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## Tau phosphorylation at Alzheimer's disease-related Ser356 contributes to tau stabilization when PAR-1/MARK activity is elevated

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### Abstract

Abnormal phosphorylation of the microtubule-associated protein tau is observed in many neurodegenerative diseases, including Alzheimer's disease (AD). AD-related phosphorylation of two tau residues, Ser262 and Ser356, by PAR-1/MARK stabilizes tau in the initial phase of mistreatment, leading to subsequent phosphorylation events, accumulation, and toxicity. However, the relative contribution of phosphorylation at each of these sites to tau stabilization has not yet been elucidated. In a *Drosophila* model of human tau toxicity, we found that tau was phosphorylated at Ser262, but not at Ser356, and that blocking Ser262 phosphorylation decreased total tau levels. By contrast, when PAR-1 was co-overexpressed with tau, tau was hyperphosphorylated at both Ser262 and Ser356. Under these conditions, the protein levels of tau were significantly elevated, and prevention of tau phosphorylation at both residues was necessary to completely suppress this elevation. These results suggest that tau phosphorylation at Ser262 plays the predominant role in tau stabilization when PAR-1/MARK activity is normal, whereas Ser356 phosphorylation begins to contribute to this process when PAR-1/MARK activity is abnormally elevated, as in diseased brains.

### Keywords

tau; phosphorylation; PAR-1/MARK; Alzheimer's disease; neurodegeneration; *Drosophila*

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## INTRODUCTION

Tau is a microtubule-associated protein predominantly localized in axons, where it regulates microtubule stability. However, in the brains of patients suffering from disorders such as Alzheimer's disease (AD), frontotemporal dementia with parkinsonism associated with chromosome 17 (FTDP-17), progressive supranuclear palsy, corticobasal degeneration, and chronic traumatic encephalopathy, tau is detached from microtubules and accumulates in the cytosol to form neurofibrillary tangles. Several lines of evidence suggest that tau misfolding and accumulation is the common molecular mechanism underlying the pathogenesis of tauopathies [1].

Tau is phosphorylated at more than 45 sites in paired helical filaments (PHF) [2–5], and phosphorylation of specific sites plays critical roles in regulating the protein's aggregation and neurotoxicity [6–8]. Ser262 and Ser356 are located in the KXGS motifs in the first and fourth microtubule-binding repeats, respectively. Tau phosphorylated at these sites is the epitope of antibody 12E8, an established marker for tau pathology in the early stages of AD [9, 10]. Ser262 and Ser356 are phosphorylated by PAR-1/MARK1-4 [11], a widely conserved family of kinases [12, 13].

PAR-1/MARK activity is elevated in several neurodegenerative conditions, including AD [14–17]. *In vitro* studies have shown that phosphorylation of tau by PAR-1/MARK causes detachment of tau from microtubules [18]. In cultured neurons, PAR-1/MARK phosphorylation can induce mislocalization of tau from the axon to dendrite [19, 20], an early marker of tau abnormality under disease conditions. Tau phosphorylation at Ser262 and Ser356 due to MARK overexpression increases tau toxicity, whereas preventing tau phosphorylation at these residues by introducing unphosphorylatable alanine substitutions reduces tau toxicity in cellular and *Drosophila* models [8, 13, 21]. Moreover, tau toxicity is elevated under pathological conditions such as  $\beta$ -amyloid accumulation and mitochondrial dysfunction [22–26], and increases in PAR-1/MARK activity, as well as the level of tau phosphorylated at Ser262 and Ser356, correlate with tau toxicity [24, 25, 27]. Recently, we showed in a *Drosophila* model that tau phosphorylation at Ser262 and Ser356 by PAR-1 stabilizes tau at the initial step of mistreatment, thereby promoting tau toxicity [28]. However, the relative contribution of tau phosphorylation at each of these sites to tau stabilization remained unclear.

In this study, we sought to investigate how Ser262 and Ser356 contribute individually to stabilization of tau when PAR-1/MARK activity is normal or abnormally elevated. Using a *Drosophila* model of tau toxicity [25, 29], we found that tau phosphorylation at Ser262 plays a predominant role in tau stabilization. Tau phosphorylation at Ser356 was not detectable under normal conditions; however, when PAR-1 was overexpressed, tau was phosphorylated at Ser356, and this phosphorylation contributed to tau stabilization. These results suggest that tau phosphorylation at Ser356 plays a role in stabilizing tau under pathological conditions in which PAR-1 activity is very high.

## MATERIALS AND METHODS

### Fly stocks

Flies were maintained in standard cornmeal media at 25 °C. The transgenic fly lines carrying the human 0N4R tau, which has four tubulin-binding domains (R) at the C-terminal region and no N-terminal insert (N), include a gift from Dr. M. B. Feany (Harvard Medical School) [29] and the lines established in our labs following the standard method [27, 30] by using human 0N4R tau (a gift from Dr. Mike Hutton (Mayo Clinic Jacksonville)). The transgenic fly line carrying UAS-S262Atau and UAS-S2Atau are established in our labs and reported previously [27, 28, 30]. GMR-GAL4 was obtained from the Bloomington Stock Center. UAS-PAR-1 is a gift from Dr. Bingwei Lu (Stanford University) [31]. All experiments were performed using female flies at 3–5 day-old after eclosion. Genotypes are described in Table S1.

