagate pathology in proteopathies.

Homologous seeding has been widely cited for many proteins involved in proteopathies, namely many neurodegenerative diseases, including tau, Aβ, α-synuclein, and huntingtin, [12] and is more efficient than heterotypic seeding [128,130]. However, cross-seeding may be critical for disease pathophysiology and may explain the coexistence of different protein aggregates in mixed pathology diseases and the worsening of symptoms when more than one aggregated protein is present in disease [12]. Additionally, the ability of amyloids to efficiently cross-seed may depend upon the aggregation state of the protein. Multiple *in vitro* studies have demonstrated cross-seeding among several amyloidogenic proteins. Due to its critical involvement in AD, Aβ remains one of the most widely cited and studied proteins. Numerous studies have shown an interaction between Aβ and other amyloidogenic proteins, including tau, a-synuclein, and prion proteins [14,105,128,129,131-133]. All of these studies have collectively revealed an important characteristic of cross-seeding: the misfolded proteins involved may influence aggregation bidirectionally. For example, pre-aggregated Aβ can induce the aggregation of prion protein and α-synuclein that can then, in turn, accelerate the misfolding of Aβ, forming a self-perpetuating toxic cycle [134,135].

However, some cross-seeding interactions have only been demonstrated unidirectionally, such as in the case of Aβ and IAPP. *In vitro* studies have shown that Aβ can effectively seed IAPP, but IAPP aggregates have negligible effects on soluble Aβ aggregates. Interestingly, cross-seeding can also lead to the opposite result, inhibition of protein aggregation. For example, depending on the experimental conditions, apolipoprotein A2 and serum amyloid A can both cross-seed and cross-inhibit amyloid formation [134].

Unlike homologous seeding, not all amyloid proteins are known to cross-seed, as certain proteins may be restricted due to differences in protein sequence or molecular conformational compatibility between the seeds and the soluble monomer [12,130]. Sequence similarity has been shown to be critical to hen egg-white lysozyme in its ability to cross-seed with other proteins. However, the proteins Aβ and IAPP, which share high sequence similarity, show poor cross-seeding, highlighting the importance of conformational compatibility [136]. Moreover, in 2013, Guo et al. performed a study investigating the effect of distinct synthetic α-synuclein strains on tau aggregation. They showed that efficient seeding was dependent on the α-synuclein strain *in vitro*, which emphasizes the importance of minute structural differences in amyloid aggregation [136,137]. Likewise, mutated tau and α-synuclein monomers form diverse fibrillar structures with varying ability to cross-seed between one another [136,138]. Similarity between the conformation of seeds and monomers is essential for seed elongation and will determine the type of aggregate formed. Additionally, the elongation characteristics of the seeds are dependent on the type of seed [136]. Both Aβ and α-synuclein fibrils have characteristic molecular conformations; thus, differences between seeds/fibrils may be critical for determining the cross-seeding efficiency between proteins with or without high sequence similarity, as monomers must directly add onto the end of filaments in the template-assisted growth model [136]. However, these differences may be less important to the cross-seeding efficiency of oligomers that may form on an independent pathway from fibrils, as we have previously shown that both Aβ and α-synuclein oligomers are effective seeds for the aggregation of

tau *in vitro* [129,139,140], suggesting that sequence and conformational similarity are critical for fibrillization steps, but that cross-seeding can effectively occur in oligomerization stages with diverse proteins (**Figure 1**).

Alterations to proteins can also lead to the formation of diverse structures with differing seeding efficiency (**Figure 2**). Hu et al. recently demonstrated that hyperphosphorylation plays a role in the seeding potency of pathological tau and in the spread of tau pathology *in vivo* by injecting

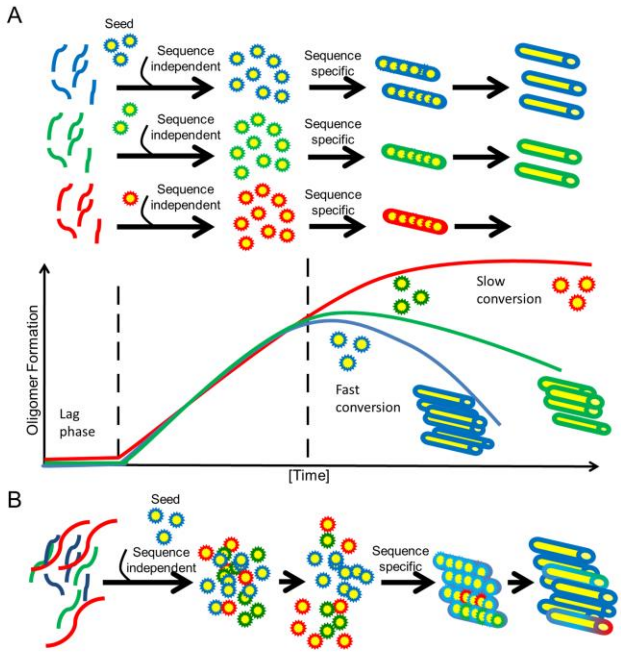

Figure 1: Hypothetical model for differential seeding efficiency, based on the aggregation species.

(A) The formation of oligomeric aggregates from different protein monomers is a sequence-independent process, leading to the formation of intermediate or independent aggregates, leading to the formation of fibrils. However, at the fibrillization stage, complimentary sequences are important for the addition of protein monomer to the fibril chain. Proteins that are slow to convert to fibrils, such as tau, will form fibrils at a later stage than proteins that more rapidly fibrillize, such as Aβ. **(B)** Cross-seeding of proteins using oligomeric seeds efficiently leads to the sequence-independent oligomerization of diverse monomeric proteins. However, efficiency to fibrillize is much slower, with sequence complementary proteins more rapidly and effectively forming fibrils. The formation of hybrid aggregate structures may also occur. (adapted from Farmer K et al. Isr. J. Chem. 2017)

(intracerebral) AD oligomeric hyperphosphorylated tau (P-tau) into the brains of mice transgenic for non-mutated human tau. The brains of these mice showed extensive P-tau tangles and neuropil

threads in the cerebral cortices and hippocampi. When the same experiment was repeated with AD oligomeric dephosphorylated P-tau, it caused a dramatic reduction in tau pathology, tau seeding, and tau lesions from neurofibrillary tangles (NFTs) to switch to a non-AD-like morphology. The authors suggested that hyperphosphorylation caused tau to become more negatively charged, which may initiate the formation and/or maintenance of seeding. They also found that this type of seeding caused tau pathology to spread locally and through axonally connected areas [141]. On the other hand, acetylation has been shown to slow rates of tau fibrillization [142].

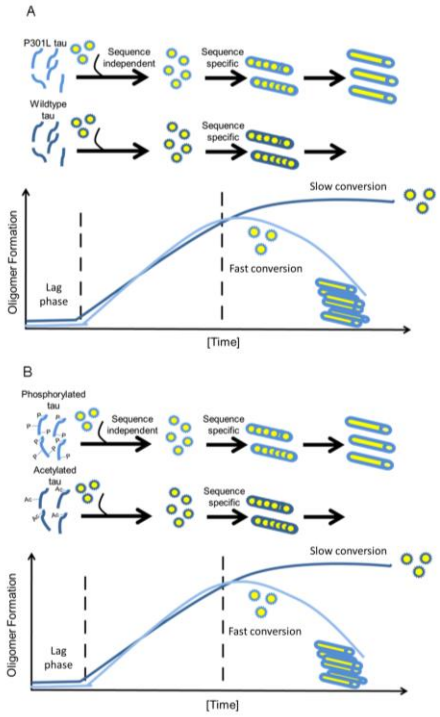

Figure 2: Hypothetical model for the formation of diverse protein species with differential rates of seeding due to protein modifications.

(A) The model described in Figure 1 can be applied to important modifications of tau and other amyloid proteins. Mutations can alter conversation rate and seeding efficiency, thus impacting toxicity. P301L tau more rapidly forms aggregates than wild-type tau. **(B)** Posttranslational modifications of tau differentially regulate aggregation. Tau phosphorylation, though dependent on the epitope, induces more rapid conversion of tau from monomer to fibril, while acetylating tau inhibits fibrillization. (adapted from Farmer K et al. Isr. J. Chem. 2017)

Spreading of Oligomers

One of the greatest roadblocks to the understanding of neurodegenerative disease, and thereby to the development of an effective treatment, is the lack of knowledge of the mechanism by which amyloid pathology spreads from initially affected brain regions to others. The seeding of aggregation-prone proteins by a small number of aggregate seeds of homologous and heterologous proteins may partially explain this stereotypic spread of neurodegeneration; however, the question of why certain cell populations are affected and not others, as well as the mechanism of protein spreading from one cell to another, remain unanswered. Recent results from resting-state functional magnetic resonance brain imaging (fMRI) have led to the

development of the hypothesis of functional connectivity, which contends that specific networks of neurons that are wired together and have synchronous activity levels may also degenerate together [143]. However, the compilation of numerous independent investigations, showing that amyloidogenic proteins, including Aβ, α-synuclein, and tau, are capable of being transported from one cell to another, suggests that the physical spread of seeds to vulnerable brain regions may underlie the pattern of spreading [144-148].

Given the potential importance of the spread of aggregates to the pathophysiology of disease, understanding the characteristics of seeds that more efficiently spread may be critical to preventing disease. Similarly, to toxicity studies, oligomeric aggregates of various amyloid proteins have been shown to be more effective at spreading than fibrillar aggregates [149] .When the relative ability of tau monomers, oligomers, and fibrils to enter cultured neurons and be transported to other cells was compared, oligomers alone were capable of entering cells [150]. Thus, it is likely that amyloid aggregates that maintain their oligomeric conformation, rather than rapidly proceeding to a fibrillar structure, may be more toxic, due to a heightened ability to seed the spread of pathology. Studies showing that increased cell activity elevates the amount of tau aggregates that are transported from cell to cell *in vitro* and enhances the spread of tau pathology *in vivo* suggest that the movement of aggregates through the brain may depend upon synaptic transmission [151-153].

p53 Protein

The p53 protein was discovered 40 years ago by three independent groups in 1979 [154-156] and is today, the most studied gene/protein in scientific research [157]. The popularity of p53 can largely be attributed to the finding that p53 is mutated in 50 percent of all cancers [158,159]. Furthermore, patients with Li-Fraumeni syndrome who contain an autosomal dominant mutation in

the Tp53 gene are predisposed to cancer [160] and p53 KO mice develop cancer at a young age [161], all confirming p53 acts as an important tumor suppressor [162-165]. However, over the years, p53 has been found to act as more than just a tumor-suppressor. Two years after its discovery, one of the founders, Lane et al coined the term, "guardian of the genome," [166] due to its involvement in DNA damage response [167] , cell cycle arrest [167], and apoptosis [166,168]. Others have called p53 a "molecular policeman" [166] or the "emergency brake" for the cell, halting many functions until repair or apoptosis can take place in order to protect the genome from accumulating excess mutations [159]. Since then, p53 function has grown even more encompassing with p53 acting as a central hub protein for various complex signaling networks [169]. P53 acts in many critical cell functions outside of those previously mentioned such as modulating autophagy, innate immunity, inflammation, stem cell maintenance, maturation of microRNAs [170], various cellular metabolic processes, growth cone formation, anti-oxidant balance, and senescence. Hence, it is not surprising that since p53 regulates such a broad and diverse set of biological processes, any kind of impairment in p53 expression or activity results in a wide-range of disorders including cancer, metabolic diseases, and neurodegeneration [171,172].

P53 Protein-Expression, Activation, Regulation

P53 protein derives from the Tp53 gene, located on chromosome 17 in the human genome. P53 is a highly conserved protein and crucial to multicellular organisms. It is expressed in every cell in the human body [173]. Since p53 functions in a wide range of critical functions for cell homeostasis, p53 expression and activity are finely and tightly regulated by many mechanisms to ensure a coordinated response to a broad range of cellular stressors. This tight regulation of p53 is demonstrated through its short half-life of 5-20 min such that p53 is in a steady state of being rapidly degraded or held in a latent form in unstressed cells. This continuous expression and

degradation enables p53 to constantly survey the cell, to try and detect cellular stress or injury [174]. P53 expression is regulated almost at every level including: transcriptional, post-transcriptional, pre-translational, and post-translational [174,175]. Furthermore, p53 activation and regulation involve complex post-translational modifications, conformational changes, binding partners, and other protein-protein interactions that can rapidly change to meet the needs of the cell. Different cell types can also affect this regulation as post-mitotic neurons do not typically divide or have the same immune system response or metabolic needs as other cells.

P53 Activation

P53 activity can be induced by the detection of many stressful factors including: hypoxia, DNA damage (both single and double strand breaks), mitochondrial dysfunction, oxidative stress, viral infection, osmotic shock, ribonucleotide depletion, hypoglycemia, and nutrient deprivation [176,177]. Once activated, it will act as a transcription factor [176,178] and bind to the promoters of a broad range of target genes to either activate or repress activity. P53 can induce or inhibit over 150 genes including CDKN1A (encoding p21; involved in cell-cycle arrest), MDM2 (negative feedback, inhibitor of p53), RRM2B (also referred to as p53R2B; involved in DNA repair), p53-upregulated modulator of apoptosis (PUMA; involved in apoptosis), and BAX (involved in apoptosis) [19,179]. However, increases in p53 levels and activation are complex. For example, mouse models show elevated, but properly regulated, p53 can resist stress and suppress tumors. However, a consistently elevated and active p53 can cause accelerated aging and/or death [180,181]. Thus, p53 expression and cellular response are not a simple linear relationship, but a complex multifactorial process.

P53 Inhibition

There are many inhibitors of p53 which serve to regulate and/or stop p53 activity when no longer needed. The first protein found to bind p53 after its discovery, was murine double minute-2 (MDM2), which is the primary inhibitor and E3 Ubiquitin ligase of p53 [166,182,183]. Evidence to suggest that p53 was still being degraded in the cells of Mdm2 null mice [184] led to the eventual discovery of other p53 inhibitors including Mdm4 (also referred to as Mdmx) [185], COP1[186], Arf-BP1 [187], and Pirh2 [188-190]. This wide range of inhibitors and E3 ubiquitin ligases suggest the importance of limiting p53 activity.

Typically, under normal physiological conditions, p53 protein is kept at low basal levels in the cell by direct binding with Mdm2 at the N-terminal transactivation domain (TAD) of p53 [182]. Mdm2 serves multiple functions in regulating p53. Mdm2 inhibits the transcriptionally activity of p53 by directly binding it and preventing formation of a tetramer. Mdm2 also binds p53 in the nucleus and shuttles p53 from the nucleus to the cytoplasm where it then mediates its E3 ubiquitin-ligase activity to mark p53 for degradation by the proteasome [191-197]. Therefore, Mdm2 regulates p53 through direct binding, degradation, and physically shuttling it from the nucleus to the cytoplasm. The Mdm2 gene is also a transcriptional target of p53 and is activated by DNA damage, which produced the finding of an auto-regulatory negative feedback loop between p53 and Mdm2 [177,198-200].

