Cell, Vol. 116, 671–682, March 5, 2004, Copyright ©2004 by Cell Press

PAR-1 Kinase Plays an Initiator Role in a Temporally Ordered Phosphorylation Process that Confers Tau Toxicity in *Drosophila*

Isao Nishimura, Yufeng Yang,[1] and Bingwei Lu[1,*]
Laboratory of Developmental Neurobiology
Rockefeller University
1230 York Avenue
New York, New York 10021

Summary

Multisite hyperphosphorylation of tau has been implicated in the pathogenesis of neurodegenerative diseases including Alzheimer's disease (AD). However, the phosphorylation events critical for tau toxicity and mechanisms regulating these events are largely unknown. Here we show that *Drosophila* PAR-1 kinase initiates tau toxicity by triggering a temporally ordered phosphorylation process. PAR-1 directly phosphorylates tau at S262 and S356. This phosphorylation event is a prerequisite for the action of downstream kinases, including glycogen synthase kinase 3 (GSK-3) and cyclin-dependent kinase-5 (Cdk5), to phosphorylate several other sites and generate disease-associated phospho-epitopes. The initiator role of PAR-1 is further underscored by the fact that mutating PAR-1 phosphorylation sites causes a much greater reduction of overall tau phosphorylation and toxicity than mutating S202, one of the downstream sites whose phosphorylation depends on prior PAR-1 action. These findings begin to differentiate the effects of various phosphorylation events on tau toxicity and provide potential therapeutic targets.

Introduction

Accumulation of proteins with abnormal conformation is a feature shared by many neurodegenerative diseases. In tauopathies, the microtubule (MT) binding protein tau forms prominent intracellular aggregates known as neurofibrillary tangles (NFT) (Feany and Dickson, 1996; Goedert, 1998; Lee et al., 2001). Tau is normally a highly soluble protein enriched in axons, but it becomes abnormally phosphorylated and insoluble in NFT. While these observations provide circumstantial evidence that tau abnormalities may lead to neuronal dysfunction and degeneration, direct evidence was not available until the discovery that mutations in tau are associated with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Foster et al., 1997; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). NFT is also a pathological hallmark of AD, where NFT often but not always surround amyloid plaques composed of amyloid β peptides (Braak and Braak, 1991; Mandelkow and Mandelkow, 1998). It is still controversial as to which pathology, the NFT or amyloid plaque, is the primary cause of neuronal loss in AD (Davies, 2000; Hardy and Selkoe, 2002). Studies in transgenic mice have shown that expression of mutant human tau (h-tau) alone could result in tangle pathology and neuronal loss without accompanying plaque pathology (Ishihara et al., 1999; Lewis et al., 2000). Induction of amyloid deposits in these animals could further promote NFT formation, suggesting that the two lesions could be causally linked (Gotz et al., 2001; Lewis et al., 2001; Oddo et al., 2003). Together, these studies support the notion that abnormality in tau can be a direct cause of neurodegeneration.

Little is known about the molecular and cellular mechanisms underlying tau-mediated cellular toxicity. The presence of abnormally phosphorylated tau in NFT suggests that an imbalance in tau phosphorylation/dephosphorylation may be pathogenic. A number of protein kinases and phosphatases have been shown to regulate h-tau phosphorylation in vitro. These include GSK-3β, MAP kinase, Cdk2 and 5, PKA, CaMKII, microtubule-affinity regulating kinase (MARK), and protein phosphatase 1, 2A, 2B, and 2C (Goedert et al., 1995; Lee et al., 2001; Mandelkow, 1999). Despite a large body of biochemical studies, surprisingly little is known about the in vivo roles of these enzymes in conferring tau toxicity. Studies using transgenic mice overexpressing Cdk5 or GSK-3 have implicated these two kinases in tau phosphorylation, aggregation, and tangle formation (Lucas et al., 2001; Noble et al., 2003), but in vivo loss-of-function analyses are needed to verify the involvement of these kinases in tau-mediated neuronal pathologies.

