# Intracellular Tau Modifications and Cell-based Sensors for Monitoring Tau Aggregation

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## 1 Introduction

Tau is a neuron-specific microtubule-binding protein that stabilizes microtubules (Kolarova *et al.*, 2012). When pathologically modified, tau dissociates from microtubules and becomes insoluble aggregates called neurofibrillary tangles (NFTs). NFTs are accumulated in neuronal perikarya or dystrophic neurites in axons and dendrites, causing degeneration of tangle-bearing neurons. The NFT formation is one of the most significant pathological signatures in Alzheimer's Disease (AD) and multiple neurodegenerative disorders classified as tauopathies. Accordingly, great efforts have been made to investigate the mechanism of tau aggregation and to identify the pathogenic tau species. Accumulating evidences suggest that soluble tau oligomers, rather than large insoluble aggregates, are the pathogenic forms responsible for neuronal degeneration and cognitive impairment. Formation of the fibrillary inclusions might serve as a neuronal defense mechanism to quarantine the toxic oligomers. In addition, tau oligomers propagate in neurons acting as a seed for native tau aggregation. Due to the pathological implication, tau oligomers become an important therapeutic target to cure tauopathies. However, progress has been slow due to the lack of understating tau aggregation mechanism. Tau is an intrinsically soluble protein. To become a neurotoxic aggregate, tau undergoes a number of harmful modifications. In this book chapter, we will look for

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various tau modifications associated with aggregation and also introduce cell models for monitoring tau aggregation in cells.

## 2 Tau and Neuro-degeneration

Tau protein is highly expressed in neurons, but also expressed in low levels in non-neuronal cells such as astrocytes and oligodendrocytes (Shin *et al.*, 1991). Tau stabilizes microtubule and promotes microtubule assembly that is critical for neuronal outgrowth (Cleveland *et al.*, 1977; Kolarova *et al.*, 2012; Mazanetz & Fischer, 2007; Obulesu *et al.*, 2011). Tau also has a role in anchoring microtubules to other cytoskeletal filaments and cytoplasmic organelles such as mitochondria for the structural supports (Jung *et al.*, 1993; Miyata *et al.*, 1986). Full-length human tau contains four tandem repeat seqeunces (R1–R4) containing a number of lysine residues (Figure 1). The postively charged lysine residues are critical for binding to microtubules, which are highly negatively charged (20 to 30 electrons per $\alpha\beta$-tubulin dimer) (Kolarova *et al.*, 2012). Thus, the repeat sequences are called as a microtubule-binding domain.

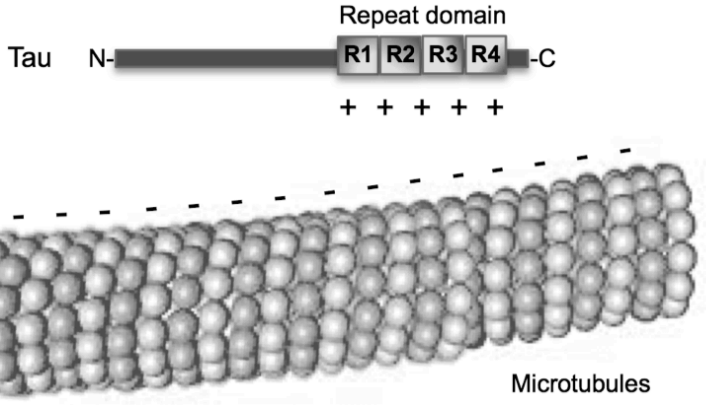


**Figure 1:** The schematic representation of tau and its repeat domain (R1–R4). The positively charged repeat domain binds to hightly negatively charged microtubules.

Microtubules are highly dynamic structures that continuously assemble and dissemble in cells. To maintain microtubule dynamics, tau's binding affinity to microtubules is tightly controlled by a number of kinases and phosphatases. By introducing one or two phosphates, tau controls its binding affinity to a microtubule (Brandt *et al.*, 2005; Kenessey & Yen, 1993) (Figure 2). However, when tau is abnormally hyperphosphorylated, tau loses its binding affinity to a microtubule and become aggregated into NFTs (LaPointe *et al.*, 2009; Mandelkow *et al.*, 2003).

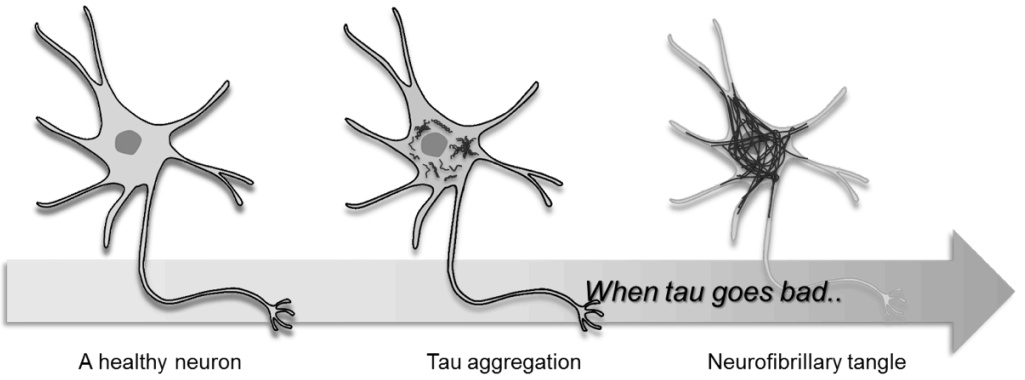


**Figure 2:** Tau physiological conditions. In healthy neuron, tau is associated with microtubules, promotes axonal outgrowth and synaptic vesicle transportation. In diseased neuron, tau becomes dissociated from microtubules, loses its function and form neurotoxic oligomers to aggregates.