The interaction between p53 and Mdm2 has been proven to be critical from previous reports that showed Mdm2 KO mice are embryonic lethal due to excess apoptosis, but can be completely rescued by simultaneous deletion of the TP53 gene [201,202]. Moreover, in adult mice with MDM2 KO and a tamoxifen-inducible p53 knock in, mice died shortly after restoration of p53 with defects in multiple tissues. Interestingly, liver, kidney, and brain tissue were not found to have defects, suggesting inhibitory effects of MDM2 are tissue specific and may be due to the different levels of endogenous p53 that can be restored [184,203]. Inhibition of Mdm2 expression or activity also causes

WT p53 to relocate from the cytoplasm to the nucleus, where in come cancers, p53 nuclear exclusion cannot occur due to loss of Mdm2 activity [204,205]. Furthermore, many cancers from diverse origins are attributed to p53 loss of function through overexpression of p53 inhibitors, including Mdm2 [186,206-208].

Therefore, inhibitors of p53 play a key role in p53 localization, concentration, and function. Many post-translational modifications that activate p53 also break the interaction between p53 and its inhibitors. Generally, N-terminal phosphorylation at Ser15 and Ser20 stabilize p53 by breaking the interaction with MDM2, allowing p53 to translocate to the nucleus and bind the DNA.

P53 Post-Translational Modifications-Phosphorylation and Acetylation

p53 has numerous post-translational modifications both during normal homeostasis and in stress-induced responses. An estimated 36 of the 393 amino acids of p53 have been reported to be modified. Those modifications include: phosphorylation, ubiquitination, acetylation, methylation, sumoylation, neddylation, glycosylation, and ribosylation, which can all influence the stability, activity, and localization of p53. For the purposes of this thesis, I will mainly describe p53 phosphorylation and acetylation as both modifications are critical to activation of p53 by inhibiting the Mdm2-p53 interaction.

There are several phosphorylation events directly involved in p53 localization, p53 stability, and nucleocytoplasmic translocation. Two groups of protein kinases are known to target p53 transcriptional activity: 1) protein kinases from the MAPK family (JNK1-3, ERK1-2, p38 MAPK) which are involved in many types of cell stress and 2) protein kinases (ATM, ATR, CHk1, CHk2, DNA-PK) which are associated with genome integrity and DNA damage [209,210]. In response to DNA damage, several kinases will phosphorylate p53 at Ser15, Ser20, and Thr18 to destabilize the interaction with Mdm2 and increase the affinity for CBP/p300. This results in an increase in p53 transcriptional activity in the nucleus [211-213]. Phosphorylation of p53 also plays a role in stabilization

of the tetramer with phosphorylation at Ser20 and Ser392 associated with tetramer formation and phosphorylation at Ser315 causing tetramer destabilization [214]. Furthermore, it has been demonstrated that phosphorylation of p53 at Ser15, Ser33, and Ser36 leads to transcriptional up-regulation of genes involved in apoptosis [215].

P53 modifications by acetylation and ubiquitination compete for sites on the C-terminus of p53 to affect p53 stability. Acetylation of the p53 C-terminus blocks many of the p53 ubiquitin sites used by Mdm2 for p53 degradation. Previous *in vitro* studies have shown that purified acetylated p53 cannot be ubiquitinated by Mdm2 and ubiquitination is significantly reduced following induction of p53 acetylation [216,217]. Therefore, acetylation of p53 inhibits its ubiquitination by MDM2 [216]. CREB-binding protein (CBP)/p300 acetylates all six C-terminal lysines of p53 [218], but more recent work cites the need for eight different lysine residues on p53 to be acetylated in order to block p53-Mdm2 interaction [219]. Cellular stress causes an increase in acetylated p53 levels, thereby promoting p53 stabilization and activation [220] [221,222]. Following p53 activation, some previous studies have shown p53 acetylation can lead to apoptosis or neurite outgrowth and axonal regeneration [223]. Interestingly, p53 acetylation at Lys373 is associated with apoptosis and p53 acetylation at Lys320 leads to neurite outgrowth and axonal regeneration. [224,225]. Therefore, acetylation can lead to pro-survival or pro-death decisions.

P53 Regulation- Redox

In addition, p53 function is also redox-regulated, meaning the intracellular environment can affect the activity of p53. Previous reports have demonstrated that an oxidative environment can disrupt the conformation of WT p53 by affecting several cysteine residues in the core DNA binding domain, thereby affecting the ability of p53 to structurally bind the DNA and perform its role as a transcription factor [226-228]. Furthermore, reducing conditions were found to positively affect the zinc

ion in the p53 core domain, allowing the appropriate conformation for DNA binding. Therefore, the intracellular redox state and zinc bioavailability of the cell can cause p53 to undergo subtle variations in the levels of activity [159,226,229,230]. This would have major implications in cells undergoing severe or persistent stress causing the formation of increased oxygen radicals, where p53 activity, and thus DNA binding, would need to be in working order.

P53 Structure

The wide range of functions and regulation of p53 stem from its structure and conformational changes. The human WT full length p53 protein contains 393 amino acids [231] and forms homo-tetramers [232] described as a dimer of dimers that are crucial to its function as a transcription factor [233]. The full length p53 protein consists of five main domains: amino (N)-terminal transactivation domain (TAD; amino acid residues 1-42 major, residues 55-60 minor), Proline-rich domain (PRD; amino acid residues 61-92), the central core DNA binding domain (DBD; amino acid residues 100-300), tetramerization domain (TD; amino acid residues 323-358), and the multi-functional C-terminal basic domain (BD; amino acid residues 363-393) [159,234,235] (**Figure 3**). The central DBD comprises four conversed pentanucleotide repeats that can directly bind the p53 consensus DNA-binding site [236]. The three-dimensional co-crystal structure of p53 bound to the DNA published in 1994 by Chen et al demonstrates that the four conserved regions within the core DNA binding domain are responsible for contacting the major and minor grooves of the p53-binding site of the DNA while the less conserved regions form a β-sandwich scaffold to support the DNA-binding regions [159,237].

The Tp53 gene produces 15 isoforms in total, but the four major isoforms include: a full length p53 and three truncated forms Δ40p53, Δ133p53, and Δ160p53, which lack the N-terminus required for DNA binding [238-242]. Thus, full length p53 is the only isoform capable of forming transcription-competent tetramers that can bind to the p53-responsive elements in DNA or

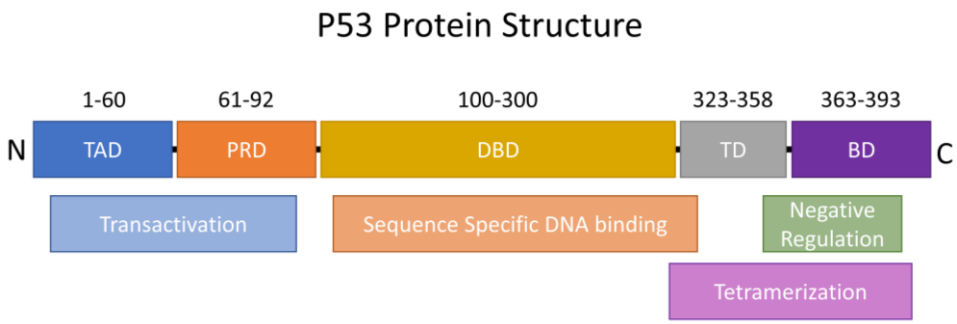

Figure 3: P53 Protein structure and domains
The human full length p53 protein contains 393 amino acids and consists of five main domains: amino (N)-terminal transactivation domain (TAD; amino acid residues 1-42 major, residues 55-60 minor), Proline-rich domain (PRD; amino acid residues 61-92), the central core DNA binding domain (DBD; amino acid residues 100-300), tetramerization domain (TD; amino acid residues 323-358), and the multi-functional C-terminal basic domain (BD; amino acid residues 363-393). Some of the major functions and regulations of these domains are found below.

translocate to the mitochondria in response to cell stress [239,243]. Full length p53 forms tetramers irreversibly through the tetramerization domain, which also regulates the oligomeric state of p53. P53 tetramer formation is regulated by the protein concentration of p53, PTMs, and protein-protein interactions. The inactive p53 monomer is in equilibrium with the active tetramer, therefore the protein concentration of p53 will affect its oligomeric state and activity [169,244]. If p53 protein concentration is high, then equilibrium will shift towards the active tetramer state, resulting in increased p53 activity. Stabilization of the p53 tetramer via phosphorylation of Ser392 at the C-terminal basic domain is associated with an active p53 tetramer [169]. Since tetramer formation is critical for the ability of p53 to bind to the DNA and ultimately act as a transcription factor, any small subtle destabilization of the tetrameric structure could result in p53 dysfunction [245]. This has been emphasized in the cancer field where most p53 mutations occur in regions that affect the p53 scaffold or structural element of the DNA-binding domain, causing loss of p53s ability to bind the DNA and activate expression of target genes [178,246-248]. Even a single point mutation in the TD, such as the p53 mutant R337H associated with adrenocortical carcinoma, affects a specific salt bridge with another residue of an adjacent p53 subunit, causing instability of the TD and ultimately p53 loss of function [249,250]. This has led to cancer related p53 mutations to be classified as

conformational mutants or contact mutants [159,237]. Together, these observations suggest that small subtle changes of p53 (by even single point mutations, PTMS, cell environment, binding partners, protein-protein interactions) can each affect the conformation of the entire p53 protein, thereby altering the structure and function of p53 [251].

P53-Intrinsically Disordered Protein

P53 structure, conformation, and the ability to form tetramers are heavily affected by a host of interactions. In order to be able to bind so many different partners and PTM's, p53 remains as a labile or intrinsically disordered protein to accommodate all the subtle changes in conformation that ultimately affects its function. While most proteins form a tertiary structure after translation, an intrinsically disordered protein lacks stable tertiary and/or secondary structure at baseline homeostasis *in vitro* [19]. Intrinsically disordered proteins are highly flexible, interact with large numbers of binding partners typically with high specificity and low affinity, can adopt a series of different conformations, and usually have crucial biological functions. Furthermore, intrinsically disordered proteins fold in order to carry out function, but that resulting conformation could be either tightly folded or relatively noncompact (ie with both regions of folded and disordered regions) [19]. In p53, the DBD, N-terminal domain, and C-terminal basic domains are all intrinsically disordered [252,253]. Furthermore, about 70% of p53 interactions are mediated by intrinsically disordered regions of the protein, with an even higher percentage (over 86%) at sites of PTMs. Therefore, p53 uses intrinsically disordered regions extensively to mediate and modulate interactions with other proteins [19,254].

While this is great in theory for the cell to use one protein for a host of functions, proteins that are intrinsically disordered are also prone to protein misfolding, which can cause a variety of consequences such as protein aggregation, loss of normal function, and gain in toxic function [19,235].

In fact, three domains of the WT full length human p53 protein including: the N-terminal transactivation domain [255], core DNA-binding domain (DBD) [256], and tetramerization domain [257,258] have all been found to misfold and form fibrillar aggregates *in vitro.* Aggregates formed from the WT p53 N-terminal transactivation domain [255] or the WT p53 DNA binding domain [259] were also both found to be cytotoxic *in vitro*. Furthermore, evidence to suggest that specific amino acid residues 251-258 of the WT p53 DBD is a nucleation site for aggregate formation was found after bioinformatic scanning of the p53 sequence [260-263]. This prompted further studies to demonstrate that this short p53 fragment alone, using synthetic peptides, could form amyloid-like aggregates. Moreover, these p53 DBD fragment aggregates were found to seed WT p53 causing the formation of hetero-tetramers and causing loss of function as measured by transcriptional activity of p53. P53 DBD fragment aggregates were also shown to cross-seed with the p53 paralogs, p63 and p73, thereby inhibiting their functions [260,261].

Moreover, in 2012-13, two groups were able to show that the full length, WT p53 protein could spontaneously aggregate and form oligomers and fibrils through physiological conditions *in vitro* [100,259]. Lasagna-Reeves et al further showed that these WT p53 full length oligomers, and not fibrils or monomer, were cytotoxic to basal cell carcinoma (BCC) and SH-SY5Y cells, suggesting a toxic gain of function for these p53 oligomers. The p53 oligomers also showed loss of function through the inability to bind the DNA [100]. Numerous publications concerning mutant or WT p53 aggregates stemming from domain fragments or full-length p53 were also positive for amyloid properties such as Bis-ANS, Th-T and congo red binding [100,256-259]. Overall, this suggests that three out of the five main domains of p53 can individually aggregate and then WT full length p53 can form oligomers and fibrils under physiological conditions as well. Furthermore, these aggregates have amyloid properties and can lose normal function and gain toxic function in vitro similar to other aggregation-prone protein implicated in other diseases.

P53 Aggregates- Spread of Pathology

More recent publications have also started to analyze whether p53 aggregates have the ability to spread from one cell to another. Forget et al demonstrated that full length p53 and N-terminally truncated p53 could form aggregates that could penetrate two different cell lines through micropinocytosis. Moreover, once internalized, the p53 aggregates could seed endogenous p53 *in vitro* [264]. This suggests, the more alarming possibility that p53 aggregates can spread in a prion-like manner [259,262,264,265]. Prions concern a protein that adopts a misconformation that can then convert other proteins to the same misconformation and then propagate between cells, and therefore spread pathology [266,267]. Many other toxic aggregate proteins have also been shown to spread in a prion-like manner in several neurodegenerative diseases [268-272]. This may suggest a common mechanism of spreading, regardless of the aggregated protein.

P53 and Alzheimer's Disease

The gradual cell death seen in AD has been primarily attributed to protein aggregates from the proteins tau and amyloid beta (Aβ), whose aggregation forms the hallmark plaques and neurofibrillary tangles seen in AD brains. However, recent studies suggest that other essential proteins form aggregates that interact and contribute to toxicity. The transcription factor p53 shares many characteristics with other aggregation-prone proteins involved in neurodegenerative diseases, suggesting the involvement of p53 in AD pathogenesis [215,273].

Given that numerous publications have demonstrated that p53 shares many characteristics with other aggregation-prone proteins involved in AD: 1) it is an intrinsically disordered protein making it prone to aggregation [274]; 2) can form oligomers and fibrils [100,259,262]; 3) oligomers are toxic to cells [100]; 4) oligomers can seed and cross-seed other proteins [100,259-261]; and 5) p53 aggregates can be taken up by cells and spread in a prion-like manner [259,262,264,265]. All these things

together highly implicate p53 in the pathogenesis of AD and yet no one has investigated if p53 aggregates in AD-a disease with hallmark aggregates that also share in the same properties just listed related to aggregation. So what is already known about p53 in AD?