### Western blotting

Western blotting was carried out as described previously [27, 28]. Multiple membranes were prepared with the same samples, and one of the membranes was probed with anti-tubulin and used as the loading control for other blots. Anti-tau monoclonal antibody (Tau46, Zymed), anti-tau phospho-Ser262 (AbCam), phospho-Ser356 (Biosource), TAU1 (Millipore), and anti-tubulin (Sigma) were purchased. Anti-tau pS202 (CP13) was a gift from Dr. Peter Davis (Albert Einstein College of Medicine, USA), and anti-tau polyclonal antibody (tauC) was a gift from Dr. A. Takashima (Gakushuin University, Japan) [32]. The signal intensity was quantified using ImageJ (NIH). Western blots were repeated a minimum of three times with different animals.

### Phosphatase treatment

As described previously [28], twenty fly heads were homogenized in NEBuffer1 for PMP (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, pH 7.5) supplemented with 1 mM MnCl<sub>2</sub> (NEB) and 0.4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc) (Wako Chemicals), 10 µg/ml leupeptin (Peptide Institute), and incubated with λ protein phosphatase (NEB) for 3h at 37°C.

### RNA extraction and quantitative real-time PCR analysis

Heads from more than thirty flies were mechanically isolated, and total RNA was extracted using ISOGEN (NipponGene) followed by reverse-transcription using PrimeScript RT reagent kit (Takara). The resulting cDNA was used as a template for PCR with THUNDERBIRD SYBR qPCR mix (TOYOBO) on a Thermal Cycler Dice real time system TP800 (Takara). Expression of genes of interest was standardized relative to rp49. Relative expression values were determined by the deltaCt method.

Primers were;

htau for 5'-CAAGACAGACCACGGGGCGG-3'

htau rev 5'-CTGCTTGCCAGGGAGGCAG-3'

rp49 for 5'-GCTAAGCTGTCGCACAAATG-3'

rp49 rev 5'-GTTTCGATCCGTAACCGATGT-3'

## Statistics

Statistics was done with Microsoft Excel (Microsoft) with Student's t.

## RESULTS

### Tau phosphorylation at Ser262 is critical for stabilization of tau in a *Drosophila* model of human tau toxicity

Wild-type human 0N4R tau proteins expressed in the fly retina under the control of the eye-specific GMR-Gal4 driver were detected as two major bands by western blotting with pan-tau antibody (Figure 1, total tau). The difference in migration speed between these two bands is related to their phosphorylation levels: tau in the slower-migrating band ( $\tau^{\text{upper}}$ ) is more highly phosphorylated than tau in the faster-migrating band ( $\tau_{\text{lower}}$ ) [28]. In addition,  $\tau_{\text{lower}}$  can be distinguished by TAU-1 antibody (TAU-1-positive tau) [28], which recognizes tau that is non-phosphorylated at the AD-related sites Ser195, 198, 199 and Ser202 [33]. The levels of  $\tau_{\text{lower}}$  and TAU-1-positive tau are prominently decreased by introduction of S262A/S356A double mutation (S2A) or knockdown of PAR-1, the major kinase that phosphorylates tau at both sites [28].

Wild-type human 0N4R tau overexpressed in fly retina was phosphorylated at Ser262 ([8, 13, 21, 25] and Figure 1, pS262), whereas phosphorylation at Ser356 was not detectable (Figure 1, pS356). However, Ser356 was strongly phosphorylated when PAR-1 was co-expressed (Figure 3A), confirming that the antibody used in this study can recognize tau phosphorylated at this residue. These results suggest that tau phosphorylation levels at Ser356 are very low in fly retina.

To investigate the role of Ser262 in stabilization of tau, wild-type human 0N4R tau or tau in which Ser262 was replaced with unphosphorylatable alanine (S262A) was expressed in the fly retina. qRT-PCR analysis revealed that mRNA levels of S262A tau were higher than those of wild-type tau (Figure 2A). However, western blotting revealed that S262A tau was significantly less abundant than wild-type tau (Figure 2B, quantitation, top, total tau). Interestingly, in contrast to wild-type tau, S262A tau exhibited a major band corresponding to  $\tau^{\text{upper}}$  (Figure 2B, total tau,  $\tau^{\text{upper}}$ ). The signal intensities of  $\tau_{\text{lower}}$  normalized to total tau was significantly lower for S262A tau than for wild-type tau (Figure 2B, quantitation, middle,  $\tau_{\text{lower}}$ ), whereas that of  $\tau^{\text{upper}}$  was not significantly different (Figure 2B, quantitation, middle,  $\tau^{\text{upper}}$ ). Moreover, western blotting with TAU-1 antibody, which only detects  $\tau_{\text{lower}}$  [28], revealed that the level of TAU-1-positive tau normalized to total tau was significantly lower for S262A tau than for wild-type tau (Figure 2B, TAU-1; also see quantitation, bottom, TAU-1). Because  $\tau_{\text{lower}}$  or TAU-1 tau represents less phosphorylated forms of tau, S262A mutation might reduce the levels of  $\tau_{\text{lower}}$  or TAU-1-positive tau by promoting tau phosphorylation. However, this was not the case: the levels of tau phosphorylated at Ser202 normalized to total tau were also significantly lower in S262A tau than in wild-type tau (Figure 2B, pSer202; also see quantitation, bottom,

pSer202). Taken together, these results indicate that Ser262 phosphorylation plays a major role in stabilizing tau, especially the less extensively phosphorylated forms.