Accumulation of tau aggregates is one of the most significant pathological events in tauopathies including AD. Hence, there has been great effort to identify the pathogenic tau aggregates that cause neurodegeneration. Tau induces neuro-degeneration in various mechanisms. Firstly, neuronal degeneration is initiated with the hyper-phosphorylated tau. The hyper-phosphorylated tau destabilizes microtubules and the disruption of microtubule directly induces neuronal dysfunctions (Reddy, 2011). Second, neuro-degeneration is mediated by diverse tau aggregates. Accumulation of NFTs in neuron might be toxic by acting as physical barriers in the cytoplasm. However, it is become apparent that soluble tau oligomers, rather than the large filamentous aggregates, are the pathogenic forms that initiate and also propagate tau pathogenesis in a brain (Kopeikina *et al.*, 2012; Lasagna-Reeves *et al.*, 2012). In addition, intracellular accumulation of phosphorylated tau might be a burden to the endoplasmic reticulum (ER), leading to induce ER-stress or unfolded protein response (Grune *et al.*, 2004).

## 3 Post-translational Modifications of Tau

Contradictory to the pathological aggregation, tau is a naturally ''unfolded'' protein, which is highly soluble in physiological conditions. To become a susceptible substrate for paired helical filaments (PHFs), tau protein undergoes a series of abnormal modifications and conformational changes (Garcia-Sierra *et al.*, 2008). Phosphorylation is the most studied tau modifications as numerous studies have suggested that tau aggregation is initiated by tau phosphorylation (Kim *et al.*, 2012). Tau hyper-phosphorylation might be important for initiating tau pathology by detaching tau from microtubules, however, hyper-phosphorylated tau does not aggregate spontaneously in cells. Diverse post-translational modifications are required to facilitate tau aggregation (Liu *et al.*,

2002; Reynolds *et al.*, 2007; Walker *et al.*, 2012). Recent evidences show that intermolecular disulfide cross-linking is critical in generating tau oligomers that serve as a building block for higher-order aggregates (Haque *et al.*, 2014). Also, a proteolytic cleavage is known to facilitate filamentous tau aggregation by removing the fluctuating N-and C-terminal ends (von Bergen *et al.*, 2006).

Cells also activate diverse defense mechanisms to prevent or reduce tau aggregates. In healthy neurons, tau phosphorylation residues are pre-sealed with glycosylation, thus the phosphorylation sites are protected from kinase attack. Also, hyperphosphorylated tau and its aggregates are degraded by ubiquitin-proteasome pathway or autophagosis (Sergeant *et al.*, 2008; Wang *et al.*, 2009). Here we will review diverse tau modification that promote or prevent tau aggregation (Figure 3).

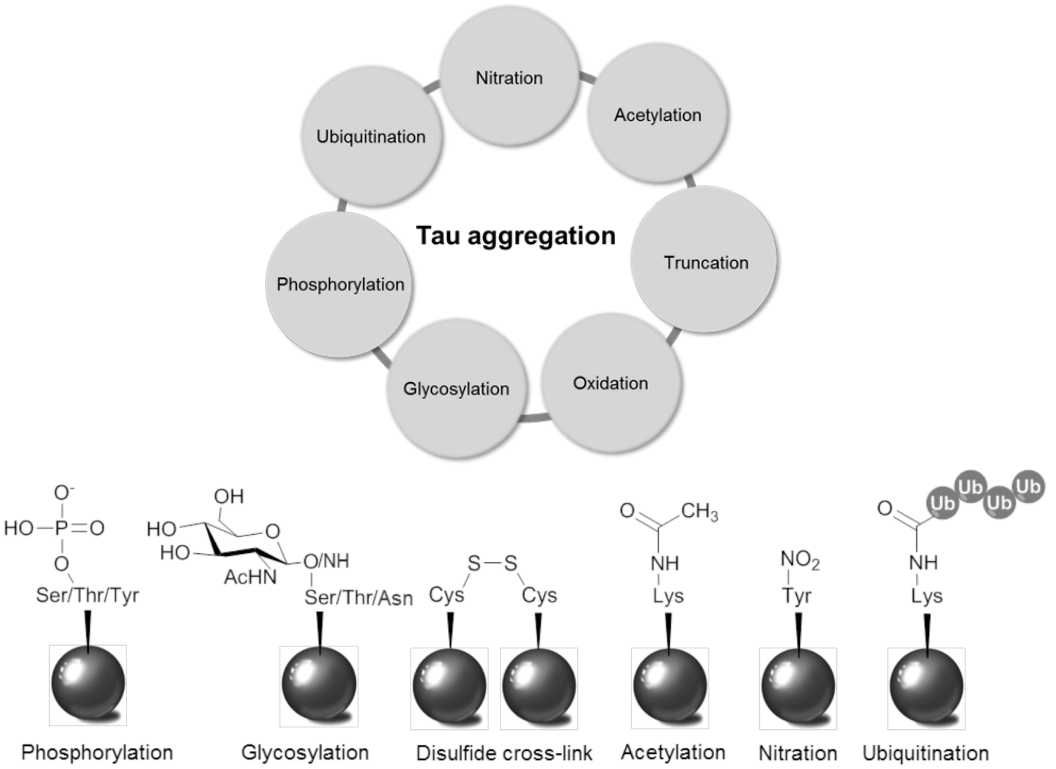


**Figure 3:** Schematic diagram of tau post-translational modifications.

## 3.1 Phosphorylation

Full-length human tau contains total 85 putative phosphorylation sites; 45 serine (ser), 35 threonine (thr), and 5 tyrosine (tyr) residues. Among the 85 residues, so far 67 residues were experimentally identified as phosphorylation sites of tau; 38 ser, 26 thr, and 3 tyr (Table 1). In neurons, tau phosphorylation is tightly regulated by multiple protein