P53 is upregulated almost 2-fold in patients with AD in comparison to age matched controls [18,275-277]. Elevated p53 expression in numerous mouse models causes premature aging [180] and aging is one of the primary risk factors for AD [3]. Furthermore, overexpression of a short isoform of p53 in mice causes premature aging, abnormal tau phosphorylation, synaptic deficits, and cognitive decline, linking increased p53 activity to hallmarks of AD [278,279]. Excessive neuronal DNA damage [280-282], altered DNA repair [283,284], oxidative stress [285,286], lethal cell-cycle re-entry in neurons [72,287,288], and altered neuronal death [289-292] have all been associated with AD pathogenesis [293] and interestingly, p53 regulates all of these functions [166]. Moreover, despite many reports describing high p53 levels and an environment rich in pro-apoptotic stimuli, most neurons in AD do not die by controlled apoptosis, instead going through a prolonged and progressive neurodegenerative process that continues for many years [294]. Interestingly, Uberti et al has published numerous publications suggesting that a conformationally altered form of p53 exists in AD patients [295-297]. Together, this would highly suggest that p53 is altered and likely involved in the pathogenesis of AD.

P53 Protein and Amyloid-β

In addition, p53 has been associated with many proteins implicated in AD. Amyloid-β (Aβ) is a small protein fragment derived from the proteolytic cleavage of amyloid precursor protein (APP). Aggregated Aβ makes up the characteristic fibrillary plaques that occur in AD brains [2,20]. Aβ can also affect p53. Oxidative DNA damage causes Aβ42 to localize to the nucleus and directly activate the p53 promoter and cause transcriptional elevation of p53 [18]. Moreover, accumulation of p53 and Aβ42 have been found together in degenerating neurons in both humans and transgenic

mouse models [18]. Numerous studies have also pointed to the intimate relationship between p53, APP, and its proteolytic products, suggesting they may have an important role in the control of p53 levels and activity and, in consequence, with the plethora of cellular processes regulated by p53 [298-303].

P53 Protein and Tau

Previous studies have investigated p53 and interaction with tau/microtubules. Giannakakou et al showed *in vitro* that p53 associates with cellular microtubules and accumulates in the nucleus after DNA damage only in cells with a functional microtubule network. They further demonstrated that disruption of normal microtubule dynamics caused impaired nuclear accumulation of p53 after DNA damage [304]. This research has huge implications for AD because the microtubule stabilizing protein, tau, is disrupted, causing disassembly of the microtubules and intracellular trafficking issues. Therefore p53 transport to the nucleus, where it serves its function as a transcription factor, may be disrupted in AD. Furthermore, Eftekharzadeh et al demonstrated that AD-related tau impairs nuclear import and export in human AD brain tissue through disruption of nuclear pores [305]. These two publications together would suggest that p53 transport may be impaired in AD, possibly through pathological effects of tau and a disrupted microtubule network.

Other studies have investigated how p53 expression affects tau. Hooper et al demonstrated that p53 overexpression could indirectly increase tau phosphorylation in HEK293 cells [277] while another study demonstrated p53 null mice displayed reduced tau phosphorylation [306]. Hyperphosphorylated tau has long been associated with cell death and neurofibrillary tau tangles are a hallmark of AD [307]. However, NFTs have largely been found to be non-toxic and do not correlate with cognitive deficits or disease progression in AD patients and mouse models. Hooper et al also suggested that due to their separate compartments, p53 and tau would likely never interact

[277]. However, the research mentioned above by Giannakakou et al as well as other work demonstrating a compromised nucleocytoplasmic transport system in neurodegeneration [308] would contradict this. In fact, there is already evidence of mislocalization of other proteins, such as Fus or TDP-43 (another DNA-binding protein) in neurodegeneration [309-312].

Lastly, although tau is predominantly associated with the cytoskeleton and cytosol, it can also be found in the nucleus [22,313,314]. While the role of nuclear tau remains unresolved, it is postulated to be involved in DNA damage. Tau has been demonstrated to protect against DNA damage [315]. Conversely, pathological tau has been shown to induce chromatin relaxation, which subsequently leads to DNA damage and global changes in transcription [313,316]. p53 is activated and transported to the nucleus in the event of DNA damage where its plays a fundamental role in the DNA damage response [177]. Therefore, an interaction between tau and p53 in the nucleus may occur during increased DNA damage, an early sign of AD [282]. It is also possible that pathological tau is causing DNA damage while p53 is trying to induce transcription of repair, causing conflicting signals for DNA damage repair.

Together, this would suggest that p53 stands to play an even bigger role than ever seen before in AD and needs to be further investigated.

Gaps in Knowledge

Does p53 aggregate in AD and does it contribute to pathology?

Chapter 2: Materials and Methods

Animals

This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care, and all experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. Tg2576 (Taconic APPSWE-Model 1349), C57BL/6J (The Jackson Laboratory #000664) and hemizygous human tau (Htau) mice (The Jackson laboratory stock #005491) [317] were bred at UTMB. Mice were housed at the UTMB animal care facility and maintained according to U.S. Department of Agriculture standards (12-hour light/dark cycle with food and water available *ad libitum*).

Human Tissue

Frozen Alzheimer's disease and age-matched control frontal cortex brain tissue were obtained from the Institute for Brain Aging and Dementia (University of California at Irvine, Irvine, California, USA) and University of Kentucky Alzheimer's Disease Center Tissue Bank (University of Kentucky Lexington, KY, USA). Brain tissue was collected with patient consent and protocols were approved by the Institutional Review Board of University of California at Irvine and University of Kentucky. All samples were examined by neuropathologists for diagnosis.

Immunofluorescence (IF) for Frozen Mouse and Human Tissue

Frozen sections used for immunofluorescence were fixed in 100% chilled methanol, washed three times in 1X PBS, and blocked in 5% goat serum for 1 hour. All human sections were immersed in 70% ethanol for 5 minutes, followed by a 5-minute incubation with autofluorescence eliminator reagent (Millipore) and three washes in 70% ethanol to block lipofuscin fluorescence in aging brain tissue prior to blocking. Sections were labeled with antibodies: Total p53 (human tissue: Total p53 Anti-Rabbit Abcam #32389 or Total P53 Anti-Mouse Abcam #1101; Mouse tissue Abcam #ab26), Phospho-p53 Ser15 (Cell Signaling #9286), Total Tau (Tau13 Biolegend), I11, T22 and incubated overnight at 4˚C. The following day, sections were washed in PBS three times for 10 minutes each and incubated with goat anti-mouse IgG Alexa-488 (1:500; Invitrogen) for 1 hour. Sections were then washed in PBS three times for 10 minutes each and incubated with DAPI (Invitrogen) to label nuclei for 5 minutes at room temperature, washed three times for 5 minutes each, and were mounted with Fluoromount G. The sections were examined using a Nikon A1R MP laser scanning microscope or a Keyence BZ-800 Microscope.

Proximity Ligation Assay (PLA)

Detection of protein-protein interaction is conducted by in-situ proximity ligation assay. The method depends on the recognition of target molecules in close proximity (<40 nm) by pairs of affinity probes, giving rise to an amplifiable detection signal. Briefly, PLA in human brain tissue was performed using Duolink® PLA in Situ Red starter kit mouse/rabbit (Sigma Aldrich, DUO92101) per manufacturer's protocol. Concentration of antibodies was established from IF protocol. Primary antibodies used for in–situ proximity assay include: Total p53 (Abcam #32389 1:100) and Total Tau (Tau13 Biolegend 1:1,000). Amplified red signal was detected and imaged using Keyence BZ-800 Microscope.

Purification of Human Recombinant p53

The plasmid pet15b/p53 containing the cDNA of human N-terminally His-tagged full length p53 was transformed into Eschichia coli strain BL21DE3. The resulting bacteria was grown at 37˚C to an OD_{600}=0.4-0.6 before 3-4 hr induction at 37˚C with 0.5 mM isopropyl β-D-thiogalactosidase (IPTG). After induction, cells were harvested by centrifugation and stored at -20 until use. Cell pellet was resuspended with 10 mL of column buffer containing protease inhibitor and then sonicated for 5 bursts for 30 seconds each. Sonicated cells were then centrifuged at 10,000 RPM for 10 min on Ultracentrifuge. Purification was performed using chromatography columns: the resin was washed with deionized water 3x pellet volume and re-calibrated with 30 mL column buffer (TritonX 100, 1 M Tris HCl (pH 8), 1 M NaCl, 1 M imidazole, and deionized water). Supernatant was incubated at RT with His-Pur Ni-NTA resin (Thermo Scientific #88221) for 1 hr. Two elution fractions were collected using 50 mL of column buffer containing 250 mM (Elution 1) and 300 mM imidazole (Elution 2). Elutions were then dialyzed in 30 mM Tris HCl (pH 8) overnight to remove imidazole. Dialyzed elutions were then placed in 30 K Amicon Ultra centrifugal filters (Millipore) to remove any degraded p53 protein and any leftover imidazole. P53 elution concentration was measured with Nanodrop using p53 extinction coefficients of ε_{280}=17,130 cm/M and then were tested by western blot before being lyophilized using Labconco Benchtop Freeze Dryer for long term storage.

Western Blot

Purified human recombinant p53 elution samples were loaded on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. Gels were subsequently transferred onto nitrocellulose membranes and blocked overnight at 4°C with 10% nonfat dry milk. Membranes were then probed for 1 hour at room temperature with Total P53 (Abcam #1101 1:1000) diluted in 5% nonfat dry milk. Total p53 was detected with an HRP-conjugated anti-mouse IgG (1:10,0000, GE Healthcare) diluted in 5% milk. ECL (Advansta) was used to visualize the bands.

Size Exclusion Chromatography (SEC) on Fast Protein Liquid Chromatography (FPLC)

Recombinant p53 samples were separated by SEC using Amersham Biosciences AKTAexplorer FPLC system fitted with Superdex 75 10/300 GL column (tricorn). L × I.D. 10 × 300 mm, 13 μm particle size from GE Healthcare. Molecular grade water was used as mobile phase, flow rate 0.5mL/min. Gel filtration standard (Bio-Rad 51-1901) was used for calibrations. Excitation and emission wavelengths used for fluorescence detection were 280 nm and 350 nm, respectively. Desired p53 monomer and oligomer peaks were collected and tested by western blot and AFM for further confirmation.

Atomic Force Microscopy (AFM)

The morphology of p53 monomers and oligomers and fibrils were assessed by AFM as previously described [129] Briefly, samples were prepared by adding 10 μL of p53 monomer or oligomers on freshly cleaved mica and allowed to adsorb to the surface. Mica were then washed three times with distilled water to remove unbound protein and impurities followed by air-drying. Samples were then imaged with Multimode 8 AFM machine (Veeco, CA) using a noncontact tapping method (ScanAsyst-Air).

Tau Oligomer (TauO) and p53 Oligomer (p53O) production, labeling, and cell treatments

The tau oligomer (TauO) were produced and characterized following established and published protocols [87]. TauO and p53 oligomer (p53O) labeling was conducted as follows: 1 mg of Alexa Fluor™ 568 NHS Ester (Invitrogen, #A20003) was dissolved in 0.1 M sodium bicarbonate to make the final concentration 1 mg/ml. The Alexa Fluor dye was then incubated with TauO and p53O in a

1:2 (w/w) ratio. The mixture was rotated overnight at 4°C on an orbital shaker. The following day, the solution was centrifuged (30 min, 15,000*g*) using 10 kDa Amicon Ultra-0.5 Centrifugal Filter Units to remove unbound dye. The oligomers were then washed with 1× PBS until the flow through solution was clear. The filter compartment was then flipped and centrifuged to collect the concentrate. The oligomers were reconstituted to their original volume. Alexa Fluor labeled TauO and p53O were re-suspended in complete DMEM to obtain 1 µM final concentration solutions. The cells were treated with TauO or p53O for 1 hr at a controlled temperature of 37°C and 5% CO_2. Afterwards, the medium was removed and the cells collected for immunofluorescence assays.

Primary Cortical Neuron Culture

Primary cortical neuronal cultures were prepared and maintained as described previously [318]. Briefly, cortical neurons were isolated from embryonic day 16–18 C57BL/6 mice (The Jackson Laboratory #000664) using Accutase® solution (A6964-100Ml Sigma-Aldrich). Dissociated neurons were plated at a density of 2×10^5 cells/ml in a 24-well plate containing high glucose DMEM (Corning) supplemented with 2% B27 (A3582801, Gibco), 10,000 units/ml penicillin, 10,000 µg/ml streptomycin, and 25 µg/ml amphotericin B (15290018, Gibco). After 2 hr, plating medium was removed from cells and replenished with Neurobasal™ medium (12348017, Gibco) plus 2% B27, 0.5 mM L-glutamine (SH30034.01, HyClone), 10,000 units/ml penicillin, 10,000 µg/ml streptomycin, and 25 µg/ml amphotericin B supplement. 50% of medium changes were performed every 3–5 days. Cells on day *in vitro* 10 were used for all experiments.

Immunofluorescence of Fixed cells and Confocal Microscopy

Cells on a 24-well coverslip were fixed with 0.5 ml of 4% PFA/PBS for 15 min. The cells were washed three times in phosphate-buffered saline (PBS), for 5 min intervals. They were then

permeabilized in 0.5 ml PBS/0.2% Triton X-100 in phosphate-buffered saline with Tween 0.5% (PBST) for 5 min. Blocking was done in 0.5 ml of 5% NGS Serum in PBST for 1 hr. Primary antibody was diluted in 5% NGS/PBST for overnight incubation at 4°C and then washed three times in PBST, for 10 min intervals. Secondary antibody diluted in 5% NGS/PBST was incubated for 2 hr at room temperature. All the secondary antibodies were purchased from Thermo Fisher Scientific and used at 1:800 dilution for staining. After 2° antibody, cells were incubated in DAPI (nuclei staining) diluted 1:10,000 in PBST (5 mg/ml stock solution) for 5 min after the first wash. They were then washed two times with PBST, and once with PBS, 10 min each, prior to mounting coverslips. Coverslips were mounted on glass microscope slides using 8–10 µl Prolong Gold Antifade mounting media with DAPI (Invitrogen, P36941) per coverslip. Slides were naturally dried in a fume hood (or store at 4°C until ready to dry in fume hood). The primary antibodies used in this study for immunocytochemistry are as follows: β-III-Tubulin, Total p53 (Abcam ab26), Total Tau (Tau13 Biolegend). After three washes with PBS, cells were probed with mouse and rabbit-specific fluorescent-labeled secondary antibodies (1:200, Alexa Fluor 488 and 546, Life Technologies). The single frame images and Z-stacks for 3D rendering and orthogonal view were collected using a Keyence Confocal Microscope and processed with ImageJ Software.