### **Tau phosphorylation at Ser356 contributes to stabilization of tau when PAR-1 activity is abnormally elevated**

Phosphorylation at Ser356 was not detectable normally (Figure 1); accordingly, it is unlikely to play a major role in stabilizing tau [28]. However, tau was phosphorylated at Ser356 when PAR-1 was overexpressed (Figure 3A) [28], suggesting that Ser356 contributes to stabilization of tau when PAR-1 activity is abnormally elevated. Therefore, we asked whether blocking tau phosphorylation at Ser262 would be sufficient to suppress the increase in tau levels caused by co-expression of PAR-1 (Figure 3A). To this end, we analyzed tau levels by western blotting with a pan-tau antibody. To rule out the possibility that changes in tau phosphorylation affect the affinity of a pan-tau antibody, lysates used for quantitation were treated with phosphatase prior to western blotting. We found that introduction of the Ser262Ala mutation alone partially, but not completely, suppressed increases in tau levels (Compare Figure 3A and B). Because co-expression of PAR-1 significantly increased phosphorylation levels of S262Atau at Ser356 (Figure 3B, pSer356), Ser356 phosphorylation may contribute to tau stabilization under this condition. Consistent with this idea, introduction of alanine mutations at both Ser262 and Ser356 (S2A) completely blocked the increase in tau levels caused by PAR-1 overexpression (Figure 3C and [28]). These results suggest that tau phosphorylation at both Ser262 and Ser356 contributes to stabilization of tau under conditions in which PAR-1 activity is very high.

## **DISCUSSION**

Multiple residues in tau are phosphorylated in brains of tauopathy patients, but the roles of each site in disease pathogenesis remain incompletely understood. Ser262 and Ser356 are both disease-specific phosphorylation sites [2] and have been implicated in structure, turnover, intracellular distribution, and promotion of tau toxicity in disease pathogenesis [8, 13, 18–27]. In this study, we investigated the effect of Ser262 and Ser356 on the stabilization of various tau species and the accumulation of tau caused by PAR-1 overexpression. Our results revealed that phosphorylation at Ser262 plays the predominant role in the stabilization of TAU-1-positive, less extensively phosphorylated forms of tau (Figures 1 and 2). This observation is consistent with a previous report that introduction of a single substitution of Ser262 to unphosphorylatable alanine is sufficient to suppress neurodegeneration caused by tau overexpression in the *Drosophila* model [25]. By contrast, Ser356 phosphorylation was only detectable when PAR-1 was overexpressed, suggesting that phosphorylation at this residue contributes additively to tau stabilization under this condition in our *Drosophila* model (Figure 3). Together, these results suggest that tau phosphorylated at Ser356 is associated with abnormal tau metabolism and represents a more advanced stage of tau pathology than tau phosphorylated at Ser262 (summarized in Figure 4).

PAR-1/MARK activity is elevated in several pathological contexts, including AD [14–17]. MARK4 is co-expressed with tau phosphorylated at Ser262 in a Braak stage-dependent

manner [14]. The level of active MARKs is increased by  $\beta$ -amyloid, which is thought to be the primary causative agent of AD pathology and to act upstream of tau toxicity [23, 24, 34]. The level of active MARKs is also increased by mitochondrial abnormality [35], stimulation of NMDA receptor [36], and ischemic events [37], all of which also promote pathological changes in tau. High MARK activity is associated with AD risk factors such as obesity: MARKs regulate the metabolic rate, and MARK activity is elevated in animals with diet-induced obesity [38, 39]. These reports suggest that neurons in AD brains have higher levels of active MARKs than those in normal brain, and tau is stabilized more often by Ser356 phosphorylation. In addition to MARK, tau phosphorylation at Ser356 is regulated by several kinases, including AMPK, AKAP14, DYRK1A, S6K, and PKA, phosphorylase kinase, and PKR [22, 40–43]. Disruption of the regulatory mechanisms of these kinases and/or phosphatases may also contribute to the stabilization of abnormal tau in disease neurons.

How does tau phosphorylation at Ser262 and Ser356 stabilize tau proteins? Ser262 and Ser356 are located in KXGS motifs in the first and fourth microtubule-binding repeat domains, respectively. In addition to directly binding microtubule [44], the microtubule-binding domain affect the structure, metabolism, and intracellular distribution of tau [18–20, 45–48]. Interestingly, the microtubule-binding domain is also critical for self-association of tau [49]. Because both the binding of tau to microtubules and its self-association/aggregation could affect its stability, tau phosphorylation at both Ser262 and Ser356 is expected to affect these processes [18, 20, 22, 28, 45, 50].