Drosophila has recently established itself as a model system for studying human neurodegenerative disorders. Fly models of tauopathy have been created by expressing wild-type or FTDP-linked mutant forms of h-tau (Jackson et al., 2002; Wittmann et al., 2001). Using such models and based largely on overexpression experiments, it was shown that Shaggy (GSK-3) can promote NFT pathology in photoreceptor neurons (Jackson et al., 2002). Whether GSK-3 and NFT are necessary for tau-mediated neurodegeneration, however, remains uncertain. Other studies have shown that tau-mediated neurodegeneration could occur without NFT and that GSK-3β-induced tau hyperphosphorylation in mice could correlate inversely with neuropathology (Spittaels et al., 2000; Wittmann et al., 2001).

Critical testing for a functional role of phosphorylation in tau-mediated neuropathology will require identifying the physiological tau kinase and assessing the consequence of removing this kinase activity on the disease process. Through loss-of-function and overexpression genetic studies and biochemical analysis, we now show that PAR-1 is a physiological tau kinase that plays a central role in regulating tau phosphorylation and toxicity in *Drosophila*. PAR-1 is a Ser/Thr kinase originally identified in *C. elegans* for its role in regulating cell polarity and asymmetric cell division (Guo and Kemphues, 1995). PAR-1 homologs have been found in eukaryotes ranging from yeast to mammals and exert essential cellular and developmental functions. MARK kinase, the

*Correspondence: bingwei@stanford.edu
[1]Present Address: Department of Pathology, Stanford University School of Medicine, Stanford, California 94305.

mammalian homolog of PAR-1, regulates MT dynamics, epithelial cell polarity, and neuronal differentiation (Biernat et al., 2002; Bohm et al., 1997; Drewes et al., 1997). *Drosophila* PAR-1 plays important roles in MT organization, oocyte differentiation, anterior-posterior axis formation, and Wingless signaling (Cox et al., 2001; Shulman et al., 2000; Sun et al., 2001; Tomancak et al., 2000). While analyzing the neuronal function of PAR-1, we found that *Drosophila* PAR-1 is a physiological kinase for fly tau and h-tau. Overexpression of PAR-1 leads to elevated tau phosphorylation and enhanced toxicity, whereas removing PAR-1 function or mutating PAR-1 phosphorylation sites in tau abolishes tau toxicity. Furthermore, we have uncovered an initiator role for PAR-1 in a multisite phosphorylation process that generates pathogenic forms of tau. In this process, phosphorylation by PAR-1 precedes and is obligatory for downstream phosphorylation events, including those carried out by GSK-3 and Cdk5, to generate toxic tau. Consistent with PAR-1 playing an initiator role in the process, mutating PAR-1 phosphorylation sites causes a much more dramatic reduction of overall tau phosphorylation and toxicity than mutating one of the downstream Cdk5/GSK-3 phosphorylation sites. These findings have important implications for understanding the biogenesis of pathogenic tau in neurons and for developing mechanism-based therapeutic strategies.

Results

Overexpression of PAR-1 in *Drosophila* Eye Results in a Degeneration Phenotype

To investigate the function of *Drosophila* PAR-1 in the nervous system, we overexpressed the longest isoform of PAR-1 in the eye using the *UAS-GAL4* system (Brand and Perrimon, 1993; Sun et al., 2001). Targeted overexpression of PAR-1 to photoreceptor neurons using the *sevenless-GAL4* line resulted in eye degeneration in newly emerged adult flies. Strong PAR-1 expression lines exhibited severely reduced eyes, with fused ommatidia and missing inter-ommatidial bristles, as revealed by scanning electron microscopy (SEM) analysis (Figure 1C). Weak or mild expression lines had slightly reduced eyes, which appeared rough and had disordered ommatidia and occasional missing bristles (Figure 1B). However, in flies that express two copies of the weak PAR-1 transgene, the eye degeneration phenotype became more severe, similar to that observed in the strong expression line (data not shown). Thus, overexpression of PAR-1 using the *sevenless-GAL4* driver causes eye degeneration in a dosage-dependent manner. No effect was observed when PAR-1 KN, which contains an inactivating point mutation in the kinase domain, was expressed under *sevenless-GAL4* control (Figure 1D). This indicates that the kinase activity of PAR-1 is required for inducing the eye degeneration phenotype.