Chapter 3: P53 Aggregates in Human AD and Transgenic Mouse Models

Background:

WT p53 is an intrinsically disordered protein making it prone to aggregate. Indeed, our laboratory and others have demonstrated that WT p53 can spontaneously aggregate and form oligomers and fibrils *in vitro*. P53 oligomers have been shown to cause loss of function and gain of toxic function *in vitro* [100,255,259,260]. Exogenous p53 aggregates can seed and cross-seed in a prion-

like manner. However, none of these studies have investigated p53 aggregates in neurodegenerative diseases- diseases specifically known for hallmark aggregates. In Alzheimer's disease, p53 is up-regulated, but little is known about its specific subcellular localization, which is vital to understanding whether p53 is functional and which other cellular processes may be affected. Furthermore, little has been demonstrated to show where p53 resides in neurons. p53 is a versatile protein with a diverse set of critical functions. Thus, post translational modifications and the subcellular location of p53 can be informative about which specific p53 signaling pathways are active, thereby contributing to functional outcomes.

Therefore, we investigated whether p53 aggregates occur in human AD as well as transgenic mouse models of AD. Our overall goal, to observe and characterize natively folded p53 and p53 oligomers in AD tissues to gain a better understanding of p53 status in AD.

Results:

Human frontal cortex from AD and age-matched control patients were stained with immunofluorescent markers for total p53 and I11, a polyclonal antibody that detects the conformation of oligomers, antibodies and evaluated by confocal imaging. Colocalization between p53 and I11 oligomers are shown in the AD tissue, but not in control tissue (**Figure 4A**). Zoomed

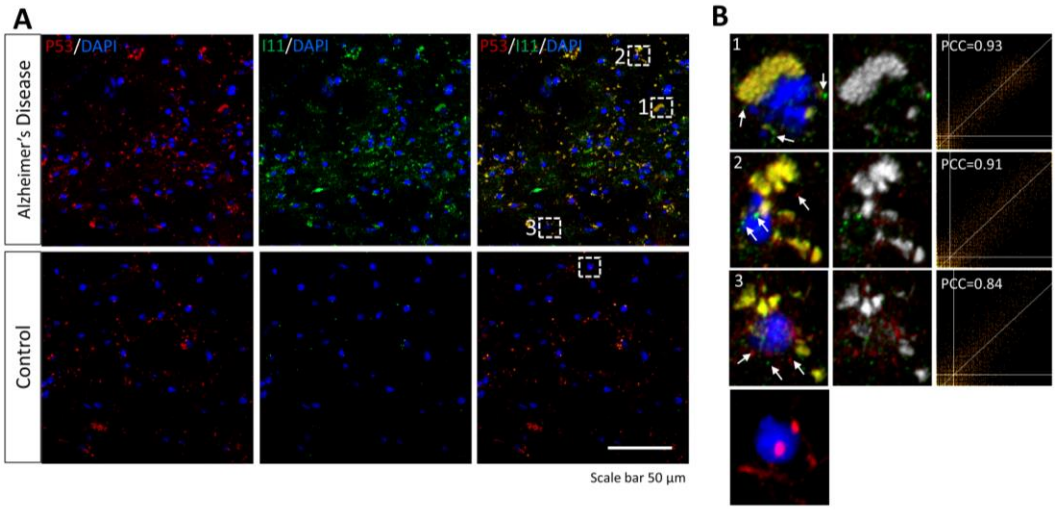

Figure 4: p53 oligomers are found in the frontal cortex of human AD patients, but not control patients

(A) Representative confocal images of human AD and age-matched control frontal cortex immunofluorescence stained with DAPI (blue), Anti-Total p53 antibody (red) and Anti-I11 antibody (green; polyclonal oligomer-specific), and merge panel (right). **(B)** Left: Zoomed regions of interest from merged panels in A. Colocalization between p53 and I11 (yellow) is shown in the AD tissue, but not in control tissue. Middle: colocalized pixel maps show peri-nuclear overlap of signal with other green oligomers and red p53 in vicinity. Right: Intensity scatterplot with a Pearson's Correlation Coefficient (PCC), suggesting that p53 oligomers occur in the brains of AD patients. No colocalized pixel map or PCC calculated for control as there is no detected colocalization between p53 and I11 signal. Nikon Confocal Microscope 60X Magnification. Scale bar=50 µm

regions of interest with colocalized pixel maps demonstrate peri-nuclear overlap of signal with other oligomers and p53 in the vicinity (**Figure 4B**). Intensity scatterplots with strong Pearson's Correlation Coefficient (PCC) also indicates a high degree of colocalization between fluorophores (**Figure 4B**), suggesting the detected p53, is in an oligomeric conformation, in the brains of AD patients, which has never been shown before.

Next, we wanted to access if oligomerization affected the ability of p53 to become activated through post-translational modifications. Human frontal cortex from AD and age-matched control

patients were stained with immunofluorescent markers for phosphorylated p53 at Ser15 and oligomers with I11 antibody (**Figure 5A**). P53 becomes active when phosphorylated at Ser15 in response to DNA damage and also prevents p53 export from the nucleus. Colocalization between phosphorylated p53 and I11 is found in AD tissue but not control tissue. Regions of interests show peri-nuclear colocalization with other unidentified oligomers present in the vicinity (**Figure 5B**). A colocalized pixel map and strong PCC support this colocalization (**Figure 5C**). Conversely, in control tissue, phosphorylated p53 forms distinct puncta in the nucleus (**Figure 5D**) suggesting it is active and functional as this Ser15 phosphorylation is specific to DNA damage, which would cause p53 to move to the nucleus. Together, this would suggest that p53 oligomers can form despite the presence of this post-translational modification. P53 mislocalization may also be apparent here as p53 phosphorylation at this specific Ser15 would suggest that p53 is being activated to assist in DNA damage repair.

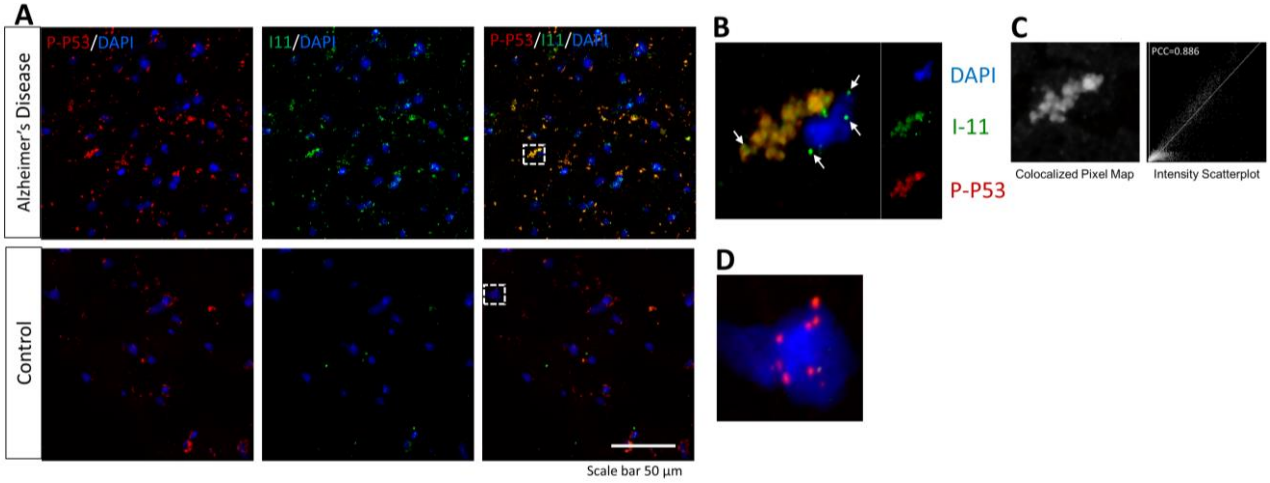

Figure 5: Phospho-p53 oligomers are found in the frontal cortex of human AD patients, but not control patients

(A) Representative confocal images of human AD and age-matched control frontal cortex immunofluorescence stained with DAPI (blue), Anti-Phospho-p53 Ser15 antibody (red, left) and Anti-I11 antibody (green, middle; polyclonal oligomer-specific), and merge panel (right). **(B)** Zoomed region of interest from AD merged panels in A. Colocalization between p53 and I11 (yellow) is shown in the AD tissue with other green oligomers in vicinity. **(C)** Colocalized pixel map shows peri-nuclear overlap of signal. Intensity scatterplot with Pearson's Correlation Coefficient (PCC) of 0.886 indicates a high degree of colocalization, suggesting that Phospho-p53 oligomers can form despite post translational modifications in the brains of AD patients. (D) Zoomed region of interest from control merged panel in A. P-P53 forms distinct punctate overlapping with DAPI stain. No colocalized pixel map or PCC calculated for control as there is no detected colocalization between p53 and I11 signal. Nikon Confocal Microscope 60X Magnification. Scale bar=50 µm

We also wanted to look at mouse models of AD so we could study p53 oligomers in a controlled animal of disease. The Tg2576 mouse is one of the most well characterized and widely used mouse models of AD. These transgenic mice overexpress a mutant amyloid precursor protein, APP, culminating in elevated levels of amyloid beta, amyloid plaque formation, and cognitive impairment [319]. Cortical and hippocampal tissue slices from aged (16-month-old) Tg2576 mice were stained with total p53 and I11 and analyzed by confocal imaging. Results demonstrate colocalization in the cortex, CA1 neurons of the hippocampus, and dentate gyrus region of the hippocampus (**Figure 6A**). Regions of interest show colocalized peri-nuclear structures with other

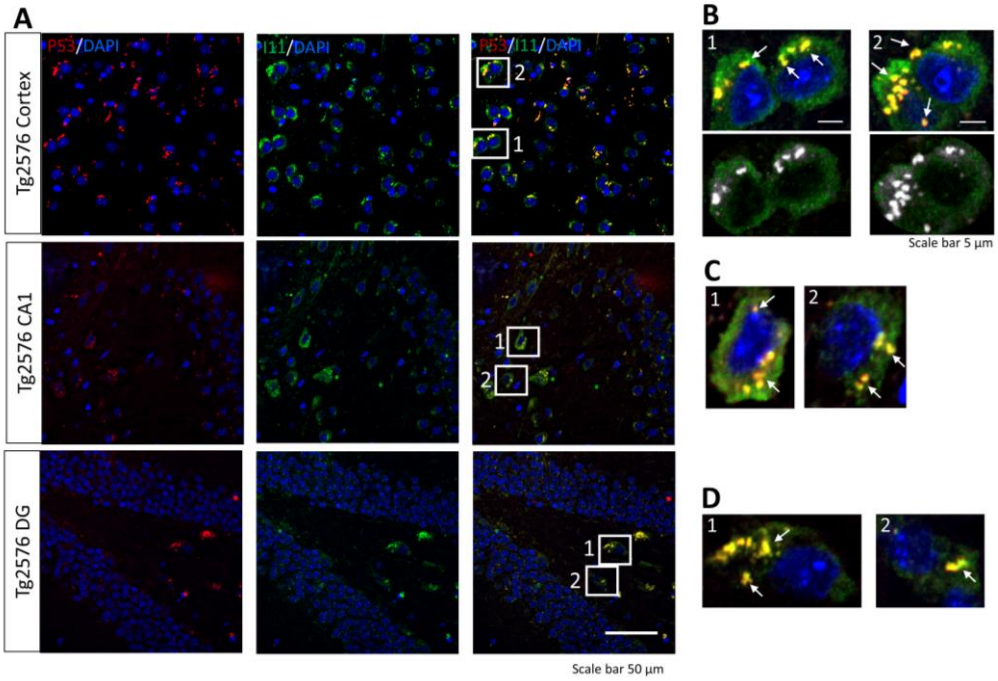

Figure 6: p53 oligomers are found in the cortex and hippocampus of Aged Amyloid Mouse Model

(A) Representative confocal images of cortex and hippocampal (CA1 neurons and dentate gyrus) regions of 16-month-old Tg2576 AD mouse model. Brain tissues immunofluorescence stained with DAPI (blue), Anti-Total p53 antibody (red, left) and Anti-I11 antibody (green, middle; polyclonal oligomer-specific), and merge panel (right). **(B)** Top: Zoomed regions of interest of cortex from merged panel in A. Colocalization between p53 and I11 (yellow) is shown. Bottom: colocalized pixel maps show peri-nuclear overlap of signal with other green oligomers in the vicinity **(C)** Zoomed regions of interest of CA1 neurons from merged panel in A. Peri-nuclear colocalization between p53 and I11 (yellow) is shown with other green oligomers in vicinity. **(D)** Zoomed regions of interest of dentate gyrus (DG) neurons from merged panel in A. Peri-nuclear colocalization between p53 and I11 (yellow) is shown with other green oligomers in vicinity. Nikon Confocal Microscope 60X Magnification. Scale bar=50 µm

unidentified oligomers in proximity (**Figure 6B**), suggesting that p53 oligomers can be found in numerous brain regions of the Tg2576 AD mouse model (**Figure 6B-D**).

In addition, we wanted to look at a tau mouse model to see how tau may affect p53 oligomer formation. The HTAU mouse model overexpresses all 6 isoforms of human Tau and develops paired helical filaments at 9 months of age with full neurofibrillary tangle detected at 15 months of age. Abnormal spatial memory has been documented by 6 months of age with more major memory deficits detected by 12 months of age. Cortical tissues from 14-month-old HTAU mice and 9-month-old C57Bl/6 mice were stained with antibodies against Total p53 and I11 (**Figure 7A**). Peri-nuclear colocalization between p53 and I11 is shown in HTAU mice, but not WT mice and can be more clearly seen in zoomed regions of interest (**Figure 7B**). This indicates that p53 oligomers can be found in the cortex of aged HTAU mice, but not in control mice.