In brains of tauopathy patients, tau exists in multiple forms associated with distinct modifications, binding partners, and/or cellular locations, and its toxicity is likely to be qualitatively and quantitatively heterogeneous [51]. Therefore, targeting tau species in the initial phase of abnormal metabolism represents a promising therapeutic strategy. This study underscores the importance of tau phosphorylation at Ser356 in stabilizing tau when PAR-1/MARK activity is abnormally elevated. Further study of the mechanisms underlying dysregulation of Ser262 and Ser356 phosphorylation, as well as the molecular mechanisms underlying tau stabilization, will identify therapeutic strategies to delay onset and progression of tauopathies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

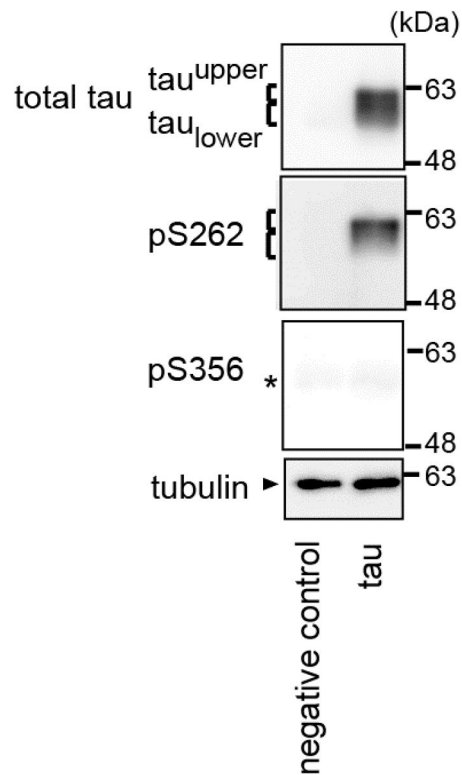
1. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002; 297(5580):353–6. [PubMed: 12130773]
2. Hasegawa M, Morishima-Kawashima M, Takio K, Suzuki M, Titani K, Ihara Y. Protein sequence and mass spectrometric analyses of tau in the Alzheimer's disease brain. *J Biol Chem*. 1992; 267(24):17047–54. [PubMed: 1512244]
3. Hanger DP, Betts JC, Loviny TL, Blackstock WP, Anderton BH. New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nanoelectrospray mass spectrometry. *J Neurochem*. 1998; 71(6):2465–76. [PubMed: 9832145]
4. Hanger DP, Brion JP, Gallo JM, Cairns NJ, Luthert PJ, Anderton BH. Tau in Alzheimer's disease and Down's syndrome is insoluble and abnormally phosphorylated. *Biochem J*. 1991; 275(Pt 1):99–104. [PubMed: 1826835]
5. Morishima-Kawashima M, Hasegawa M, Takio K, Suzuki M, Yoshida H, Titani K, et al. Proline-directed and non-proline-directed phosphorylation of PHF-tau. *J Biol Chem*. 1995; 270(2):823–9. [PubMed: 7822317]
6. Wang JZ, Grundke-Iqbal I, Iqbal K. Kinases and phosphatases and tau sites involved in Alzheimer neurofibrillary degeneration. *Eur J Neurosci*. 2007; 25(1):59–68. [PubMed: 17241267]
7. Sergeant N, Bretteville A, Hamdane M, Caillet-Boudin ML, Grognet P, Bombois S, et al. Biochemistry of Tau in Alzheimer's disease and related neurological disorders. *Expert Rev Proteomics*. 2008; 5(2):207–24. [PubMed: 18466052]
8. Steinhilb ML, Dias-Santagata D, Fulga TA, Felch DL, Feany MB. Tau phosphorylation sites work in concert to promote neurotoxicity in vivo. *Mol Biol Cell*. 2007; 18(12):5060–8. [PubMed: 17928404]
9. Seubert P, Mawal-Dewan M, Barbour R, Jakes R, Goedert M, Johnson GV, et al. Detection of phosphorylated Ser262 in fetal tau, adult tau, and paired helical filament tau. *J Biol Chem*. 1995; 270(32):18917–22. [PubMed: 7642549]
10. Augustinack JC, Schneider A, Mandelkow EM, Hyman BT. Specific tau phosphorylation sites correlate with severity of neuronal cytopathology in Alzheimer's disease. *Acta Neuropathol*. 2002; 103(1):26–35. [PubMed: 11837744]
11. Drewes G, Ebnet A, Preuss U, Mandelkow EM, Mandelkow E. MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell*. 1997; 89(2):297–308. [PubMed: 9108484]
12. Matenia D, Mandelkow EM. The tau of MARK: a polarized view of the cytoskeleton. *Trends Biochem Sci*. 2009; 34(7):332–42. [PubMed: 19559622]
13. Nishimura I, Yang Y, Lu B. PAR-1 kinase plays an initiator role in a temporally ordered phosphorylation process that confers tau toxicity in *Drosophila*. *Cell*. 2004; 116(5):671–82. [PubMed: 15006350]
14. Lund H, Gustafsson E, Svensson A, Nilsson M, Berg M, Sunnemark D, et al. MARK4 and MARK3 associate with early tau phosphorylation in Alzheimer's disease granulovacuolar degeneration bodies. *Acta Neuropathol Commun*. 2014; 2:22. [PubMed: 24533944]
15. Gu GJ, Wu D, Lund H, Sunnemark D, Kvist AJ, Milner R, et al. Elevated MARK2-dependent phosphorylation of Tau in Alzheimer's disease. *J Alzheimers Dis*. 2013; 33(3):699–713. DOI: 10.3233/JAD-2012-121357 [PubMed: 23001711]
16. Chin JY, Knowles RB, Schneider A, Drewes G, Mandelkow EM, Hyman BT. Microtubule-affinity regulating kinase (MARK) is tightly associated with neurofibrillary tangles in Alzheimer brain: a fluorescence resonance energy transfer study. *J Neuropathol Exp Neurol*. 2000; 59(11):966–71. [PubMed: 11089574]