Staining of eye sections revealed that loss of photoreceptor neurons is associated with the eye degeneration phenotype (Figures 1E and 1F). Since *sevenless-GAL4* is expressed in the early developing eye disc and is later expressed in photoreceptor neurons as well as non-neuronal pigment and cone cells, the loss of photoreceptor neuron phenotype could be due to progressive neurodegeneration or developmental perturbation of cell patterning, which often leads to secondary neuronal apoptosis. To distinguish between these possibilities, we first tested whether overexpression of PAR-1 specifically in postmitotic neurons is sufficient to cause photoreceptor degeneration. For this we used the *elav-GAL4* driver to direct PAR-1 expression. Resulting *elav-GAL4>UAS-PAR-1* flies exhibited a mild rough eye and loss of photoreceptor neuron phenotype (Figures 1H and 1J). To further address the cell type specificity and to distinguish between neurodevelopmental or neurodegenerative mechanisms of PAR-1-induced toxicity, we used a *heat shock-GAL4* driver to direct PAR-1 overexpression. Induction of ubiquitous PAR-1 overexpression by daily heat shock treatment starting from first instar larval stage resulted in animals with rough eyes and accompanying photoreceptor loss (Figures 1K and 1N). The animals are otherwise normal externally. Heat shock induction starting from pupae or young adult stages, when photoreceptor neurons are fully differentiated, also resulted in a photoreceptor loss phenotype (Figures 1L, 1O, and 1P). Together, these results indicate that PAR-1 overexpression-induced toxicity is relatively specific to photoreceptor neurons and that this toxicity is mediated at least in part through a progressive degeneration mechanism.

Genetic Interaction between PAR-1 and Tau

The degeneration phenotype induced by PAR-1 overexpression could be mediated by abnormal phosphorylation of its substrate(s). MARK kinase was previously shown to phosphorylate tau in vitro (Drewes et al., 1997; Jenkins and Johnson, 2000). This, together with the fact that expression of h-tau in *Drosophila* photoreceptor neurons caused similar eye degeneration (Jackson et al., 2002; Wittmann et al., 2001), led us to investigate the relationship between tau and PAR-1 in the process. A fly tau homolog was recently shown to be expressed in the nervous system (Heidary and Fortini, 2001), but no information on its loss-of-function or overexpression phenotype is available. We used a chromosomal deficiency associated with Tp(3;Y)R97 to test possible genetic interaction between endogenous fly tau and PAR-1. Through single embryo PCR analysis, we confirmed that this deficiency deletes fly tau. In flies overexpressing the strong PAR-1 transgene and heterozygous for the chromosomal deficiency, the eye degeneration phenotype was partially suppressed (Figure 2B). This suggests that endogenous fly tau may mediate PAR-1-induced eye degeneration phenotype.

We next tested for possible genetic interaction between fly PAR-1 and h-tau. Overexpression of the four C-terminal tandem repeats (4R) isoform of h-tau containing the R406W mutation (henceforth referred to as h-tauM), which is associated with a familial form of FTDP-17 (Hutton et al., 1998; Wittmann et al., 2001), caused a moderate eye degeneration phenotype (Figure 2C). Coexpression of the weak PAR-1 transgene, which by itself had a small effect on eye morphology (Figure 1B), enhanced h-tauM toxicity and resulted in smaller sized eyes (Figure 2D). The effect of the genetic interaction is also reflected by the extent of photoreceptor neuron loss. As shown in Figure 2G, PAR-1 and h-tauM coexpressing flies showed a greater neuronal loss (15%, $n = 1050$, 5 samples) than files expressing h-tauM (6%,