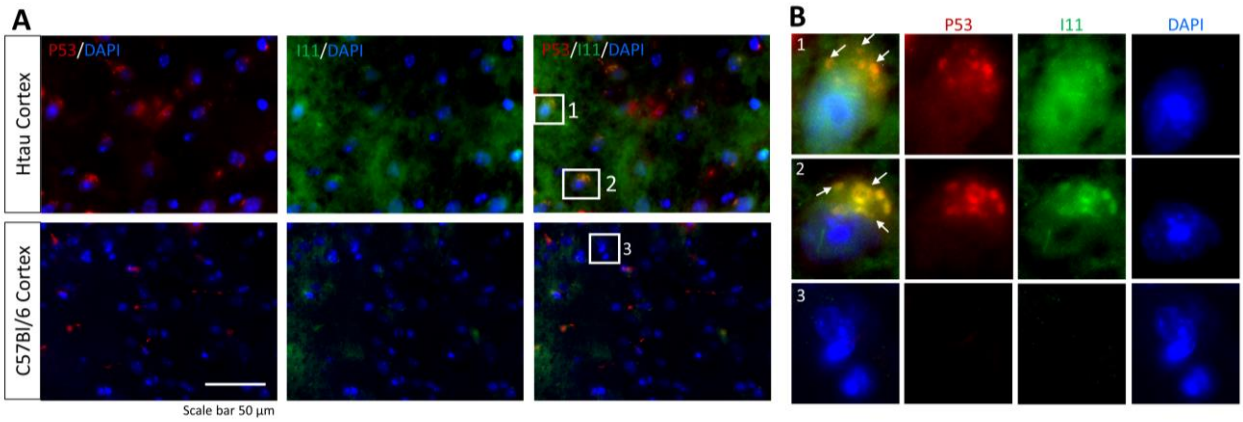

Figure 7: p53 oligomers are found in the cortex of aged Tau mouse model, but not in cortex of aged control mice

(A) Representative confocal images of cortex of 14-month-old HTAU transgenic mouse model and cortex of 9-month-old C57Bl/6 mice. Brain tissues immunofluorescence stained with DAPI (blue), Anti-Total p53 antibody (red, left) and Anti-I11 antibody (green, middle; polyclonal oligomer-specific), and merge panel (right). **(B)** Zoomed regions of interest of cortex from merged panel in A. (ROI 1 and 2) Peri-nuclear colocalization between p53 and I11 (yellow) is shown with other green oligomers in the vicinity. (ROI 3) No colocalization between p53 and I11 is detected in C57Bl/6 mice. Keyence Microscope 100X magnification. Scale bar=50 µm

Discussion:

The overall goal of this chapter was to identify p53 aggregates, define the extent of p53 aggregation and oligomerization in human AD brain tissue and transgenic mouse models. A focus was placed on the oligomer formation because this soluble intermediary species has been reported to be the most toxic species, can affect the misfolding of other proteins through cross-seeding, and therefore affects the spread and severity of pathology. Overall, our evidence suggests that the aggregate-prone protein, p53 is indeed forming oligomers in human AD as well as transgenic mouse models associated with AD, but not in controls. This demonstrates that p53 function may be disrupted and needs to be further investigated.

Overall, peri-nuclear distribution of p53 oligomers is consistent across human AD, Tg2576, and HTAU mouse model tissues. It is also consistent with previous publications showing p53 aggregates in cancer [100,259,260,320]. This would suggest that the peri-nuclear distribution of p53 aggregates is a common factor across species and diseases. Numerous other labs, in addition to our own lab, have reported discovery of p53 aggregates in cancer tissues using the closely related A11 antibody from our laboratory. [100,259,321]. Therefore, we are using the same techniques established in the field to determine p53 aggregates.

It is also interesting to note that in the human tissue, the activated form of p53, phosphorylated p53 at Ser15, also shows oligomerization and mislocalization. P53 has many post-translational modifications that alter its activity. Since phosphorylation of p53 did not prevent p53 from forming oligomers, it is possibly that the many other PTM's may also be able to form oligomers. The presence of this Ser15 phosphorylation also demonstrates that DNA damage is likely occurring in these AD neurons and excessive DNA damage has been reported as an early marker in mild cognitive impairment (MCI) and AD patients. It is possibly that this excessive DNA damage may be due to p53 forming oligomers and losing normal function. This is especially

interesting given that the activated phosphorylated p53 oligomers appeared mislocalized outside the nucleus. This specific phosphorylation site at Ser15 is in response to DNA damage and causes p53 to break away from its inhibitor, Mdm2, and also prevents p53 nuclear export while performing its function in the nucleus. The peri-nuclear distribution of Phospho-p53 oligomers in AD but not in control would suggest mislocalization. Evidence of mislocalization may indicate that oligomer formation is preventing p53 from entering the nucleus- possibly through changes in the nuclear localization signal that may be necessary to gain entrance to the nucleus or through interactions with other proteins or aggregates. This interaction with other oligomer may be a real possibility as other unidentified oligomers were found in proximity to the p53 oligomers shown in our human and mouse models. Overall, p53 oligomers may cause a loss of function (LOF), which is a core finding in p53 aggregates in cancer. One mechanism of p53 LOF in cancer is through p53 cytoplasmic sequestration which has been documented in human neuroblastomas [322]. In cancer, a loss of p53 allows the tumor cells to become immortal and spread disease. Given prior evidence that many neurons in AD do not die by apoptosis, but rather a slow progressive death over many years would suggest that the ability of p53 to induce apoptosis may be disrupted.

Furthermore, it was reported this year, α-synuclein is capable of binding to DNA and modulating DNA repair. α-synuclein forms abnormal cytoplasmic aggregates in Lewy body disorders such as Parkinson's disease. One of the mechanisms proposed was that cytoplasmic aggregation of α-synuclein reduced its nuclear levels, causing nuclear LOF thereby causing increased lethal double strand breaks and contribution to cell death [323]. Given our data presented here, it is plausible to speculate that we see a similar LOF of nuclear p53 in AD.

The mouse models associated with AD also demonstrate p53 oligomer formation is affected by the two major proteins implicated in AD-Tau and amyloid beta (Aβ). Together, with the Tg2576 Aβ mouse model and the Htau tau mouse model, we may conclude that both tau and amyloid beta

can separately affect p53 oligomerization. A mouse model that contains both tau and Aβ pathology, such as the 3xTg mouse, may help ascertain if the combination of both aggregate proteins causes even more p53 oligomers to be produced.

Chapter 4: P53 interacts with tau and tau oligomers in human AD and in vitro

Background:

Previous publications from our laboratory and others have shown that oligomers are the most toxic protein species in AD [64,324,325]. Although most research has concentrated on tau and Aβ, p53 could be a novel protein implicated in the disease, as oligomers of p53 have been shown to be cytotoxic [100,255,259]. However, this toxicity has not been addressed in an AD model. Moreover, oligomers from tau, Aβ, and α-synuclein have been observed to cross-seed and induce oligomerization of other proteins *in vitro* and *in vivo* [12,83]. Mutations in p53 have also been shown to induce the oligomerization of p53 and its paralogs p63 and p73 [260,326,327]. Certain protein-protein interaction has also been shown to increase toxicity [64,139,140,328]. Tau oligomers (tauO) have been shown to cause varying degrees of toxicity based on those protein-protein interactions [329]. To date, no study has investigated an interaction between p53 and tauO. Taken together, this would suggest that tau and/or Aβ oligomers may induce the oligomerization of p53 in AD or p53 oligomers may be an earlier event that induces tau or Aβ oligomerization. This would help increase our understanding of early mechanisms of disease.

Previous studies have investigated p53 and interaction with tau/microtubules. One study showed p53 overexpression indirectly caused an increase in Tau phosphorylation in HEK293 cells. Giannakakou et al showed *in vitro* that p53 associates with cellular microtubules and accumulates

in the nucleus after DNA damage only in cells with a functional microtubule network. They further demonstrated that disruption of normal microtubule dynamics caused impaired nuclear accumulation of p53 after DNA damage [304]. This publication has huge implications for AD because the microtubule stabilizing protein, tau, is disrupted and therefore p53 transport to the nucleus may be disrupted in AD. Furthermore, Eftekharzadeh et al demonstrated that AD-related tau impairs nuclear import and export in human AD brain tissue through disruption of nuclear pores [305]. These previous publications would suggest that p53 transport may be impaired in AD, possibly through pathological effects of tau and a disrupted microtubule network.

Previous *in-vitro* studies have shown that both exogenous mutant and wild-type p53 oligomers can penetrate, seed, and cause rapid aggregation of endogenous p53 in a prion-like manner [259,264]. As p53 is known to cross-seed p63 and p73 [260,261] and engage with Aβ [18] and tau [304], it is possible that aggregated p53 can affect the conformations or cross-seed tau and/or Aβ. These interactions may be dictated by the subcellular localization of p53 oligomers.

I investigated interactions between p53 and tau with their subsequent aggregation state in human AD tissue and exogenous treatment on WT mouse primary neurons. Localization of these interactions was also considered.

Results:

Human frontal cortex from AD and age-matched control patients were stained with immunofluorescent markers for total p53 and total tau antibodies and evaluated by confocal imaging (**Figure 8A**). Zoomed regions of interest from AD tissue show large peri-nuclear colocalization with no detectable p53 overlapping with the nucleus and tau in the nearby vicinity (**Figure 8B**). Conversely, control tissue shows some neurons with diffuse p53 signal (**Figure 8C**) and other neurons with a single small region of colocalization between p53 and tau (**Figure 8D**).

This would suggest that p53 interacts with tau in the human frontal cortex, but the interaction is larger and more widespread in AD. Proximity Ligation Assay (PLA) using the same Total p53 and Total tau antibodies and human tissues demonstrates a similar pattern of colocalization seen in the immunofluorescent staining (**Figure 8E-G**). Of note, this PLA only causes fluorescence when the fluorophores are within 40 nm of each other, suggesting more of a direct physical interaction rather than the two proteins merely being in the same cellular region. Together, this suggests that there is an interaction between p53 and tau in human frontal cortex with a larger degree found in AD than control brain tissue.

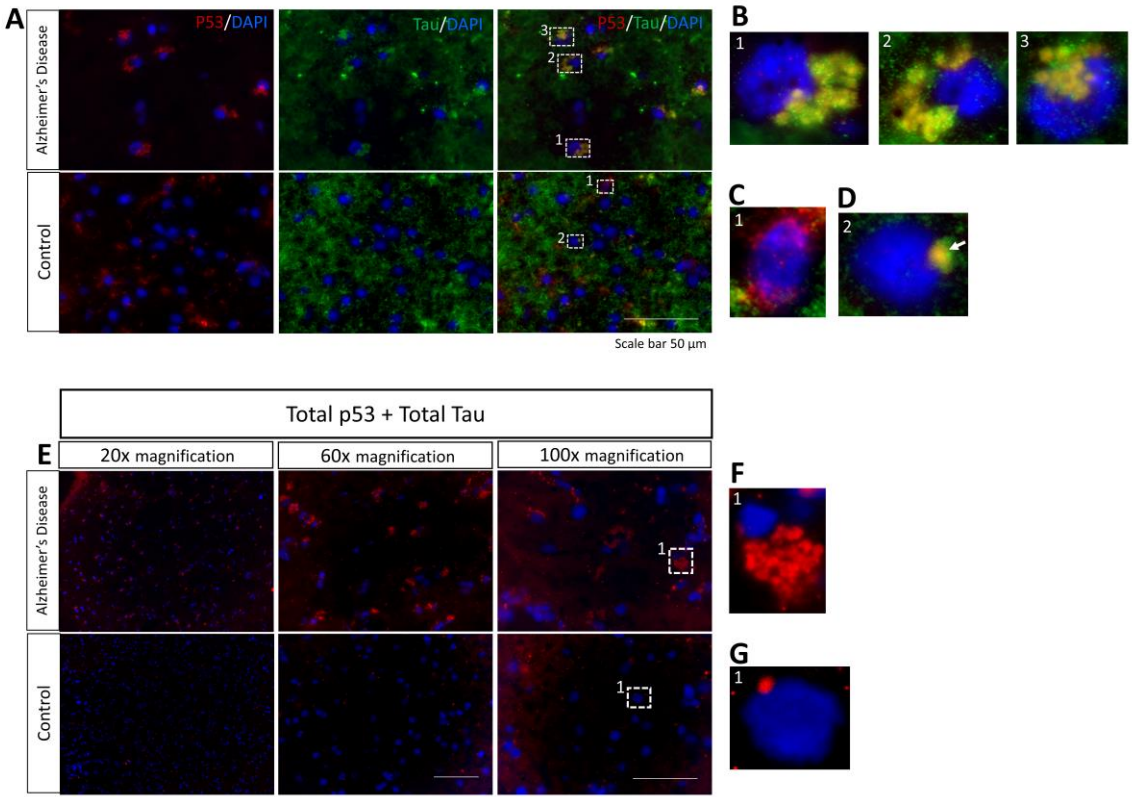

Figure 8: p53 interacts with Tau in human Frontal cortex

(A) Representative confocal images of human AD and age-matched control frontal cortex immunofluorescence stained with DAPI (blue), Anti-Total p53 antibody (red, left) and Anti-Total Tau antibody (green, middle), and merge panel (right). **(B)** Zoomed regions of interest from AD merged panels in A. Large peri-nuclear colocalization between p53 and Tau (yellow) is shown in the AD tissue with other green tau in the vicinity. **(C)** Zoomed regions of interest from control merged panels in A. Diffuse p53 signal with very little colocalization shown. **(D)** Zoomed regions of interest from control merged panels in A. Small single peri-nuclear region of colocalization between p53 and tau detected in control brain tissue with other green tau in the vicinity. **(E)** Representative confocal images of human AD and age matched control frontal cortex using Proximity Ligation Assay (PLA) with same Total p53 and Total tau antibodies used for immunofluorescence in A. Different magnifications (20-100X) are shown. **(F)** Zoomed region of interest from AD merged panel in E shows large peri-nuclear interaction similar to zoomed images shown in B. (G) Zoomed region of interest from control merged panel in E shows small single region of interaction similar to zoomed image in D. Keyence Microscope 100X magnification. Scale bar=50 µm

As we observed neurons with p53 and tau interaction, we wanted to determine if p53 was able to enter the nucleus in neurons with peri-nuclear interaction. Human AD and control frontal cortices were immunofluorescent stained with DAPI (blue), a Total p53 (red) antibody and a Total Tau (green) antibody (**Figure 9A, D**). Zoomed regions of interest from AD tissue with orthogonal view demonstrates some neurons with diffuse p53 covering the nucleus (**Figure 9B**). These neurons show detectable p53 and tau inside the nucleus confirmed by orthogonal view (**Figure 9B**). Other neurons with large peri-nuclear colocalization show no detectable p53 in the nucleus by orthogonal