17. Gu GJ, Lund H, Wu D, Blokzijl A, Classon C, von Euler G, et al. Role of individual MARK isoforms in phosphorylation of tau at Ser(2)(6)(2) in Alzheimer's disease. *Neuromolecular medicine*. 2013; 15(3):458–69. [PubMed: 23666762]
18. Schneider A, Biernat J, von Bergen M, Mandelkow E, Mandelkow EM. Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments. *Biochemistry*. 1999; 38(12):3549–58. [PubMed: 10090741]
19. Mandelkow EM, Thies E, Trinczek B, Biernat J, Mandelkow E. MARK/PAR1 kinase is a regulator of microtubule-dependent transport in axons. *J Cell Biol*. 2004; 167(1):99–110. [PubMed: 15466480]
20. Li X, Kumar Y, Zempel H, Mandelkow EM, Biernat J, Mandelkow E. Novel diffusion barrier for axonal retention of Tau in neurons and its failure in neurodegeneration. *EMBO J*. 2011; 30(23):4825–37. [PubMed: 22009197]
21. Chatterjee S, Sang TK, Lawless GM, Jackson GR. Dissociation of tau toxicity and phosphorylation: role of GSK-3beta, MARK and Cdk5 in a Drosophila model. *Hum Mol Genet*. 2009; 18(1):164–77. [PubMed: 18930955]
22. Mairet-Coello G, Courchet J, Pieraut S, Courchet V, Maximov A, Polleux F. The CAMKK2-AMPK kinase pathway mediates the synaptotoxic effects of Abeta oligomers through Tau phosphorylation. *Neuron*. 2013; 78(1):94–108. [PubMed: 23583109]
23. Zempel H, Thies E, Mandelkow E, Mandelkow EM. Abeta oligomers cause localized Ca(2+) elevation, missorting of endogenous Tau into dendrites, Tau phosphorylation, and destruction of microtubules and spines. *J Neurosci*. 2010; 30(36):11938–50. [PubMed: 20826658]
24. Yu W, Polepalli J, Wagh D, Rajadas J, Malenka R, Lu B. A critical role for the PAR-1/MARK-tau axis in mediating the toxic effects of Abeta on synapses and dendritic spines. *Hum Mol Genet*. 2012; 21(6):1384–90. [PubMed: 22156579]
25. Iijima K, Gatt A, Iijima-Ando K. Tau Ser262 phosphorylation is critical for Abeta42-induced tau toxicity in a transgenic Drosophila model of Alzheimer's disease. *Hum Mol Genet*. 2010; 19(15):2947–57. [PubMed: 20466736]
26. Fulga TA, Elson-Schwab I, Khurana V, Steinhilb ML, Spires TL, Hyman BT, et al. Abnormal bundling and accumulation of F-actin mediates tau-induced neuronal degeneration in vivo. *Nat Cell Biol*. 2007; 9(2):139–48. [PubMed: 17187063]
27. Iijima-Ando K, Sekiya M, Maruko-Otake A, Ohtake Y, Suzuki E, Lu B, et al. Loss of axonal mitochondria promotes tau-mediated neurodegeneration and Alzheimer's disease-related tau phosphorylation via PAR-1. *PLoS Genet*. 2012; 8(8):e1002918. [PubMed: 22952452]
28. Ando K, Maruko-Otake A, Ohtake Y, Hayashishita M, Sekiya M, Iijima KM. Stabilization of Microtubule-Unbound Tau via Tau Phosphorylation at Ser262/356 by Par-1/MARK Contributes to Augmentation of AD-Related Phosphorylation and Abeta42-Induced Tau Toxicity. *PLoS Genet*. 2016; 12(3):e1005917. [PubMed: 27023670]
29. Wittmann CW, Wszolek MF, Shulman JM, Salvaterra PM, Lewis J, Hutton M, et al. Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. *Science*. 2001; 293(5530):711–4. [PubMed: 11408621]
30. Iijima K, Chiang HC, Hearn SA, Hakker I, Gatt A, Shenton C, et al. Abeta42 mutants with different aggregation profiles induce distinct pathologies in Drosophila. *PLoS One*. 2008; 3(2):e1703. [PubMed: 18301778]
31. Wang JW, Imai Y, Lu B. Activation of PAR-1 kinase and stimulation of tau phosphorylation by diverse signals require the tumor suppressor protein LKB1. *J Neurosci*. 2007; 27(3):574–81. [PubMed: 17234589]
32. Ueno H, Murayama O, Maeda S, Sahara N, Park JM, Murayama M, et al. Novel conformation-sensitive antibodies specific to three- and four-repeat tau. *Biochem Biophys Res Commun*. 2007; 358(2):602–7. [PubMed: 17493585]
33. Szendrei GI, Lee VM, Otvos L Jr. Recognition of the minimal epitope of monoclonal antibody Tau-1 depends upon the presence of a phosphate group but not its location. *J Neurosci Res*. 1993; 34(2):243–9. [PubMed: 7680727]