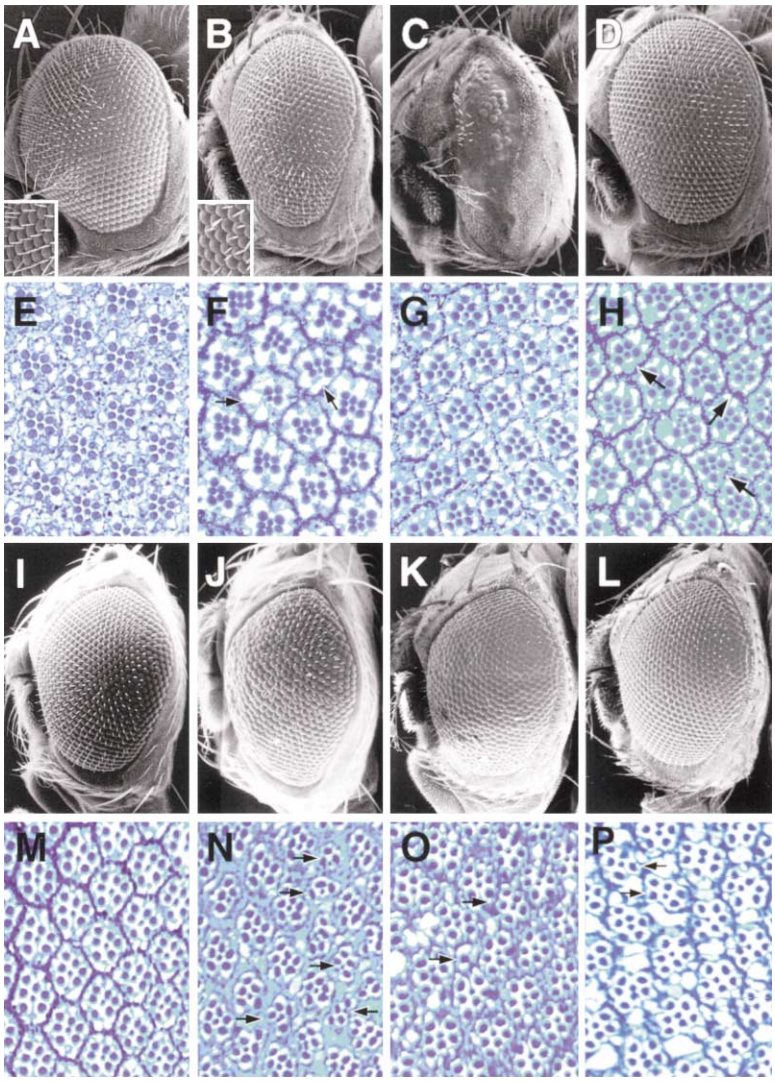

Figure 1. Eye Degeneration Phenotypes Caused by Overexpression of PAR-1

(A–D) SEM of a wild-type eye (A) and transgenic eyes overexpressing a weak PAR-1 transgene (B), a strong PAR-1 transgene (C), or PAR-1 KN (D). Insets in (A) and (B) are higher magnification views of ommatidia and bristles.

(E–H) Toluidine blue staining of photoreceptor neurons. The genotypes are wild-type (E), *sev-GAL4>UAS-PAR-1* (F), *elav/+* (G), *elav-GAL4>UAS-PAR-1* (H). Arrows point to ommatidia with missing photoreceptor neurons.

(I–L) SEM showing eye phenotypes induced by *elav-GAL4*- or *heat shock-GAL4*-driven PAR-1 overexpression. The genotypes are: *elav/+* (I), *elav-GAL4>UAS-PAR-1* (J), *heat shock GAL4>UAS-PAR-1* (K and L). The sample in (K) is heat shocked around first instar larvae stage, whereas the one in (L) at pupae stage.