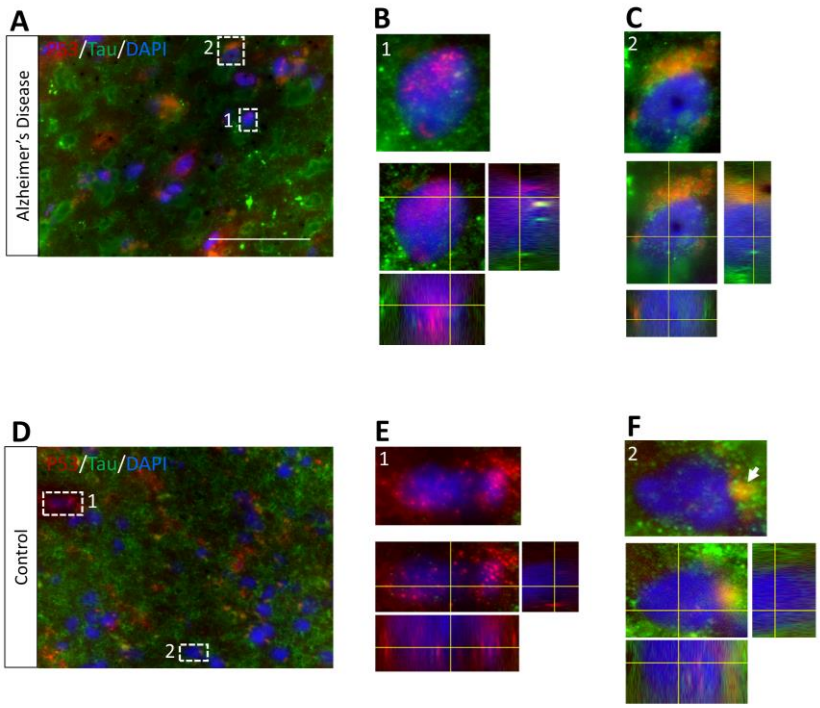

Figure 9: p53 interacts with Tau in human frontal cortex

(A,D) Representative confocal images of human AD and age-matched control frontal cortex immunofluorescence stained with DAPI (blue), Anti-Total p53 antibody (red) and Anti-Total Tau antibody (green) in a merged image. **(B)** Top: Zoomed region of interest from AD merged panels in A with Bottom: orthogonal view. Some neurons show diffuse p53 covering the nucleus and some detectable tau inside the nucleus. **(C)** Top: Zoomed region of interest from AD merged panels in A with Bottom: orthogonal view. Other neurons with large peri-nuclear colocalization between p53 and tau show no detectable p53 in the nucleus. **(E)** Top: Zoomed region of interest from control merged panels in D with Bottom: orthogonal view. Some neurons show diffuse p53 covering the nucleus and p53 inside the nucleus by orthogonal view. **(F)** Top: Zoomed region of interest from control merged panels in D with Bottom: orthogonal view. Other control neurons show small single peri-nuclear region of colocalization between p53 and tau with little p53 detected in the nucleus. Keyence Microscope 100X magnification. Scale bar=50 µm

view, demonstrating that, in AD, most of the p53 is outside the nucleus with tau (**Figure 9C**). In control tissue, zoomed regions of interest show some neurons with diffuse p53 covering the nucleus

and p53 inside the nucleus by orthogonal view (**Figure 9E**). Other control neurons with a single region of colocalization between p53 and tau also show very little p53 detected in the nucleus (**Figure 9F**). Together, this would suggest that neurons with interaction between p53 and tau are associated with less p53 in the nucleus.

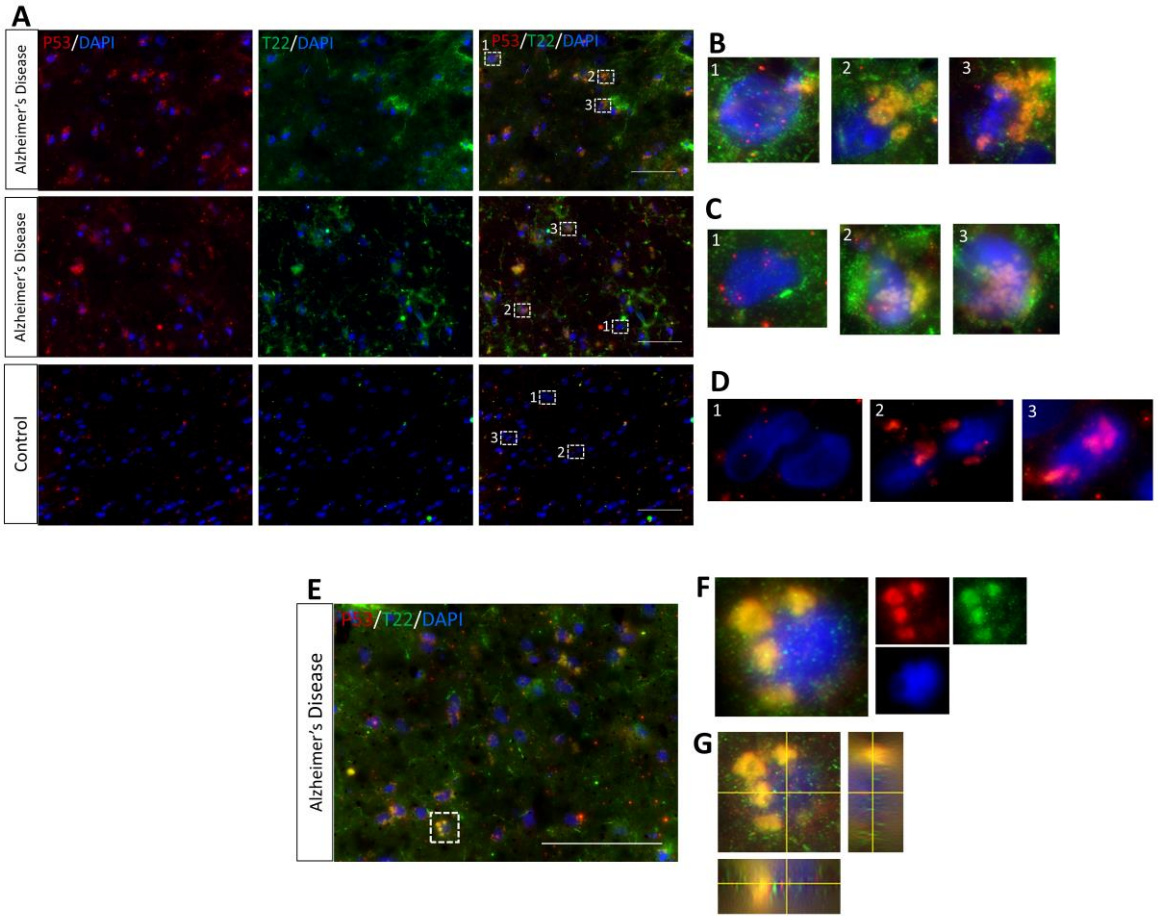

Figure 10 : Tau Oligomers interact with p53 in human AD patients, but not in control patients

(A) Representative confocal images of human AD and age-matched control frontal cortex immunofluorescence stained with DAPI (blue), Anti-Total p53 antibody (red, left) and Anti-T22 antibody (green, middle; tau oligomer specific) antibodies, and merge panel (right). **(B)** Zoomed region of interest from AD merged panels in A. ROI1 shows neurons with small region of colocalization of p53 and T22 also show detectable p53 and tau covering the nucleus. ROI2,3 show large peri-nuclear colocalization between p53 and T22 (yellow) with other green tau in the vicinity. **(C)** Zoomed regions of interest from another AD merged panel in A. Zoomed region of interest show similar results to images in C, but colocalization between p53 and T22 is smaller and overlapping with the nuclear staining more. **(D)** Zoomed regions of interest from control tissue in A. ROI1 shows some nuclei with little detectable p53 while ROI2,3 show neurons where p53 signal is detected and converges with the nuclear stain. **(E)** Representative confocal image of human AD with same staining as those in A. **(F)** Zoomed region of interest shows large peri-nuclear colocalization between p53 and T22. **(G)** Orthogonal view of zoomed regions of interest in F shows a large amount of p53 is localized outside the nucleus where it heavily colocalizes with T22. Furthermore, other tau oligomers are shown in the near vicinity and appear inside the nucleus by orthogonal view. Keyence Microscope 60X magnification. Scale bar=50 µm

Since we were able to show an interaction between p53 and tau, we also wanted to determine if tau oligomers (tauO) were part of the total tau species previously observed, as tauO have been shown to cause toxicity and cross-seed other proteins. Immunofluorescent staining of human AD and control frontal cortices with total p53, T22 (polyclonal antibody that specifically recognizes tau oligomers), and DAPI (nucleus) show peri-nuclear colocalization in AD tissue but not control tissue (**Figure 10A**). Zoomed regions of interest also highlight other tau oligomers in the vicinity (**Figure 10B, C**). Zoomed regions of interest in the control tissue shows some neurons with little p53 signal, while other neurons with more p53 signal show more colocalization with the nucleus (**Figure 10D**). Orthogonal view of AD tissue demonstrates that most of the p53 is outside the nucleus, heavily colocalizing with tauO. Orthogonal view of AD tissue also shows detectable tauO in the nucleus, which may be causing unknown pathology (**Figure 10G**). Overall, we may conclude that there is an interaction between p53 and tauO in AD tissue that is not found in controls.

Due to our cumulating evidence of p53 oligomers and interactions between p53 and tauO, we wanted to understand how exogenous treatment of p53O or tauO may affect endogenous p53 and tau in neurons. In order to do so, we produced purified human recombinant full-length p53. We started from a well-documented plasmid expressing human p53 with a His tag, which was then purified on a nickel column. Purified recombinant p53 elutions were tested by western blot using a total p53 antibody to confirm presence of p53 monomer. P53 dimers and high molecular weight aggregates were also detected by western blot (**Figure11A**). Attempts were made to destabilize these high molecular weight p53 aggregates into monomer by 8M urea and boiling, but high molecular weight p53 proved resistant (**Figure 11B**). Therefore, size exclusion chromatography (SEC) by fast protein liquid chromatography (FPLC) was performed to separate p53 monomer from

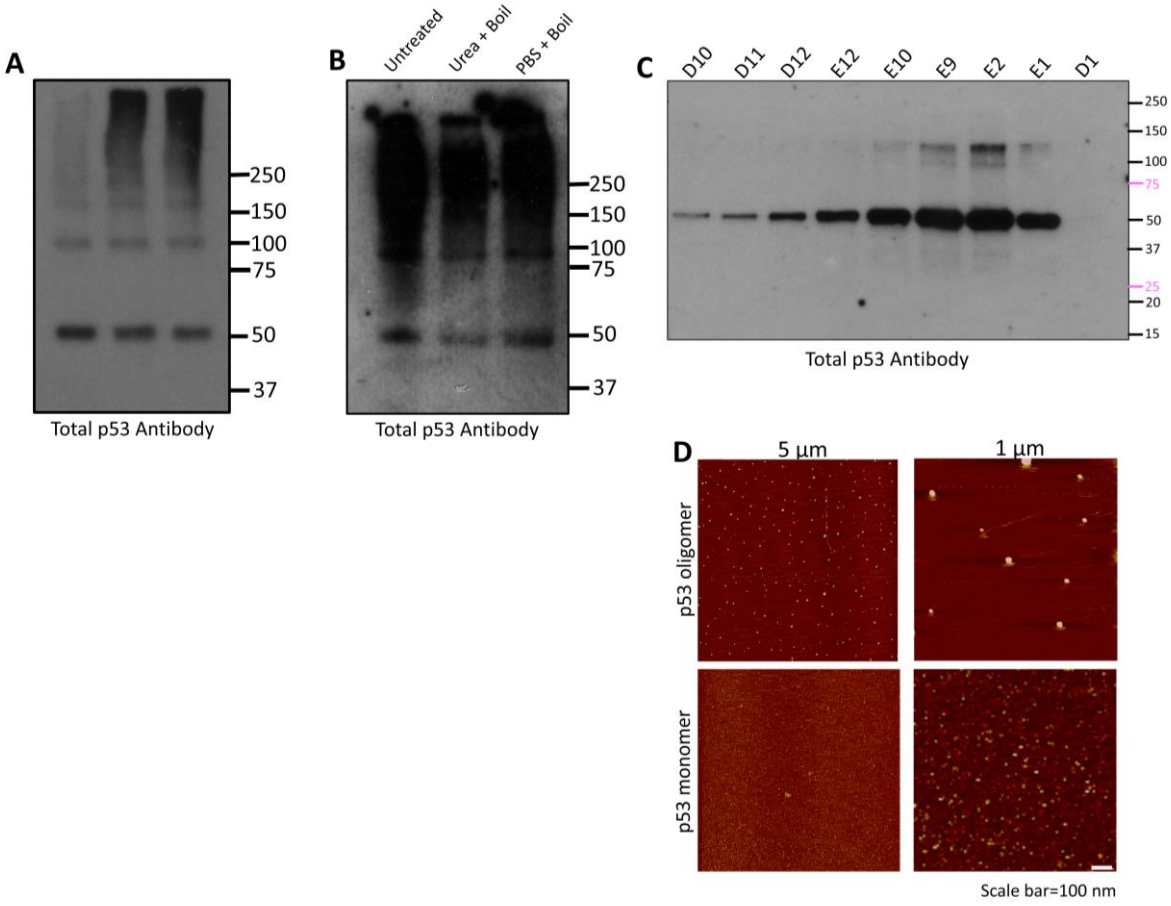

Figure 11: Purification of Recombinant Human full length p53 produces monomer and oligomers that are resistant to boiling and 8 M Urea Treatments. SEC fractionation by FPLC can be used to separate monomer from oligomers as confirmed by AFM.

(A) Representative image of different p53 purification elutions tested by western blot using anti-total p53 antibody shows detection of p53 monomer (53 kD) and higher molecular weight p53 formation. **(B)** High molecular weight p53 from purified recombinant p53 elutions are resistant to 8 M urea and boiling treatments by western blot. **(C)** Representative image of p53 fractions separated by Size Exclusion chromatography (SEC) on FPLC showing separation of p53 monomer (53 kD) from higher molecular weight bands by western blot. P53 protein fractions show monomers and size and spherical shape consistent with p53 oligomers by AFM.

higher molecular weight p53 (**Figure 11D**). p53 monomers and oligomers were additionally confirmed by atomic force microscopy (AFM) (**Figure 11C**).

Once we successfully had a stock of p53 oligomers (p53O), we Alexa-Fluor labeled the p53O (AFL-p53O) and then exogenously treated C57Bl/6 primary neurons with the labeled oligomers at 1 µm to see how p53O affected WT neurons. To determine uptake and internalization of p53O, we stained primary neurons (both treated with Alexa-fluor labeled p53O and untreated) with DAPI and antibodies against Total p53 and β-III-Tubulin (**Figure 12A,B**). β-III-tubulin is a major component of microtubules in neurons, which are exclusively intracellular. We used this signal to determine if exogenous p53 was taken up, intracellularly, by the primary mouse neurons.

In untreated cells, total p53 is detected diffusely in both the nucleus and cytoplasm, but within the confines of the β-III-tubulin signal (**Figure 12A**). Alexa-fluor labeled p53O also show within the confines of the β-III-tubulin signal, suggesting that p53O are internalized by WT primary neurons within 1 hour (**Figure 12B**). The AFL-p53O also appear to localize with the nucleus in contrast with the untreated group that shows endogenous p53 more diffusely.

After confirming that p53O could indeed be taken up by primary neurons, we investigated if exogenous p53O would interact with endogenous p53, potentially seeding the endogenous protein.

