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Recommended Citation
Ando, Kanae; Oka, Mikiko; Ohtake, Yosuke; Hayashishita, Motoki; Shimizu, Sawako; Hisanaga, Shin-Ichi; and Iijima, Koichi M., "Tau phosphorylation at Alzheimer's disease-related Ser356 contributes to tau stabilization when PAR-1/MARK activity is elevated." (2016). *Department of Neuroscience Faculty Papers.* Paper 33.
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Biochem Biophys Res Commun. Author manuscript; available in PMC 2017 November 07.

Published in final edited form as:

Biochem Biophys Res Commun. 2016 September 16; 478(2): 929–934. doi:10.1016/j.bbrc.2016.08.053.

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 mismetabolism, 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 β-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 mismetabolism, 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.

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MATERIALS AND METHODS

Fly stocks

Flies were maintained in standard cornmeal media at 25 ºC. 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 $MnCl_2$ (NEB) and 0.4 mM 4-(2-aminoethl)-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′-CTGCTTGGCCAGGGAGGCAG-3′

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rp49 for 5′-GCTAAGCTGTCGCACAAATG-3′

rp49 rev 5′-GTTCGATCCGTAACCGATGT-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^{upper}) is more highly phosphorylated than tau in the faster-migrating band (tau_{lower}) [28]. In addition, tau_{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_{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^{upper} (Figure 2B, total tau, tau^{upper}). The signal intensities of tau_{lower} normalized to total tau was significantly lower for S262A tau than for wild-type tau (Figure 2B, quantitation, middle, tau_{lower}), whereas that of tau^{upper} was not significantly different (Figure 2B, quantitation, middle, tau^{upper}). Moreover, western blotting with TAU-1 antibody, which only detects tau_{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_{lower} or TAU-1 tau represents less phosphorylated forms of tau, S262A mutation might reduce the levels of tau_{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,

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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

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manner [14]. The level of active MARKs is increased by β-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.

Acknowledgments

We thank Drs. Peter Davis, Mel B. Feany, Bingwei Lu, Mike Hutton, and Akihiko Takashima; TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) for fly stocks, cDNA and antibodies. We thank Dr. Akira Ishio for technical assistance and Dr. Akiko Maruko-Otake for critical reading of the manuscript.

FUNDING

This work was supported by grants from the National Institutes of Health [R01AG032279-A1] (to K.M.I. and K.A.), and the Alzheimer's Association [NIRG-10-173189] (to K.A.), and in part by the National Institute of Health [U01AG046170-01] (to K.M.I. and K.A.), The Research Funding for Longevity Sciences (28-26) from National Center for Geriatrics and Gerontology (NCGG), Japan (to K.M.I.), Grants in aid for Scientific Research [JSPS KAKENHI Grant number16K08637] (to K.M.I.), Takeda Science Foundation, Japan (to K.M.I.), research award

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from Hoansha foundation (to K.A.), Grant-in-Aid for Scientific Research on Innovative Areas (Brain Protein Aging and Dementia Control) [JSPS KAKENHI Grant number 15H01564] (to K.A.) and Grants in aid for Scientific Research [JSPS KAKENHI Grant number 15K06712] (to K.A.), and Takeda Science Foundation, Japan (to K.A.).

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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.

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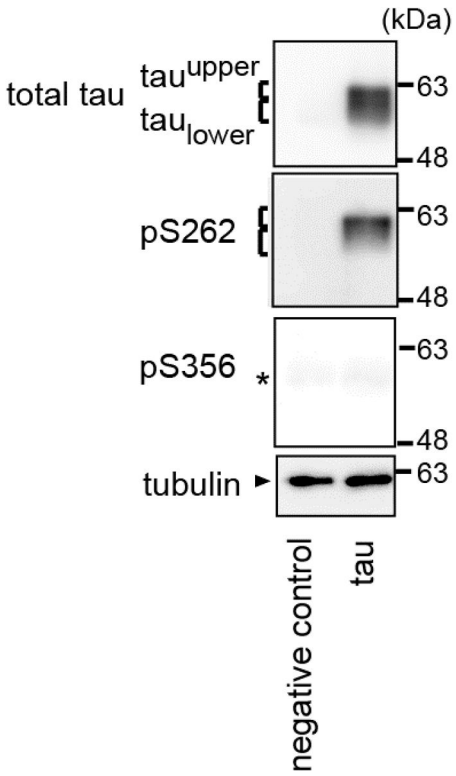

Figure 1. Wild-type human 0N4R tau overexpressed in fly retina is phosphorylated at Ser262, but not Ser356

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 (tau^{upper}) and the faster-migrating band (tau_{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.

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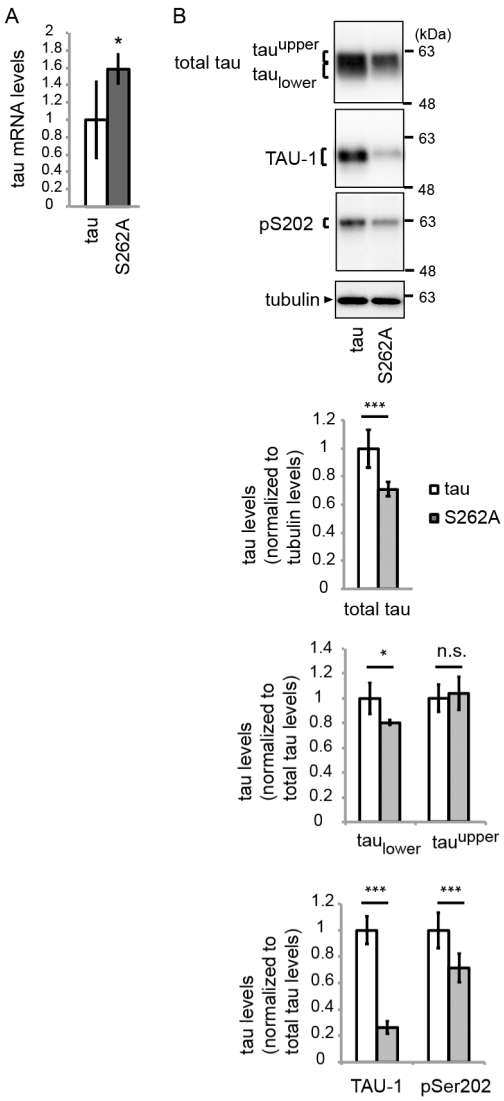