(M–P) Toluidine blue staining of photoreceptor neurons in *heat shock-GAL4>UAS-PAR-1* flies. The samples are either non-heat shocked (M) or heat shocked at first instar larvae (N), pupae (O), or adult (P) stages. Arrows point to ommatidia with missing photoreceptor neurons.

n = 1050, 5 eye samples) or PAR-1 (0.9%, n = 1050, 5 eye samples) alone. Interaction between PAR-1 and h-tauM was also observed in cholinergic neurons in the CNS, where coexpression of PAR-1 and h-tauM resulted in profound vacuole formation in both the cell bodies and neuronal processes, whereas expression of h-tauM alone caused only mild vacuole formation in cell bodies and PAR-1 alone had little effect (Figures 2H and 2I). Thus, elevated expression of fly PAR-1 enhances the toxic effects of h-tauM. We next tested whether PAR-1 and wild-type h-tau genetically interact in the eye. Overexpression of wild-type h-tau also caused a rough eye phenotype, as reported previously (Jackson et al., 2002). Coexpression of wild-type h-tau and a PAR-1 transgene resulted in an exacerbated phenotype compared to expression of either gene alone (Figures 2J–2L). Thus, increased expression of fly PAR-1 enhances the toxic effects of both wild-type and mutant forms of h-tau.

Overexpression of PAR-1 Enhances Tau Toxicity without Promoting NFT Formation

In AD and related tauopathies, NFT formed by the aggregation of fibrillar forms of tau can be identified by monoclonal antibodies such as AT100, which recognizes a disease-specific phospho-epitope (Augustinack et al., 2002; Zheng-Fischhofer et al., 1998). Using AT100 antibody, it was found that while a low level, diffused distribution of phospho-tau immunostaining was evident in photoreceptor neurons expressing h-tau alone, coexpression of GSK-3 and h-tau promoted the appearance of prominent AT100-positive, NFT-like structures (Jackson et al., 2002). In contrast, coexpression of PAR-1 and h-tauM did not produce obvious NFT-like aggregates in either photoreceptor neurons or cholinergic neurons (Figure 2M). This suggests that some soluble, nonaggregated forms of h-tauM may mediate the enhanced toxic effects of PAR-1 coexpression.

PAR-1 Directly Phosphorylates h-tau and Fly Tau In Vitro and In Vivo

In immunohistochemical analyses using a battery of phosphorylation-sensitive tau antibodies, we observed that PAR-1 overexpression resulted in a marked increase in the level of 12E8-positive h-tauM. The 12E8 antibody specifically detects pS262 and pS356 in the