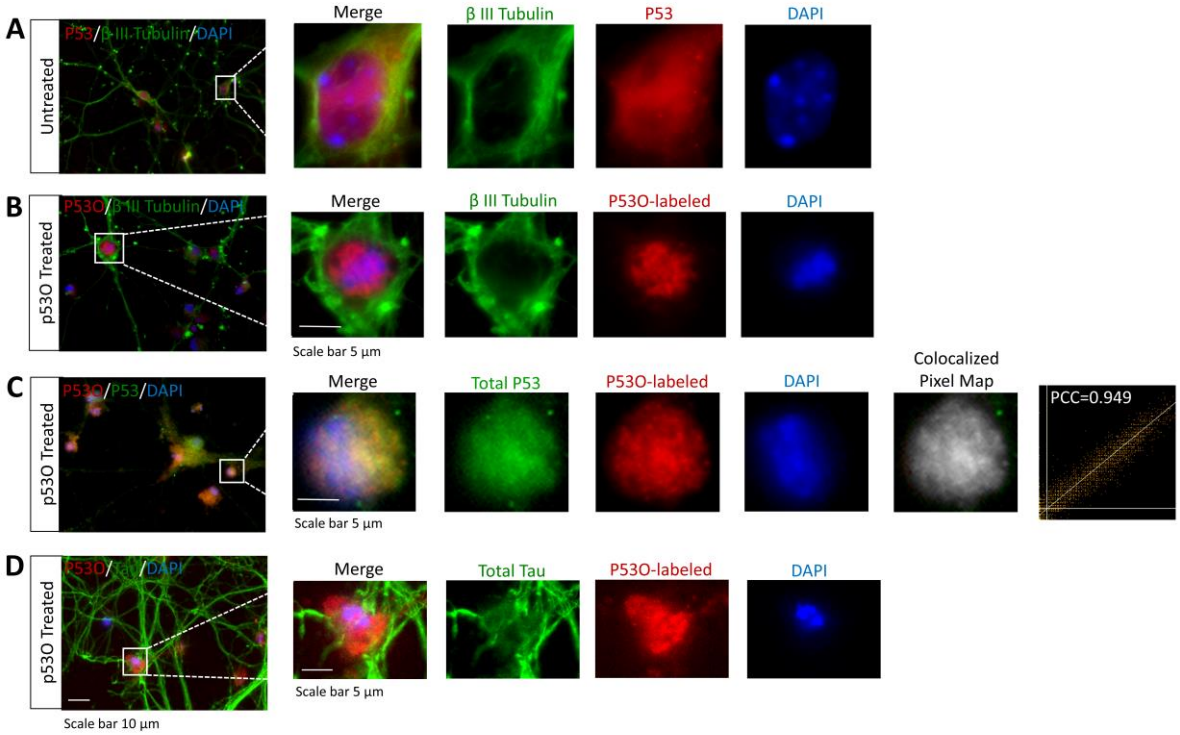

Figure 12: Alexa Fluor Labeled Recombinant human P53 Oligomers [1 µM] are internalized, localize to the nucleus, and colocalize with endogenous p53 in C57Bl/6 primary neurons after 1 hr treatment

(A-B) Representative confocal images of C57Bl/6 primary neurons (DIV 10) immunofluorescent stained with DAPI (blue), Anti-β-III-Tubulin (green), and Anti-Total p53 antibody (red; B has no red stain as it is labeled) in merged confocal images. **(A)** Zoomed region of interest from merged image of untreated C57Bl/6 primary neurons demonstrates endogenous p53 is within the confines of β-III-Tubulin, suggesting it is inside the cell and localizes to the nucleus and cytoplasm diffusely. **(B)** Zoomed region of interest from merged image of AFL-p53O treated C57Bl/6 primary neurons demonstrates AFL-p53O are within the confines of β-III-Tubulin, suggesting they are internalized by the cell and localize to the nucleus. **(C)** Representative confocal image of C57Bl/6 primary neurons (DIV 10) treated with AFL-p53O were immunofluorescent stained with DAPI (blue) and Anti-Total p53 antibody (green). Confocal merge image and zoomed region of interest demonstrate colocalization between AFL-p53O and endogenous p53. Colocalized Pixel Map and strong PCC supports this colocalization. **(D)** Confocal Imaging of C57Bl/6 primary neurons treated with AFL-p53O were immunofluorescent stained with DAPI (blue) and Anti-Total tau antibody (green) and show no colocalization between tau and AFL-p53O. Keyence microscope , 60X magnification, scale bar=10 µm and 5 µm)

C57Bl/6 primary neurons treated with AFL-p53O were stained with DAPI and an antibody against total p53 (**Figure 12C**). Confocal imaging demonstrated colocalization between AFL-p53O and endogenous p53, suggesting that exogenous p53O localize and may interact with endogenous p53 (**Figure 12C**), which sets up the potential for p53O seeding. Furthermore, we stained p53O treated

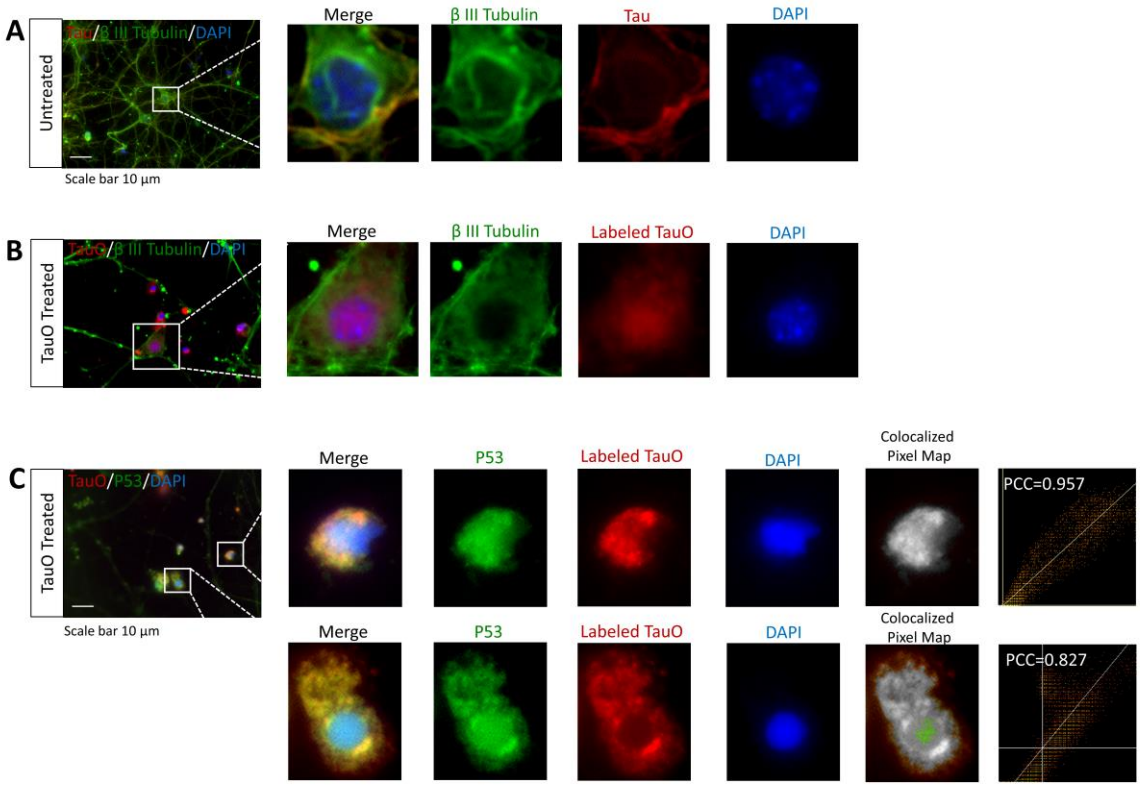

Figure 13: Alexa Fluor Labeled Recombinant human Tau4R Oligomers (AFL-TauO) [1 µM] are internalized, localize to the nucleus and cytoplasm, and colocalize with endogenous p53 in C57Bl/6 primary neurons after 1 hr treatment

(A-B) Representative confocal images of C57Bl/6 primary neurons (DIV 10) immunofluorescent stained with DAPI (blue), Anti-β-III-Tubulin (green), and Anti-Total Tau antibody (red; B has no red stain as it is labeled) in merged confocal images. **(A)** Zoomed region of interest from merged image of untreated C57Bl/6 primary neurons demonstrates endogenous tau colocalizes with β-III-Tubulin, which is expected from a microtubule stabilizing protein such as tau. **(B)** Confocal merge image and zoomed region of interest of AFL-tauO treated C57Bl/6 primary neurons **demonstrates AFL-tauO** are within the confines of β-III-Tubulin, suggesting they are internalized by the cell and localize to the nucleus and cytoplasm. **(C)** Representative confocal image of C57Bl/6 primary neurons (DIV 10) treated with AFL-p53O were immunofluorescent stained with DAPI (blue) and Anti-Total p53 antibody (green). Confocal merge image and zoomed regions of interest demonstrate peri-nuclear colocalization between AFL-p53O and endogenous p53. Colocalized Pixel Maps and strong PCCs supports this colocalization. Keyence microscope , 60X magnification, scale bar=10 µm and 5 µm)

primary neurons with DAPI and total tau to determine if p53O may interact with endogenous tau. No colocalization was found, suggesting that p53O do not interact with WT tau after 1 hr treatment at a concentration of 1 µm of p53O (**Figure 12D**).

We also wanted to consider the reverse treatment, with tauO to determine if tau oligomers could affect endogenous p53 in WT primary neurons. Therefore, we alexa fluor labeled tauO (AFL-tauO) and then exogenously treated C57 primary neurons with AFL-tauO at 1 μm for 1 hour. To determine uptake and internalization of tauO, we stained primary neurons (both treated with AFL-tauO and untreated) with Total tau, DAPI, and β-III-Tubulin (**Figure 13A, B**). In untreated cells, total tau colocalizes with the neuronal microtubule marker, β-III-Tubulin, which is expected as tau is a microtubule stabilizing protein (**Figure 13A**). However, the AFL-tauO appear to cluster near the nucleus but do show signal in the cytoplasm, but within the confines of the β-III-tubulin signal (**Figure 13B**). This suggests that tauO are also taken up by primary neurons within 1 hour.

Next, we immunofluorescent stained with DAPI and total p53 antibodies to determine if exogenous treatment with tauO could affect endogenous p53. Indeed, we did see peri-nuclear colocalization between AFL-tauO and endogenous total p53 (**Figure 13C**). A colocalized pixel map and strong PCC support this colocalization, suggesting that tauO can interact with endogenous p53.

Discussion:

The major goal of this chapter was to access interactions between p53 and tau. A focus was placed on the oligomeric forms because of previous evidence of toxic interactions and cross-seeding, which can contribute to the spread and severity of AD pathology [12,330]. Since tauO have already been documented to cross-seed and form toxic interactions with other proteins [119,139], we chose to begin by assessing an interaction in human AD tissue and then moved to exogenous treatment of oligomers and its effects on endogenous proteins. Primary neurons were chosen because many immortal cell lines contain mutated or dysfunctional forms of p53. Also, neurons behave and are structurally different from other cell-types. In fact, there is a lack of studies investigating treatment with exogenous p53 aggregates in neurons. Therefore, the data presented here, represent some of the first experiments investigating p53 aggregates in normal, primary

neurons. Overall, our evidence suggests that tauO are indeed interacting with p53 in human AD as well as primary neurons exogenously treated with tauO. This demonstrates that p53 function may be disrupted by tauO and warrants further investigation.

It has been reported that p53 is associated with microtubules in normal, healthy cells. Giannakakou et al demonstrated that disruption of the microtubule network affected p53 nuclear accumulation [304]. This was particularly interesting given that our previous evidence supported p53 mislocalization. Destabilization of the microtubule network in AD is attributed to disruption of normal tau function. This normal p53-microtubule association involving transport and localization of p53 helps us understand why our control tissue saw some defined areas of colocalization in the IF and PLA. It also increases the chances of this normal interaction being corrupted through pathological tau as the two proteins are normally in close proximity. Pathological tau may increase the stress in the cell, which could cause more p53 to move to the nucleus, setting up a perfect opportunity for tauO to interact, seed, or otherwise disrupt p53.

Overall, p53 was found to interact with tau and tauO as evidenced by the formation of large peri-nuclear colocalization in human AD brain tissue. Since tauO heavily colocalized with p53 and not in the control tissue, we postulate that a large proportion of the Total tau we saw in Figure 6 colocalizing with p53 was indeed tau oligomers. This also would make sense considering these AD brain tissues are late Braak stage when tauO concentrations would be high. It would also allow us to suggest that the colocalization between p53 and tau seen in the control tissues was largely WT tau and gives more credibility to the idea of a normal association-like p53 transport along the microtubules.

It is possible that tauO are sequestering p53 outside the nucleus as neurons that showed colocalization between p53 and tauO were found to have little to no p53 in the nucleus by orthogonal view. Interestingly, even neurons in AD with diffuse p53 signal overlapping with the

nucleus showed evidence of tau inside the nucleus with even less found in neurons with large p53-tau colocalization. Numerous publications have noted the presence of nuclear tau and many have shown a link between nuclear tau and DNA damage [313,315]. Since p53 also responds to DNA damage, it would place both inside the nucleus at the same time. The question remains however, if this is normal tau or pathological tau, which has been shown to have very different outcomes for the DNA with pathological tau causing heterochromatin relaxation and WT nuclear tau protecting the DNA [313,315].

The tauO in the nucleus of AD brain tissue could be causing unknown pathology. It is also interesting to note that the exogenously added tauO appeared to localize to the nucleus. Since p53O also localized to the nucleus, it is possible that oligomers in general are drawn to the nucleus. This could be in relation to a recent publication by Eftekharzadeh et al that showed pathological tau can disrupt the nuclear pore [305]. Other reports have shown that tau can be found in the nucleus in response to DNA damage [313,315]. The phosphorylated p53 we saw in figure 3 suggests the presence of DNA damage in AD. Tau oligomers could be disrupting the DNA and thereby disrupting transcription and RNA processing.

Tau oligomer cross-seeding of p53 is plausible as evidenced by our previous finding of p53 oligomers in the same peri-nuclear localization as the p53 and tau interactions in the human AD tissue. This would suggest that tau is involved in the oligomerization of p53, which was also supported by our primary neuron treatments with tauO. This may lead us to conclude that tauO cross-seed p53 and not the reverse, which would indicate that tau pathology comes first and then p53 disruption. Considering our primary neuron treatments with tau were only 1 hr, it is unlikely that cross-seeding would have taken place. However, the fact that tauO colocalized with p53 in just 1 hr would suggest that this is a relatively fast interaction, suggesting it would not take long for tauO to disrupt p53 function.

It also could be due to microtubule breakdown, which may be preventing p53 nuclear import, thereby causing p53 to gather outside the nucleus, which may promote p53 aggregation or tauO cross-seeding since other tauO are detected nearby. A large subset of the total tau we saw before may be tauO in AD. Since no T22 is detected in controls, we may conclude that the colocalization between p53 and total tau in controls in figure 6 and 7 are likely WT tau.