34. Lee S, Wang JW, Yu W, Lu B. Phospho-dependent ubiquitination and degradation of PAR-1 regulates synaptic morphology and tau-mediated A $\beta$  toxicity in *Drosophila*. *Nature communications*. 2012; 3:1312.
35. Iijima-Ando K, Hearn SA, Shenton C, Gatt A, Zhao L, Iijima K. Mitochondrial Mislocalization Underlies A $\beta$ 42-Induced Neuronal Dysfunction in a *Drosophila* Model of Alzheimer's Disease. *PLoS ONE*. 2009; 4(12):e8310. [PubMed: 20016833]
36. Bernard LP, Zhang H. MARK/Par1 Kinase Is Activated Downstream of NMDA Receptors through a PKA-Dependent Mechanism. *PLoS One*. 2015; 10(5):e0124816. [PubMed: 25932647]
37. Schneider A, Laage R, von Ahsen O, Fischer A, Rossner M, Scheek S, et al. Identification of regulated genes during permanent focal cerebral ischaemia: characterization of the protein kinase 9b5/MARKL1/MARK4. *J Neurochem*. 2004; 88(5):1114–26. [PubMed: 15009667]
38. Sun C, Tian L, Nie J, Zhang H, Han X, Shi Y. Inactivation of MARK4, an AMP-activated protein kinase (AMPK)-related kinase, leads to insulin hypersensitivity and resistance to diet-induced obesity. *J Biol Chem*. 2012; 287(45):38305–15. [PubMed: 22992738]
39. Hurov JB, Huang M, White LS, Lennerz J, Choi CS, Cho YR, et al. Loss of the Par-1b/MARK2 polarity kinase leads to increased metabolic rate, decreased adiposity, and insulin hypersensitivity in vivo. *Proc Natl Acad Sci U S A*. 2007; 104(13):5680–5. [PubMed: 17372192]
40. Pei JJ, An WL, Zhou XW, Nishimura T, Norberg J, Benedikz E, et al. P70 S6 kinase mediates tau phosphorylation and synthesis. *FEBS Lett*. 2006; 580(1):107–14. [PubMed: 16364302]
41. Azorsa DO, Robeson RH, Frost D, Meec hoovet B, Brautigam GR, Dickey C, et al. High-content siRNA screening of the kinome identifies kinases involved in Alzheimer's disease-related tau hyperphosphorylation. *BMC Genomics*. 2010; 11:25. [PubMed: 20067632]
42. Litersky JM, Johnson GV, Jakes R, Goedert M, Lee M, Seubert P. Tau protein is phosphorylated by cyclic AMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II within its microtubule-binding domains at Ser-262 and Ser-356. *Biochem J*. 1996; 316(Pt 2):655–60. [PubMed: 8687413]
43. Paudel HK. The regulatory Ser262 of microtubule-associated protein tau is phosphorylated by phosphorylase kinase. *J Biol Chem*. 1997; 272(3):1777–85. [PubMed: 8999860]
44. Fischer D, Mukrasch MD, Biernat J, Bibow S, Blackledge M, Griesinger C, et al. Conformational changes specific for pseudophosphorylation at serine 262 selectively impair binding of tau to microtubules. *Biochemistry*. 2009; 48(42):10047–55. [PubMed: 19769346]
45. Dickey CA, Dunmore J, Lu B, Wang JW, Lee WC, Kamal A, et al. HSP induction mediates selective clearance of tau phosphorylated at proline-directed Ser/Thr sites but not KXGS (MARK) sites. *FASEB J*. 2006; 20(6):753–5. [PubMed: 16464956]
46. Karagoz GE, Duarte AM, Akoury E, Ippel H, Biernat J, Moran Luengo T, et al. Hsp90-Tau complex reveals molecular basis for specificity in chaperone action. *Cell*. 2014; 156(5):963–74. [PubMed: 24581495]
47. Lee MJ, Lee JH, Rubinsztein DC. Tau degradation: the ubiquitin-proteasome system versus the autophagy-lysosome system. *Prog Neurobiol*. 2013; 105:49–59. [PubMed: 23528736]
48. Dou F, Netzer WJ, Tanemura K, Li F, Hartl FU, Takashima A, et al. Chaperones increase association of tau protein with microtubules. *Proc Natl Acad Sci U S A*. 2003; 100(2):721–6. [PubMed: 12522269]
49. Ksiazek-Reding H, Yen SH. Structural stability of paired helical filaments requires microtubule-binding domains of tau: a model for self-association. *Neuron*. 1991; 6(5):717–28. [PubMed: 1709023]
50. Biernat J, Gustke N, Drewes G, Mandelkow EM, Mandelkow E. Phosphorylation of Ser262 strongly reduces binding of tau to microtubules: distinction between PHF-like immunoreactivity and microtubule binding. *Neuron*. 1993; 11(1):153–63. [PubMed: 8393323]
51. Sanders DW, Kaufman SK, DeVos SL, Sharma AM, Mirbaha H, Li A, et al. Distinct tau prion strains propagate in cells and mice and define different tauopathies. *Neuron*. 2014; 82(6):1271–88. [PubMed: 24857020]