Figure 2. Blocking tau phosphorylation at Ser262 with unphosphorylatable alanine substitutions (S262A) preferentially reduces the levels of less extensively phosphorylated forms of tau

(A) Tau mRNA levels in fly heads expressing human wild-type tau (tau) and S262A tau (S262A) were quantitated by qRT-PCR (means ± 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_{lower}. Western blotting of wild-type tau or S262Atau 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^{upper} and tau_{lower} (right, middle), and relative ratio of signal intensities of TAU-1 and pS202 (right, bottom) are shown. Means ± 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.

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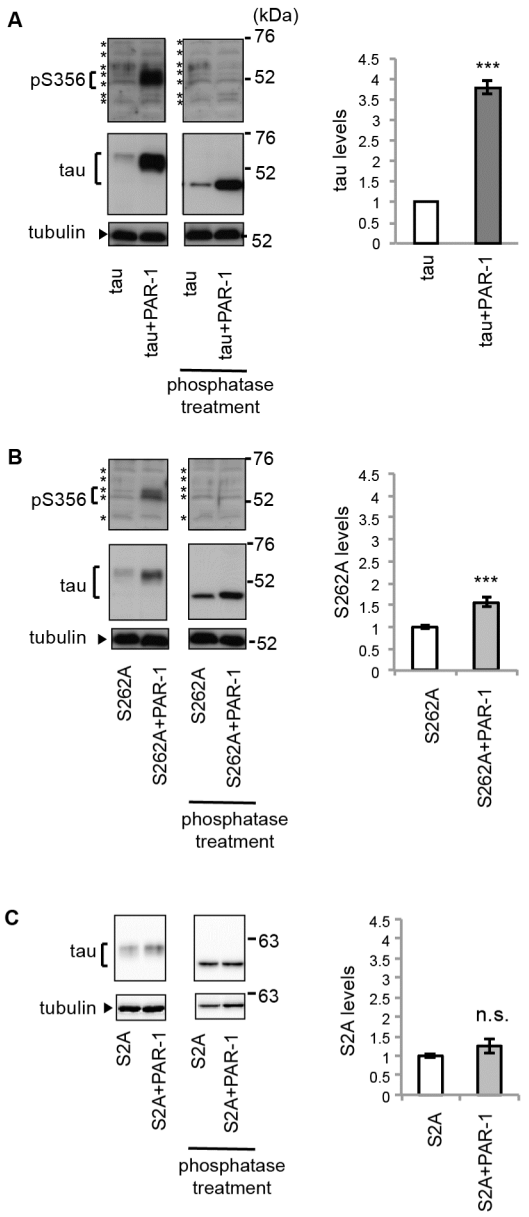

Figure 3. Tau phosphorylation at Ser356 contributes to stabilization of tau when PAR-1 activity is abnormally elevated

(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

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

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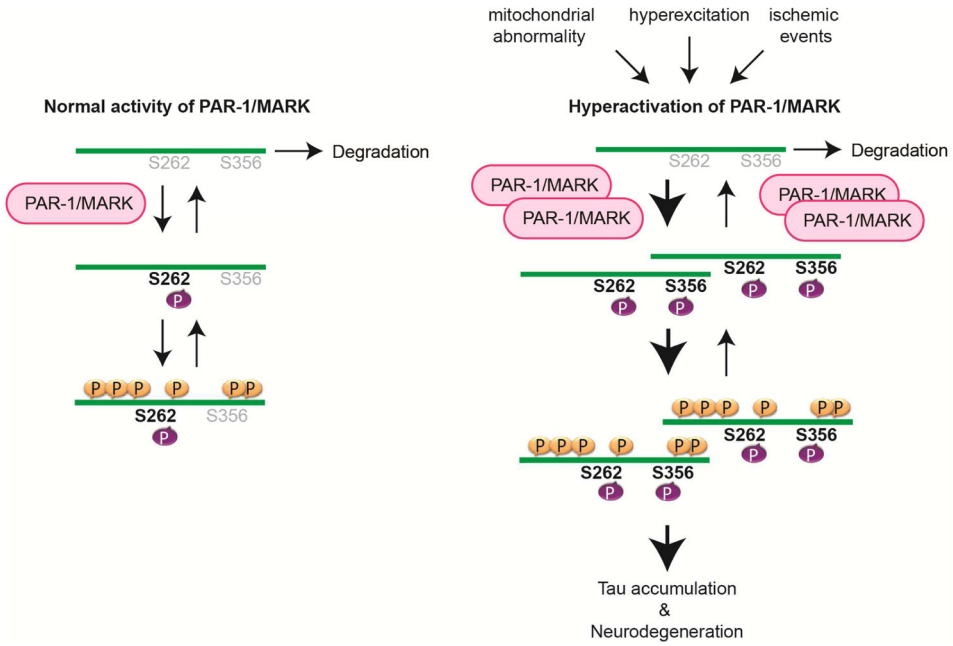

Figure 4. Working model of the roles of Ser262 and Ser356 in tau stabilization

(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.