Interestingly, two different AD patients show colocalization with p53 and tauO, but with different sized colocalizations. One AD patient demonstrated very large colocalizations in comparison to another AD patient, which showed smaller colocalization that appear less per-nuclear and more converging with the nucleus. This may represent different tauO strains, which has been previously suggested in the literature by our lab and others [139,331-333]. Different strains of tauO can cross-seed different proteins , which could be the case here, whereby they are producing different sizes or conformations of aggregates with p53.

From primary neuron studies, we can draw many interesting conclusions. First, concerning internalization, both tauO and p53O were able to be internalized by C57Bl/6 primary neurons within 1 hour. Considering this is very fast, it may suggest a specific type of internalization mechanism. Indeed, previous reports have also shown internalization by both p53O and tauO. Forget et al showed internalization by at least 15 hours in NIH3T3 and HEK cells. The authors of this publication also suggested the mechanism of uptake was through micropinocytosis, a nonspecific pathway of entry [264]. It is also interesting that both appeared to localize towards the nucleus. A longer time point may help ascertain the final location of the oligomers and if they are able to be cleared by the cells. It is possible that in the human brain, astrocytes or microglia may be able to reduce some extracellular oligomers, but if oligomerization begins within neurons, it may not be possible for the nonparenchymal cells to stop initial oligomerization.

Furthermore, although a 1 hr time point is likely too early to access seeding or cross-seeding, evidence that both p53O and tauO colocalize with endogenous p53, suggest there is a real possibility, considering they were able to colocalize and interact in such a short timeframe. This may also suggest that in AD pathology, once tauO form in the cell, it would not take long to affect p53 and cause dysfunction. The low concentration of tauO used here also indicates it may be detrimental to p53 even at miniscule concentrations. Longer time points would very likely aide in accessing seeding/cross-seeding, if tauO can sequester endogenous p53, and determine if p53O may take longer to affect endogenous WT tau. Co-treatment with both p53O and tauO may also provide clues.

Overall, we present evidence that p53 can form oligomers, mislocalize, and form interactions with tau and pathological tauO, indicating likely disfunction. A proposed mechanism for how p53 contributes to AD pathology (**Figure 14**): early or later in AD disease pathology, the cell becomes stressed leading to activation of p53 to address DNA damage and oxidative stress. However, due to breakdown of the microtubule network and tau oligomer pathology, p53 transport cannot enter the nucleus and accumulates outside the nucleus. Over time p53 may become unstable and start to aggregate. tauO near the nucleus interact with p53 and further sequester it outside the nucleus and may also cross-seed p53 to form p53O. WT p53 that cannot enter the nucleus will result in loss of function, causing further dysfunction in critical cell function such as cell cycle arrest, DNA damage repair, and apoptosis. With no repair, nor a way to perform controlled cell death, conditions in the cell will continue to deteriorate, promoting additional aggregation of intrinsically disordered proteins.

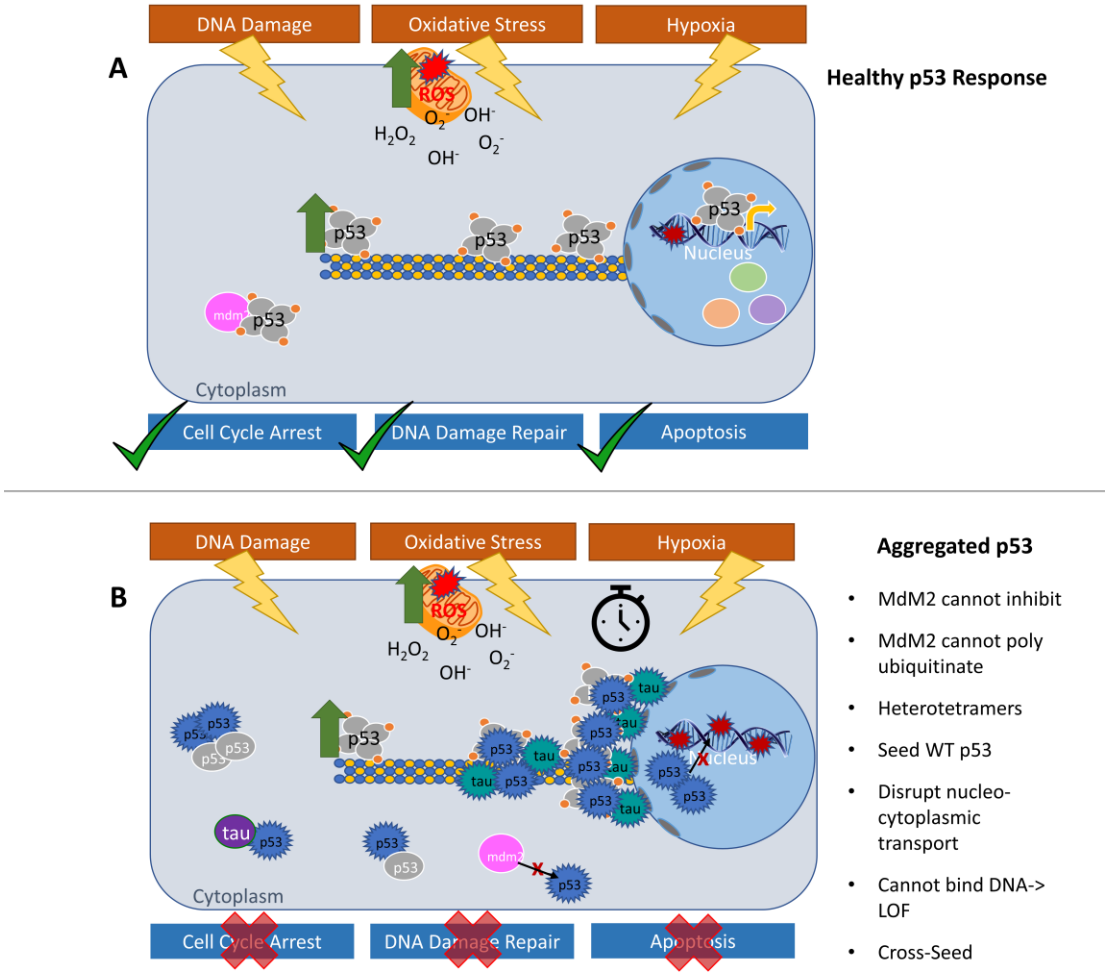

Figure 14: Proposed Mechanism of p53 in AD Disease Pathology

(A) Normal functional p53 response where cell stress occurs, p53 forms a tetramer, and induces transcription of target genes to ameliorate damage. **(B)** In AD disease pathology, the cell becomes stressed and activates p53 to address DNA damage and oxidative stress. However, due to breakdown of the microtubule network and tau oligomer pathology, p53 transport cannot enter the nucleus and accumulates outside the nucleus. Over time p53 may become unstable and start to aggregate. tauO near the nucleus interact with p53, causing sequestration and cross-seeding. P53 that cannot enter the nucleus will result in loss of function, causing dysfunction in critical cell function such as cell cycle arrest, DNA damage repair, and apoptosis. With no repair nor a way to perform controlled cell death, conditions in the cell will continue to deteriorate, promoting aggregation of intrinsically disordered proteins.

Chapter 5: Conclusions and Future Directions

Conclusions:

Alzheimer's disease is a devastating neurodegenerative disease that affects millions worldwide and remains without effective diagnostics and effective therapies despite millions of dollars in contributions to research and countless clinical trials. A large factor in this, is that the mechanism of disease pathology remains unsolved. While the breadth of studies aimed at understanding the biological mechanisms surrounding protein aggregation in AD and other neurodegenerative diseases is continuously growing, much is left to be understood.

The experiments in this study provide novel evidence of p53 oligomers in both human AD patient brain tissue as well as transgenic mouse models overexpressing the hallmark AD aggregate proteins tau and Aβ (tau overexpression in Htau and Aβ overexpression in Tg2576) in comparison to respective control tissues. Moreover, some evidence points to a mislocalization of p53 and activated p53, which could result in loss of function due to cytoplasmic sequestration-a mechanism of p53 loss of function (LOF) documented in mutant p53 associated with neuroblastomas [322]. Previous reports from mutant p53 aggregates and other aggregate-proteins would also suggest that some p53 oligomers may gain toxic function. Together, this has far-reaching effects as p53 is a critical stress sensor and stress mediator for the cell. As p53 controls many critical cell functions, affecting this transcription factor may set an irreversible course towards AD.

When p53 oligomers form in the pathogenesis of disease still needs to be addressed. Presently, we have investigated p53O in late Braak stage AD and aged transgenic mice wherein heavy aggregate pathology has already set in. If p53O spontaneously aggregate and/or are induced

earlier in the disease pathology still needs to be addressed through investigation of earlier ages in mice and earlier Braak stages.

Furthermore, we found that p53 interacts with tauO in *in vitro* and human AD brain tissue. Based on the peri-nuclear distribution and strong colocalization, we posit the p53O with peri-nuclear distribution may be due to sequestration or cross-seeding from tauO. Tau oligomers have been previously shown to cross-seed and cause increased toxicity from this interaction. This interaction and dysfunction of such a critical protein could have devastating effects for the cell- making it vulnerable to ongoing pathology that could spread. Our *in vivo* experiments indicate that tauO affect endogenous p53, within 1 hr, and not the reverse. Although, further experiments need to be performed to discern this cross-seeding ability, this evidence still provides clues as to when p53 oligomerization occurs and possibly may be a marker for worse disease outcomes. Tau-p53 cross-seeding could also play a role in different disease outcomes, or even the development of different tauopathies.

We also provide evidence to support a previous suggestion by Giannakakou et al that p53 localization in normal cells may be influenced by tau [304]. Our human control tissue shows a focal point of interaction between p53 and tau, which is to a much higher degree in AD tissue.

In conclusion, this research has contributed to the discovery of a potentially critical previously unreported protein aggregate in AD. All the proteins currently implicated in AD: tau, amyloid-β, and α-synuclein, do not demonstrate lethality when knocked out *in vitro* or *in vivo* [17,334,335]. However, if p53 is knocked out or disrupted, devastating events can occur for the cell. This makes p53, arguably, a very detrimental protein to be affected by any disease pathology. Therefore, aggregated p53 should be deemed a new, genuine contributor in AD pathogenesis- both on its own and as an interactor with other proteins that could cause a variety of severe consequences at the cellular level. How and when this protein becomes disrupted through aggregation and interactions

with tauO need to be further investigated as it may provide a valuable diagnostic tool and may provide novel cellular targets for future therapeutics.

Future Directions:

This research project has laid the foundation for many future experiments as this is the first time p53 oligomers have been found in AD. Typical questions arising from the discovery of an aggregate protein would indicate structural studies, biological, biochemical, and *in vivo* studies need to be performed. Cumulatively, this project has provided the initial insights into cell signaling and mechanisms of cell death, cell senescence, damage response, cellular stress, and cellular vulnerability in AD, but nonetheless, future experiments will provide a more complete understanding.

Until recently, p53 was the only DNA binding protein, other than TDP-43, found to aggregate. However, just this year, Schaser et al demonstrated that α-synuclein, a protein previously known to aggregate in AD, is also a DNA binding protein [323]. Thus, DNA binding proteins and their newly identified role in AD and neurodegeneration represent an enormous gap in knowledge unlike other well-known amyloids and RNA-binding proteins. It is possible that nuclear aggregates could form early and cause undue harm to the DNA or RNA in the nucleus and then spread to the cytoplasm. Therefore, a focus on DNA binding proteins could have broad implications for AD and beyond.

Experiments in the near future will aim to establish at what concentration p53O cause toxicity *in vitro*. Furthermore, experiments to determine toxicity of p53 aggregates in primary neurons as well as experiments to determine if interactions between p53 and tau cause increased toxicity will be performed. Western blot analysis, cell fractionation studies will also be performed to aid confocal evidence of p53O and mislocalization. Some downstream markers of p53 may also be investigated to determine if formation of p53O cause loss of transcription factor function. Future

studies may be conducted to determine if p53 oligomers alone can induce cognitive deficits in wild-type mice and exacerbate cognitive deficits in AD mouse models. These experiments will provide clear evidence of a new protein contributing to AD pathological mechanisms.

How p53 aggregates spread and the ramifications of gain in toxic function or loss of function would also need to be considered, especially considering the widespread and many critical functions p53 regulates. Since p53 has many different binding partners and PTM's, these all need to be considered. Previous work from the cancer field has shown that p53 aggregates lose DNA binding function. Furthermore, p53 has many inhibitors, such as MDM2, that may no longer be able to bind p53 if the binding sites are covered by a mis-conformation.

Other things to consider would be that p53 forms tetramer and a mis-conformation may cause tetramer instability similar to α-synuclein or transthyretin. Transthyretin tetramer disassembly was successfully ameliorated by the production of a chaperone that kinetically stabilizes transthyretin. The drug, Tafamidis, was successfully approved for treatment of cardiac transthyretin amyloidosis in the European Union and is used today. Concerning therapeutics, it is also important to note that there are numerous drugs available that selectively target p53 from the cancer field. Those same drugs could also be reapplied to treat AD and would take much less time to be used as a treatment, since the drugs are already FDA approved. However, our data so far would suggest that targeting tauO may be the best course of action if we find further evidence to believe that tauO causes p53 aggregation and not the reverse. Tau typically becomes involved in AD pathology after Aβ, but since we have evidence from Aβ overexpressing mice, it is possibly that p53 oligomers begin earlier, around the same time amyloid beta begins to aggregate. Future experiments to discern when exactly p53 begins to aggregate would need to be understood, before an effective time frame for therapeutics could be established.

Furthermore, considering that many aggregate-prone proteins appear to be more widespread in numerous other neurodegenerative diseases, p53 aggregates should also be investigated in other tauopathies and neurodegenerative disorders.

P53 aggregate interactions-may only interact with certain proteins, although due to its many binding partners and PTMs, p53 would likely bind with many things. P53 has already been shown to cross-seed its homologs p63 and p73 in cancer, but these proteins have also been found to have important functions in neurons and would need to also be assessed.

Other critical functions that are regulated by p53, such as DNA damage, oxidative stress, senescence, and apoptosis would also be affected and there is already substantial evidence to prove these downstream functions are already dysfunctional. However, many questions remain to be answered. High oxidative stress and excessive DNA damage is an early marker of MCI and AD, but how this is related to aggregation as cause or consequence has yet to be determined. Furthermore, despite a pro-apoptotic environment, neurons in AD are not consistently dying through programmed cell death such as apoptosis and instead are going through a long, progressive death that can last many years. P53 aggregation may play a role in this inability to induce apoptosis, forcing the sick cell to remain instead of going through controlled death. Furthermore, previous work has suggested neurons enter a senescent like-state where neurons are avoiding acute apoptosis and entering this stage that has numerous characteristics to AD. This research project has set the stage for many future avenues of study, now that p53 oligomers have been identified in AD.

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