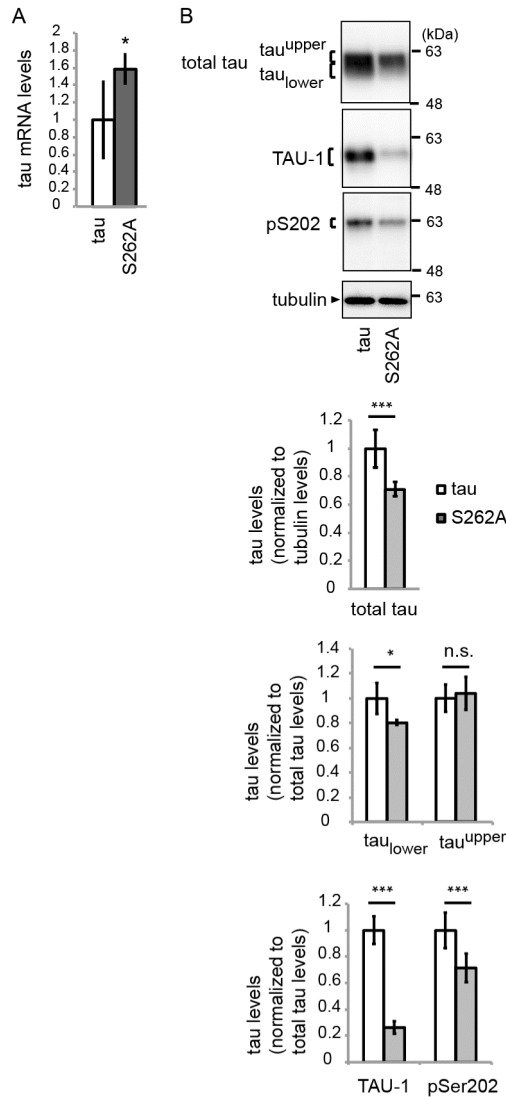
**HIGHLIGHTS**

- Ser262, but not Ser356, is phosphorylated in tau overexpressed in fly eyes.
- Ser262 phosphorylation plays a predominant role in tau stability.
- Tau phosphorylation at Ser356 occurs when PAR-1 is co-overexpressed.
- Tau phosphorylation at Ser356 stabilizes tau when PAR-1 activity is high.

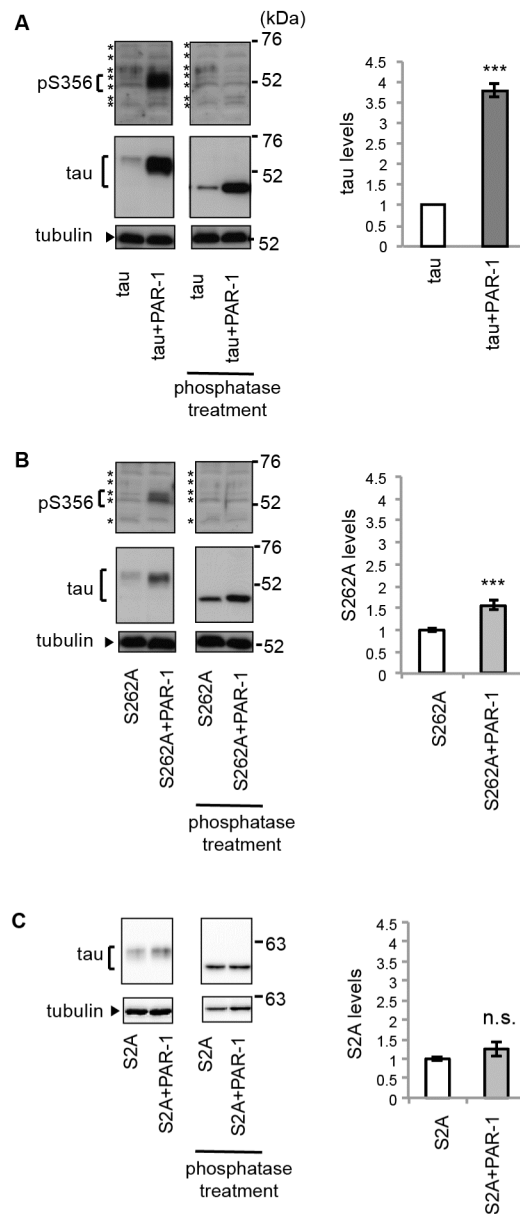


**Figure 1. Wild-type human 0N4R tau overexpressed in fly retina is phosphorylated at Ser262, but not Ser356**

Western blot analysis of fly heads carrying the eye-specific GMR-Gal4 driver alone (negative control) or expressing human tau driven by GMR-Gal4 (tau) with a pan-tau antibody (total tau) or antibodies that recognize the phosphorylation status of tau at specific sites (pSer262 and pSer356). Tau in the slower-migrating band ( $\text{tau}^{\text{upper}}$ ) and the faster-migrating band ( $\text{tau}^{\text{lower}}$ ) are indicated. The asterisk indicates a non-specific band detected by pSer356 antibody in the negative control. Tubulin was used as a loading control.