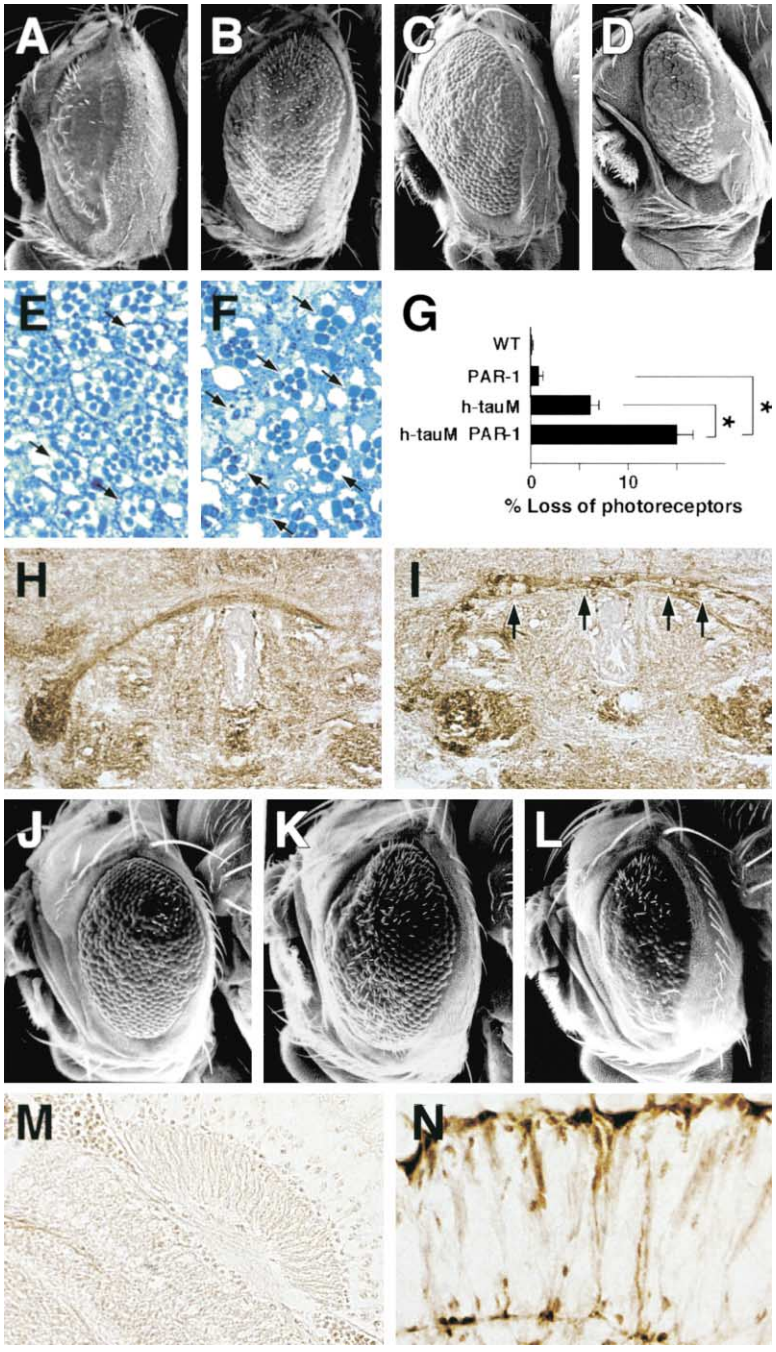

Figure 2. Genetic Interactions between PAR-1 and Fly Tau and h-tau

(A–D) SEM of transgenic fly eyes expressing a strong PAR-1 transgene (A), a strong PAR-1 transgene and heterozygous for a chromosomal deficiency that removes fly tau (B), h-tauM (C), or h-tauM together with a weak PAR-1 transgene (D).

(E–F) Toluidine blue staining of photoreceptor neurons in transgenics expressing h-tauM (E) or coexpressing h-tauM and a weak PAR-1 transgene (F). Arrows point to ommatidia with missing photoreceptors.

(G) Quantitative analysis of photoreceptor neuron loss. Asterisks indicate $p < 0.01$ in Student's t test.

(H and I) Tau immunostainings of transgenic brain expressing h-tau (H) or coexpressing h-tau and PAR-1 (I). Arrows in (I) indicate vacuoles within the processes of the giant interneuron commissure.

(J–L) SEM of transgenics expressing wild-type h-tau (J), PAR-1 (K), or h-tau and PAR-1 (L).

(M and N) AT100 immunostaining of transgenic fly brain coexpressing PAR-1 and h-tauM (M) or Shaggy and h-tauM (N). *Sevenless-GAL4* driver was used in (A)–(F), (J)–(L), and (N), *Cha-GAL4* was used in (H), (I), and (M).

MT binding domain of h-tau (Seubert et al., 1995). Biochemical and cell culture studies have implicated MARK, GSK-3, PKA, and CaMKII in phosphorylating these sites (Drewes et al., 1995; Litersky et al., 1996; Moreno et al., 1995), but no animal study is yet available to validate these kinases as the physiological kinase for these sites. We found that transgenic flies coexpressing PAR-1 and h-tauM stained robustly with 12E8 (Figure 3A). Flies expressing h-tauM alone also exhibited strong staining with 12E8, albeit at a lower level (Figure 3B). This was confirmed by Western blot analysis (Figure 3E). To estimate the level of h-tauM phosphorylation at S262/S356 sites, we used 12E8 antibody-immunoprecipitated h-tauM as standard in Western blot analysis of total brain extracts with 12E8 antibody and T14 antibody, which measures total tau level. Three independent experiments indicated that approximately 20% of total h-tau was phosphorylated at S262/S356 sites in h-tauM transgenic flies and this was increased to approximately 80% in h-tauM and PAR-1 coexpression flies (Figure 3H).