kinases and phosphatases. When the regulation is imbalanced, tau becomes hyper-phosphorylated. Evidences have showed that tau protein isolated from a healthy brain is also partially phosphorylated with an average of about 2 moles of phosphate per a mole of protein (Drechsel *et al.*, 1992; Mazanetz & Fischer, 2007). In contrast, tau isolated from the AD patient's brain contains 6 to 8 moles of phosphate per mole of protein (Mazanetz & Fischer, 2007). Hyper-phosphorylated tau dissociates from microtubules and initiates tau pathology.

Tau phosphorylation is mediated by kinases or phosphatases (Buee *et al.*, 2000; Trojanowski & Lee, 1995). Among many kinases, proline-directed kinases such as GSK-3β (glycogen synthase kinase 3), CDK5 (cyclin-dependent kinase 5) have received the most attention due to the selectivity to tau (Dhavan & Tsai, 2001; Perry *et al.*, 1999; Shelton & Johnson, 2004; Spittaels *et al.*, 2000). GSK-3β is highly expressed in brains associated with tauopathies (Bhat *et al.*, 2004) and also the elevated activity of CDK5 was observed in AD brain tissues (Augustinack *et al.*, 2002; Tseng *et al.*, 2002). This suggests that GSK-3β and CDK5 involves in the early stage of NFT formation during AD progression. Inhibition of GSK3β is known to decrease tau phosphorylation and NFT formation (Hong *et al.*, 1997; Munoz-Montano *et al.*, 1997).

Non-proline directed kinases such as PKA (protein kinase A), CK1 (casein kinase) also regulates tau phosphorylation. It is also known that CK1 is highly expressed in AD patient's brain (Li *et al.*, 2004). It suggests that CK1 might play an important role in tau phosphorylation in AD (Singh *et al.*, 1995). Besides serine and threonine residues, tau contains five tyrosine residues. Among the five-tyrosine residues, three residues are known to be phosphorylated. A Src-family kinase, Fyn, is the mostly studied tyrosine kinase that phosphorylates tyrosine-18 (Y18) of tau (Lee *et al.*, 2004). Y18-phosphorylation was also found in NFTs isolated from AD brains and also AD transgenic mice (Bhaskar *et al.*, 2010).

Among many phosphatases, protein phosphatase 2A (PP2A) is the major tau phosphatase in the AD pathology. Inhibition of PP2A induces tau hyper-phosphorylation and disrupts neuronal cytoskeleton and neuritic outgrowth (Chen *et al.*, 2008; Saito *et al.*, 1995). The decrease of PP2A also increases GSK3β level that induces tau phosphorylation (Tian *et al.*, 2004; Wang *et al.*, 2010). In addition, protein phosphatases 1 (PP1) and protein phosphatases 2B (PP2B) are also related in AD process (Chung, 2009; Martin *et al.*, 2013).

## 3.2 Glycosylation

Glycosylation is a key post-translational modification and mediates covalent attachment of oligosaccharides to the protein backbone. There are two types of glycosylation involved in tau pathology. N-glycosylation adds sugars to asparagine (Asn) residues and O-glycosylation adds sugars to serine or threonine residues (Arnold *et al.*, 1996; Wang *et al.*, 1996) (Figure 4).

Recent evidences have suggested the role of O-glycosylation in preventing tau aggregation. O-glycosylation prevents tau phosphorylation by the pre-acquisition of serine and threonine residues. Actually in AD, a negative correlation between O-