**Figure 2. Blocking tau phosphorylation at Ser262 with unphosphorylatable alanine substitutions (S262A) preferentially reduces the levels of less extensively phosphorylated forms of tau**  
 (A) Tau mRNA levels in fly heads expressing human wild-type tau (tau) and S262A tau (S262A) were quantitated by qRT-PCR (means  $\pm$  SD,  $n = 3$ ; \* $p < 0.05$ , Student's t-test). (B) Introduction of the S262A mutation decreased the levels of tau, with a more pronounced effect on tau<sub>lower</sub>. Western blotting of wild-type tau or S262A tau with pan-tau antibody (total tau) or antibodies that recognize the phosphorylation status of tau at specific sites (TAU-1 and pS202). Representative blot (left), relative ratio of signal intensities of total tau (right, top), the relative ratio of signal intensities of tau<sup>upper</sup> and tau<sub>lower</sub> (right, middle), and relative ratio of signal intensities of TAU-1 and pS202 (right, bottom) are shown. Means  $\pm$  SD,  $n = 5$ ; \*,  $p < 0.05$ ; \*\*\*,  $p < 0.005$ , n.s., not significant by Student's t-test. Tubulin was used as a loading control.



**Figure 3. Tau phosphorylation at Ser356 contributes to stabilization of tau when PAR-1 activity is abnormally elevated**

(A) Western blot of fly heads expressing tau (tau) or co-expressing tau and PAR-1 (tau+PAR-1), with or without phosphatase treatment, with anti-pan-tau antibody (tau) or an antibody that recognizes tau phosphorylated at Ser356 (pS356). The asterisk indicates a non-specific band detected by the anti-pSer356 antibody after phosphatase treatment. (B) Western blot analyses of fly heads expressing S262A tau (S262A) or co-expressing S262A and PAR-1 (S262A+PAR-1) with anti-tau antibody (tau) or anti-pSer356 antibody (pS356). (C) Western blot of fly heads expressing tau carrying the S262A/S356A double mutation (S2A) or co-expressing S2A and PAR-1 (S2A+PAR-1), with anti-tau antibody (tau). Representative blots are shown, along with the relative ratios of signal intensities of total tau

blots of phosphatase-treated samples. Means  $\pm$  SD, n = 4–5; \*\*\*,  $p < 0.005$  by Student's t-test. Tubulin was used as a loading control.

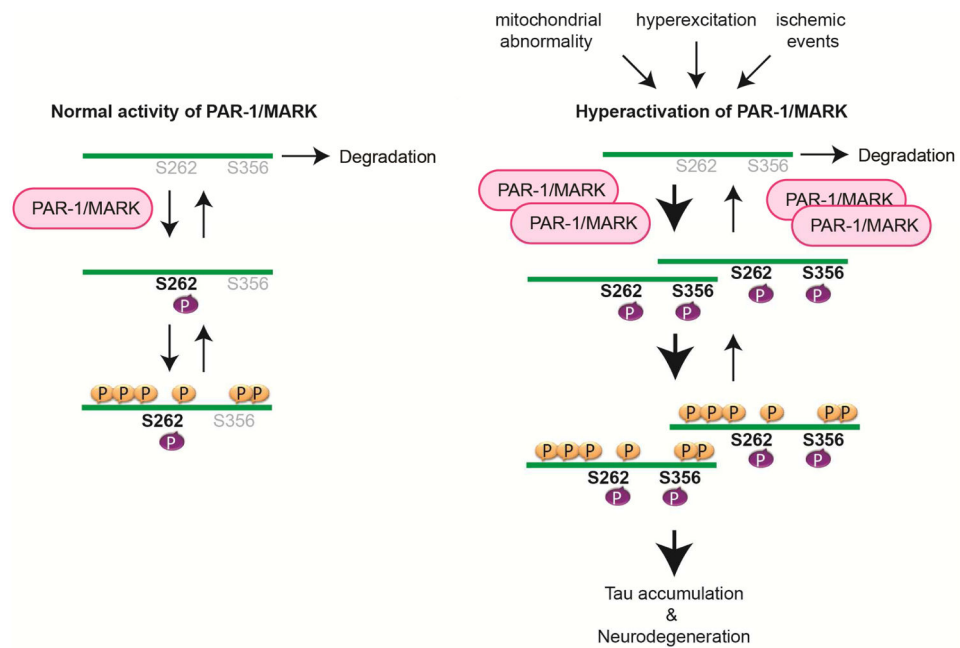
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**Figure 4. Working model of the roles of Ser262 and Ser356 in tau stabilization**

(Left) When PAR-1/MARK activity is normal, tau phosphorylation at Ser262 plays the predominant role in the stabilization of tau. (Right) By contrast, when PAR-1/MARK activity is high, tau phosphorylation at Ser356 occurs and contributes additively to tau stabilization. This process promotes subsequent tau phosphorylation, accumulation and toxicity.