| Domain | Amino acid position | Glycosylation | Phosphorylation kinase | | | | | | | | | | | | Phosphatase | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Non-proline directed | | | | | Proline directed | | | | Tyrosine | | | PP1 | PP2A | PP2B |
| | | | CK1δ | CK2 | PKA | PKC | CaMPK II | GSK-3β | CDK2 | CDK5 | MAPK | Fyn | Syk | c-Abl | | | |
| Acidic domain | T17 | | ● | ● | | | | | | | | | | | | | |
| | Y18 | | | | | | | | | | | ● | ● | | | | |
| | T39 | | | ● | | | | | | | | | | | | | |
| | S46 | | ● | | | | | ● | ● | | ● | | | | | ● | ● |
| | T50 | | ● | | | | | ● | | | ● | | | | | | |
| | T52 | | | ● | | | | | | | | | | | | | |
| | S56 | | | ● | | | | | | | | | | | | | |
| | T69 | | | | | | | ● | | | ● | | | | | | |
| | T95 | | ● | | | | | | | | | | | | | | |
| | T101 | | ● | | | | | | | | | | | | | | |
| | T102 | | ● | | | | | | | | | | | | | | |
| | S113 | | ● | | | | | | | | | | | | | | |
| | T123 | | | | | ● | | | | | | | | | | | |
| Proline-rich domain | S129 | | | | | | | | | | | | | | | | ● |
| | S131 | | ● | | | | ● | | | | | | | | | | |
| | T149 | | ● | | | | | ● | | | | | | | | | |
| | T153 | | | | | | | ● | | ● | ● | | | | | | |
| | T169 | | ● | | | | | | | | | | | | | | |
| | T175 | | | | | | | ● | | | ● | | | | | | |
| | T181 | ● | | | | | | ● | ● | ● | ● | | | | | | ● |
| | S184 | | ● | | | | | ● | | | | | | | | | |
| | S195 | | | | | | | ● | ● | ● | ● | | | | | | |
| | Y197 | | | | | | | | | | | | ● | | | | |
| | S198 | | ● | ● | ● | | | ● | | ● | ● | | | | | | |
| | S199 | ● | | | ● | | | ● | ● | ● | ● | | | | ● | ● | ● |
| | S202 | ● | | | ● | | | ● | ● | ● | ● | | | | ● | ● | ● |
| | T205 | ● | | | | | | ● | ● | ● | ● | | | | | ● | |
| | S208 | | ● | | | | | | | | | | | | | | |
| | S210 | | ● | | ● | | | ● | | | | | | | | | |
| | T212 | ● | ● | | ● | | | ● | ● | ● | ● | | | | | | ● |
| | S214 | ● | ● | | ● | | ● | ● | | ● | | | | | | | ● |
| | T217 | ● | | | ● | | | ● | | ● | ● | | | | | | |
| | T220 | | | | ● | | | ● | | | | | | | | | |
| | T231 | | | | ● | | | ● | ● | ● | ● | | | | ● | | ● |
| | S235 | | | | ● | | | ● | ● | ● | ● | | | | | | ● |
| | S237 | | ● | | | | | ● | | | | | | | | | |
| | S238 | | ● | | | | | | | | | | | | | | |

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| Domain | Amino acid position | Glycosylation | Phosphorylation kinase | | | | | | | | | | | | Phosphatase | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Non-proline directed | | | | | Proline directed | | | | Tyrosine | | | PP1 | PP2A | PP2B |
| | | | CK1δ | CK2 | PKA | PKC | CaMPK II | GSK-3β | CDK2 | CDK5 | MAPK | Fyn | Syk | c-Abl | | | |
| Microtubule-binding domain | S241 | | • | | | | | • | | | | | | | | | |
| | T245 | | | | • | | | • | | | | | | | | | |
| | S258 | | • | | • | • | | • | | | | | | | | | |
| | S262 | • | • | | • | • | • | • | | | • | | | | | | |
| | T263 | | • | | | | | | | | | | | | | | |
| | S285 | | • | | | | • | • | | | | | | | | | |
| | S289 | | • | | | | | • | | | | | | | | | |
| | S293 | | | | • | • | | | | | | | | | | | |
| | S305 | | • | | • | • | | • | | | | | | | | | |
| | S320 | | | | • | • | | | | | | | | | | | |
| | S324 | | | | • | • | | • | | | | | | | | | |
| | S341 | | • | | | | | | | | | | | | | | |
| C-terminal domain | S352 | | • | | • | | | • | | | | | | | | | |
| | S356 | • | • | | • | | • | • | | | • | | | | | | |
| | T361 | | • | | | | | | | | | | | | | | |
| | T373 | | • | | | | | • | | • | | | | | | | |
| | T386 | | • | • | | | | | | | | | | | | | |
| | Y394 | | | | | | | | | | | | | • | | | |
| | S396 | | • | • | | • | | • | • | • | • | | | | | • | • |
| | S400 | | | • | | | | • | | | | | | | | | • |
| | T403 | | | | | | | • | | | | | | | | | |
| | S404 | • | • | • | | • | | • | • | • | • | | | | • | • | |
| | S409 | | | | • | | • | • | | | | | | | | | • |
| | S412 | | • | • | • | | | • | | | | | | | | | |
| | S413 | | • | • | • | | | • | | | | | | | | | |
| | T414 | | • | • | • | | | • | | | | | | | | | |
| | S416 | | • | • | • | | • | • | | | | | | | | | |
| | S422 | • | | | | | • | | | | • | | | | | | |
| | S433 | | • | | | | | | | | | | | | | | |
| | S435 | | • | | • | | | | | | | | | | | | |

**Table 1:** Single letter amino acid abbreviations indicate the sites of important phosphorylation kinase, phosphatase and glycosylation residues in tau (S, serine; T, threonine; Y, tyrosine). Numbering is based on the sequence of the largest isoform of human central nervous system tau (441 amino acids long). (Avila, 2006; Gong *et al.*, 2005; Hanger *et al.*, 2009; Morishima-Kawashima *et al.*, 1995; Sergeant *et al.*, 2008).

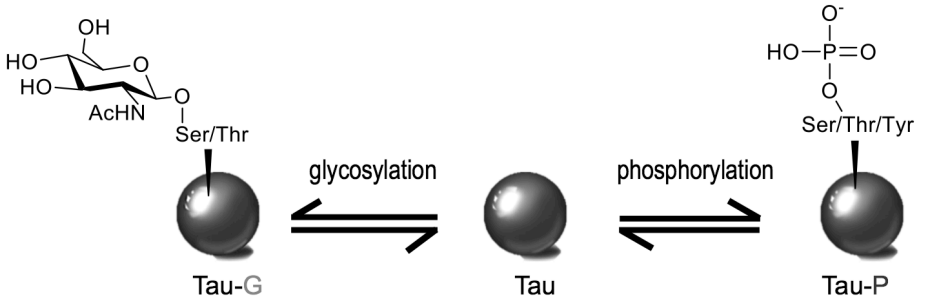



**Figure 4:** O-glycosylation prevents tau phosphorylation.

Glycosylation and tau phosphorylation has been reported (Lefebvre *et al.*, 2003; Liu *et al.*, 2009; Robertson *et al.*, 2004). It is worth mentioning that impaired glucose metabolism in AD brain results in the reduction of O-Glycosylation, leading to tau hyper-phosphorylation (Deng *et al.*, 2009; Gong *et al.*, 2006; Liu *et al.*, 2009). Therefore, O-glycosylation may protect tau from hyperphosphorylation and consequently is expected to prevent NFT formation. Compared to O-glycosylation, the role of N-glycosylation is not clear in tau pathology. However, N-glycosylation was found in PHFs isolated AD brain (Liu *et al.*, 2002), and this suggests that N-glycosylation might be associated with tau pathology (Selkoe, 2004; Suzuki *et al.*, 2006).

## 3.3 Disulfide Bond Cross-linking

Full-length human tau (441 a.a.) contains two cysteine residues that can form both intra- and inter-molecular disulfide bonds. When tau is exposed to oxidizing conditions such as $H_2O_2$, a series of inter- and intramolecular disulfide bonds are formed (Zambrano *et al.*, 2004). Intramolecular disulfide bonds lead to the formation of compact monomers that cannot form extended structure. However, intermolecular disulfide-bonds lead to form higher-order oligomers. The intermolecular cross-linked oligomers serve as ''nuclei'' for further tau aggregation (Walker *et al.*, 2012) (Figure 5).

Oxidative stress is one of the key factors contributing to neuro-degeneration (Uttara *et al.*, 2009). It is highly possible that the disruption of cellular redox potential induces tau oxidetion to form intermolecular disulfide cross-links. Although the precise role that disulfide cross-linked tau play in vivo is not clear, the cross-linked tau oligomers promote tau aggregation in vitro (Zhao & Zhao, 2013). Recent studies reported that the soluble tau oligomers are presumed to be neuro-toxic than large insoluble aggregates and propagate into other brain regions and induce aggregation of normal tau proteins. Therefore, the generation of disulfide cross-linked tau oligomer might be the most critical event in the initiation and progression of neuro-degeneration (Walker *et al.*, 2012).

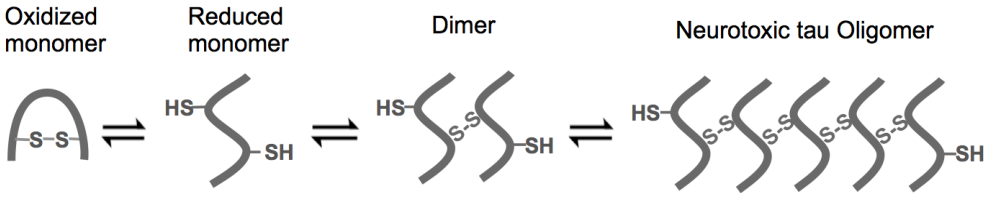



**Figure 5:** Tau disulfide cross-links generates tau oligomers (Haque *et al.*, 2014).

## 3.4 Proteolytic Cleavage (Truncation)

A number of studies have shown that tau is a substrate for various proteases and proteolysis affects tau toxicity and aggregation. Evidence exists that tau proteins isolated from AD patient's brain contain the mixture of C-truncated and N-truncated tau species (Novak *et al.*, 1993). The N-terminal of tau is highly sensitive to proteolysis that quickly digested to small peptides. In contrast, the C-terminal cleavage sites are near the microtubule-binding domain, thus the cleavage is semi-protected when tau is binding to a microtubule. When tau protein detaches from a microtubule, C-terminus is exposed and digested by proteases. Evidence also suggest that the proteolytic cleavages facilitate tau polymerization (Garcia-Sierra *et al.*, 2008). N- and C- terminal ends of tau might be too soluble to form compact aggregates (von Bergen *et al.*, 2006). By removing the flickering N- and C-terminal ends, tau could be aggregated into compact filaments.

Abnormal proteolytic cleavages occur as part of the aging process and cell death in many neurodegenerative disorders. A major participant is a family of serine-aspartyl proteases called caspases, which are activated during apoptosis (Dickson, 2004). It is known that caspases are activated and also over expressed in AD brains (Chung *et al.*, 2001; Horowitz *et al.*, 2004). Researchers have found tau truncation in AD patients' brains, especially at aspartic acids (D13 and D421), and glutamic acid (E391).

This truncation contributes to neuronal apoptosis and tau polymerization (Fasulo *et al.*, 2000; Garcia-Sierra *et al.*, 2008; Horowitz *et al.*, 2004; Martin *et al.*, 2011). In addition, tau truncation might recruit other modifications such as glycation (Ledesma *et al.*, 1994), ubiquitination (Morishimakawashima *et al.*, 1993), accumulation of hyperphosphorylated tau in tangles (de Calignon *et al.*, 2010), alterations in the organizations and functions of some membrane organelles like mitochondria (Quintanilla *et al.*, 2009) and endoplasmic reticulum (Matthews-Roberson *et al.*, 2008).

## 3.5 Acetylation

Acetylation is the introduction of an acetyl group to a lysine residue of a protein. Recently, acetylation has been highlighted as a potentially harmful modification of tau facilitating aggregation. The microtubule-binding domain of tau contains 45 lysine residues, of which positive charges are critical for microtubule binding. Acetylation removes the positive charge on tau, and accordingly acetylated tau lose its binding affinity to microtubule (Cohen *et al.*, 2011; Irwin *et al.*, 2012). Mass spectrometry analysis

identified that lysine residues (Lys280, Lys281, Lys369) in the microtubule-binding motif as the major sites for acetylation (Cohen *et al.*, 2011). Researchers also showed that the increase acetylation on Lys280 in AD patient's brain, and the acetylation on Lys280 is known to facilitate tau tangle formation (Irwin *et al.*, 2012). Acetylation impairs tau's binding affinity to microtubules, prevents tau degradation by blocking ubiquitinylation, and promotes tau aggregation (Cohen *et al.*, 2011; Min *et al.*, 2010; Wang *et al.*, 2013). Therefore, acetylation is critical for leading to tau pathology (Kolarova *et al.*, 2012; Min *et al.*, 2010).

## 3.6 Nitration

Oxidative/nitrative injury has been implicated in the pathogenesis of tauopathies (Horiguchi *et al.*, 2003). Reactive oxygen and nitrogen species produce nitrating agents that nitrates tyrosine residues of tau (Figure 3). Actually, nitration on tyrosine-29 was found in tau isolated from AD patient brain (Reynolds *et al.*, 2007; Reynolds *et al.*, 2006). Nitration could promote a conformation change in tau that may promote fibril assembly. In addition, nitration might induce o-o' dityrosine cross-linking that stabilizes tau polymers at several stages of filament maturation. Although the exact mechanism is still in debate, tau nitration is known to promote tau aggregation, and tau nitration might provide a links between the oxidative/nitrative damages and tau pathology.

## 3.7 Ubiquitination

Ubiquitin-proteasome system (UPS) has implicated in removal of mis-folded proteins in a number of neurodegenerative diseases (Korhonen & Lindholm, 2004). Recent studies also suggested that UPS is responsible for degrading abnormally modified tau protein in neurons. Several lysine residues (K254, K311, and K353) located in the microtubule-binding region have been identified as ubiquitinylation sites of tau (Cripps *et al.*, 2006; de Vrij *et al.*, 2004; Morishimakawashima *et al.*, 1993). The ubiquitinylation sites imply that when tau is binding to a microtubule, tau ubiquitinylation is prohibited. When tau is released from a microtubule via hyper-phosphorylation, the lysine residues are exposed and become ubiquitinylated. Ubiquitinylated tau protein is degraded by proteasome complex. Recent evidences have shown that the proteasome activity is actually down-regulated or inhibited in AD patients (de Vrij *et al.*, 2004; Keck *et al.*, 2003). The down-regulation of UPS system results in the accumulation of mis-folded tau in neurons.

In addition to UPS, autophagosis has been highlighted as an important pathway that participate in the degradation of tau aggregates. Autophagy is a catabolism-based mechanism that degrades unnecessary and dysfunctional components in cells. Recent evidence has shown that mutant tau and tau aggregates are degraded by autophagosis in N2a cell model (Wang *et al.*, 2009). It seems that large tau aggregates are degraded by autophagosis rather than proteasome complex (Feuillette *et al.*, 2005; Kruger *et al.*, 2012).

# 4 Cell-based Models to Investigate Intracellular Tau Interactions & Aggregation

To investigate tau aggregation processes in cells, diverse cell-models expressing tau have been developed. For the live cell observation, various fluorescence proteins (GFP, CFP and YFP) conjugated to tau were expressed in cells (Lu & Kosik, 2001; Nonaka *et al.*, 2010). The exogenously expressed tau showed aggregated phenotypes upon the diverse stimulation inducing tauopathies. However, in the fluorescence protein conjugated systems, majority of tau molecules exist as monomers presenting strong fluorescence signals in cytosol. Thus it was difficult to distinguish oligomeric tau aggregates from monomeric tau in the early stage of aggregation. It is becoming apparent that oligomeric tau species play a critical role in the initiation and progression of tau pathology. Therefore, a cell-based sensor, which is able to discriminate pathological tau aggregates in cells, would be beneficial to investigate tau pathogenesis. Here we will introduce advanced cell-based sensors that could monitor and quantify tau assembly in living cells by using fluorescence resonance energy transfer (FRET) or fluorescence complementation techniques (split-GFP and BiFC) (Figure 6).

## 4.1 FRET-based Sensor to Investigate Tau-tau Interaction

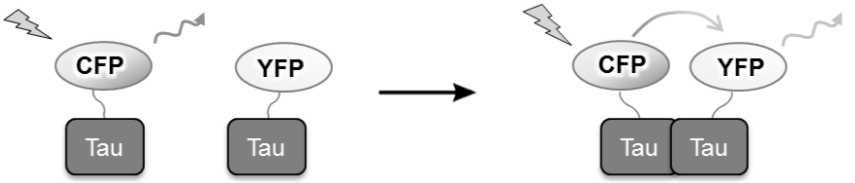
Fluorescence resonance energy transfer (FRET) is one of the most common techniques used for studying protein-protein interactions. FRET technique is based on energy transfer from a donor fluorophore to an acceptor fluorophore in close proximity. This FRET technique has been introduced to investigate tau-tau interactions in living cells by Johnson's group (Chun & Johnson, 2007). In their study, full-length tau was conjugated with a donor fluorophore (CFP) and caspase-cleaved tau was conjugated to an acceptor fluorophore (YFP), and then co-expressed in HEK293 cells (Chun & Johnson, 2007) (Figure 6a). Energy transfer between CFP and YFP occurs only when those two tau isoforms are close enough (typically 2–6 nm). FRET microscopy showed that the two different tau isoforms bind to each other when tau phosphorylation is stimulated by GSK-3β. This FRET-based approach enables to quantify tau-tau association in cells.

However, FRET technique has several limitations for studying protein-protein interaction. First, the lifetime of donor fluorophores is only in the nanosecond range so that time is too short to measure FRET. Second, background interference arising from the fluorescence of donor can result in a poor FRET signal-to-background ratio. Third, the use of quite huge fluorescence protein tagging might interfere the interaction between the proteins of interest. Therefore, there has been effort to overcome the limitations of FRET.

## 4.2 Fluorescence Complementation Assays

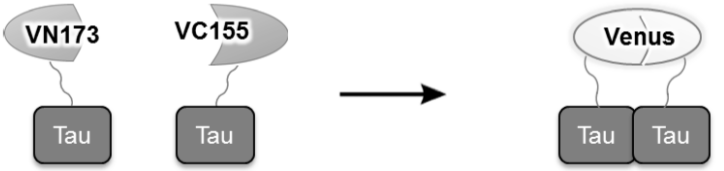
### 4.2.1 Split-GFP method to investigate tau aggregation

At first, split-GFP BiFC technique was developed to quantify tau aggregation in cells




**(a)** FRET-based sensor to investigate tau-tau interaction.


**(b)** Split-GFP-based sensor to investigate tau aggregation.




**(c)** Venus-based BiFC sensor to investigate tau-tau interaction.

**Figure 6:** Comparison of the detection of tau aggregation using fluorescence fragment. (a) FRET sensor. Tau fused to CFP or YFP. Upon tau aggregation, the FRET sensor is activated and fluorescent color is changed. (b) Split-GFP-based sensor. Tau is fused to the smaller fragment ($GFP_{11}$), and co-expressed in cells with larger GFP fragment ($GFP_{1–10}$). Active GFP is re-constituted if the two fragments re-associate; however, GFP fluorescence is decreased when tau becomes aggregate, because the tau-$GFP_{11}$ is less accessible to the larger $GFP_{1–10}$ fragment. (C) Venus-based BiFC. Tau is fused to VN173 and VC155. When tau becomes aggregate, VN173 and VC155 combine and turn on fluorescence.

(Chun *et al.*, 2007; 2011). In this assay, GFP is split into two non-fluorescent fragments; a small fragment containing the eleventh domain of GFP ($GFP_{11}$) and a large fragment containing the rest of GFP ($GFP_{1–10}$). Then, the small fragment ($GFP_{11}$) was fused to tau protein, and transiently expressed in cells with the large $GFP_{1–10}$ fragment (Figure 6b). When tau exists as a monomer, the large fragment is accessible to the small fragment, leading to the maturation of an active GFP complex. When tau becomes aggregated, the large $GFP_{1–10}$ fragment is not accessible to the small $GFP_{11}$ fragment, resulting in the de-

crease of GFP fluorescence intensity. Actually when tau aggregation was induced by the co-expression of GSK3β, the GFP fluorescence intensity decreased significantly suggesting the increased tau aggregation in cells.

As an indirect method to measure tau aggregation, the split-GFP technique provides the overall view of tau aggregation. Even though the split-GFP assay is an innovative method to quantifying the overall tau aggregation in living cells, the resolution of split-GFP technique is not sufficient enough to distinguish tau oligomers from monomers.

### 4.2.2 Venus-based BiFC Method to Investigate Tau-tau Interaction

The next generation of fluorescence complementation assay overcomes the limitation of the split-GFP method and enables to monitor tau-tau interaction from the early stage of the aggregation. As a bimolecular fluorescence complementation technique, venus fluorescence protein is split into two non-fluorescent N- and C-terminal fragments; VN173 and VC155. Then, both N- and C- terminal fragments were fused to tau protein and co-expressed in cells (Tak *et al.*, 2013). Different from the split-GFP method, the N- and C-fragments do not have any intrinsic binding affinity, thus there is little fluorescence signal when tau exists as a monomer. Only when tau proteins are aggregated, the N- and C-terminal fragments of Venus could be located closely enough to form an active venus protein complex. (Figure 6c).

In the study, okadaic acid and forskolin used to turn on the Venus fluorescence protein by inducing tau assembly. Okadaic acid and forskolin are small molecules commonly used to induce tau phosphorylation (Arias *et al.*, 1993; Liu *et al.*, 2004; Tian *et al.*, 2004; Zhang & Simpkins, 2010). Forskolin promotes tau phosphorylation by the activation of tau kinase, PKA and okadaic acid protects tau phosphorylation by inhibiting tau phosphatase, PP2A. Upon the treatment of okadaic acid and forskolin, venus fluorescence intensity increased dramatically in cells (Figure 7).

As fluorescence 'turn-on' approach, tau-BiFC approach enables to achieve spatial and temporal resolution of tau aggregation in living cells (Figure 8) (Tak *et al.*, 2013). When cells were treated with okadaic acid, venus fluorescence was observed as a concentrated linear structure. In contrast, when cells were treated with forskolin, venus fluorescence was amorphously distributed in cytoplasm (Figure 8a). Forskolin and okadaic acid are small molecules commonly used to induce tau phosphorylation, however, their contribution to tau aggrega tion has not yet been clearly identified. Even though the mechanism of tau aggregation is not clearly identified, the tau-BiFC method enables to visualize diverse forms of tau aggregates in cells. In addition the spatial resolution of tau aggregation, tau-BiFC methods provide temporal resolution of tau aggregation (Figure 8b). When a higher concentration of okadaic acid (80 nM) was treated to cells, venus fluorescence was turned on within 20 minutes and the fluorescence signals become concentrated as linear structures in cells.

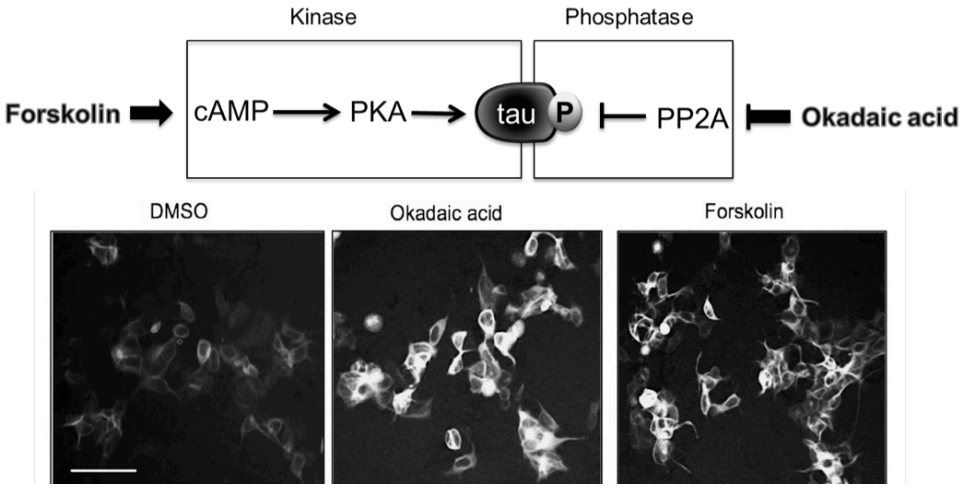
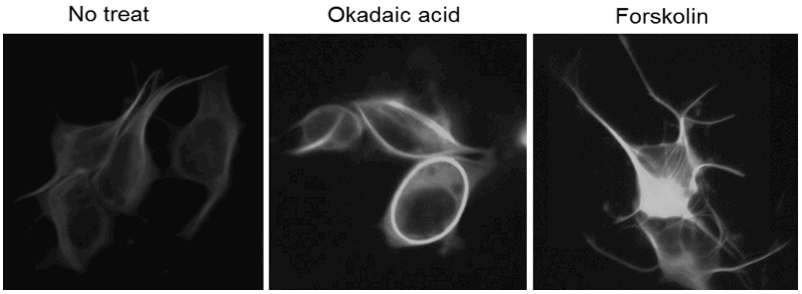
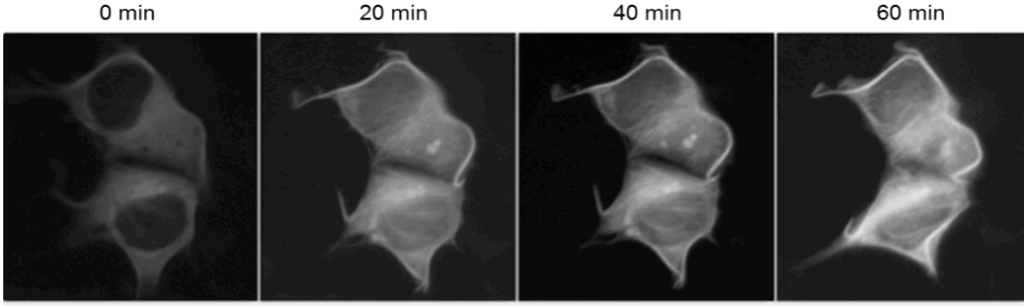



**Figure 7:** Maturation of tau-BiFC upon tau phosphorylation. Tau-BiFC cells were incubated with okadaic acid and forskolin to induce tau phosphorylation. Increase in tau-BiFC fluorescence indicates tau aggregation induced by hyperphosphorylation (Tak *et al.,* 2013).




**(a)** Spatial resolution of tau-tau interaction.




**(b)** Temporal resolution of tau-tau interaction.

**Figure 8:** Spatial and temporal resolution of tau-tau interaction in HEK293-tau-BiFC cell model (Tak *et al.,* 2013).

## 5 Conclusion

Tau is naturally a soluble protein that promotes microtubule assembly and stabilization. Diverse intracellular modifications make soluble tau to be a susceptible substrate for the soluble tau oligomers and insoluble filamentous aggregates. Due to the implications of tau pathology in many neuro-degenerative disorders, preventing the pathological tau aggregation become an important therapeutic strategy to halt the disease. However, tau aggregation is a multi-step process regulated by complicated cellular pathways. In tau pathology, diverse tau modifications including phosphorylation, oxidation, and truncation promote tau aggregation in a defected neuron. At the same time, the neuron activates cellular defense mechanisms such as glycosylation and ubiquitination. Here we reviewed diverse tau modifications that promote or inhibit tau aggregation, and also introduced cell-based sensors to investigate tau pathology.

## Acknowledgment

The authors would like to acknowledge the financial support from the R&D Convergence Program of NST (National Research Council of Science & Technology, CRC-15-04-KIST) of Republic of Korea, and the Ministry of Health & Welfare, Republic of Korea (HI14C3344).

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