The conservation of S262/S356 and their flanking residues between h-tau and fly tau suggests that 12E8 may also recognize phosphorylated fly tau. Indeed, a basal level of 12E8 staining was detected in wild-type flies (Figure 3D). In flies that overexpress PAR-1, the level of 12E8 staining was significantly elevated (Figure 3C). This

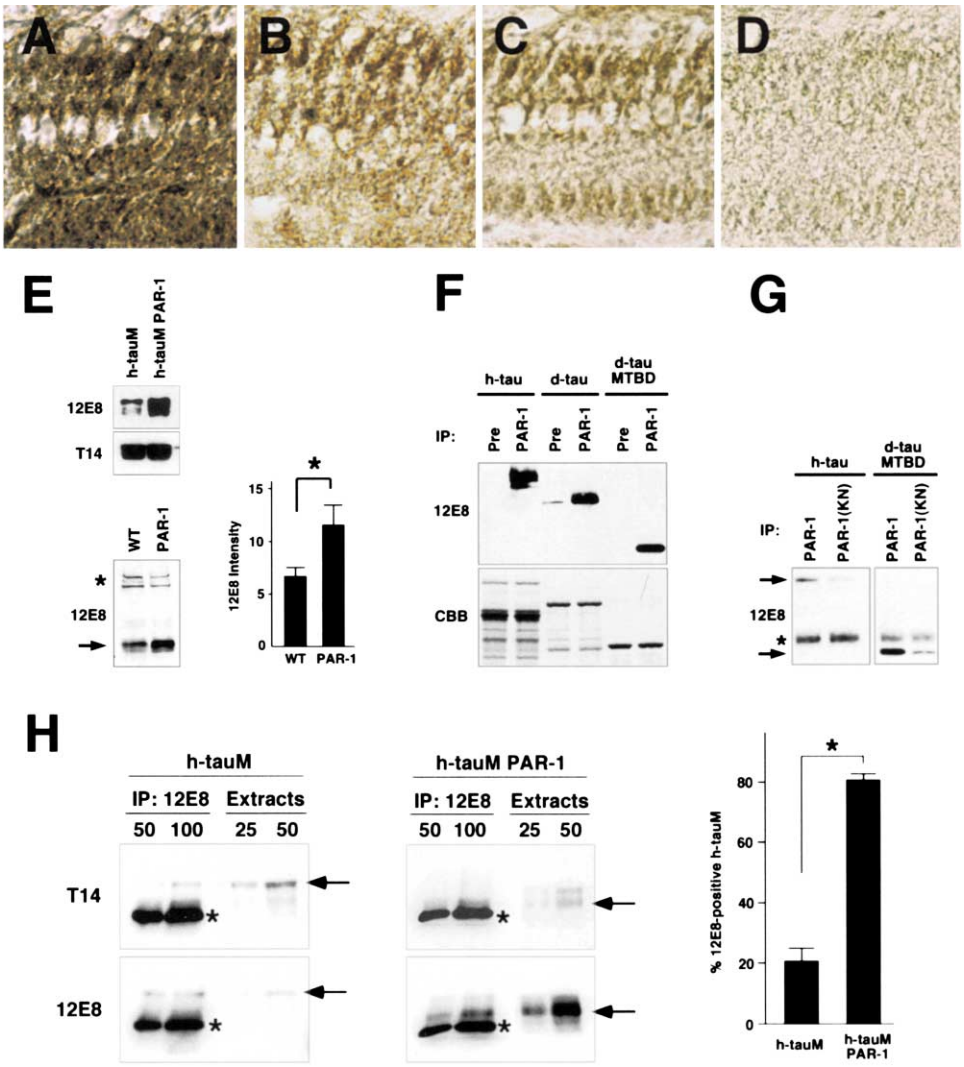

Figure 3. PAR-1 Directly Phosphorylates Fly Tau and h-tau at 12E8 Epitope Sites

(A–D) 12E8 immunostaining of cholinergic nerve terminals in the optic medulla of *Cha-GAL4*-driven transgenics expressing h-tauM and PAR-1 together (A), h-tauM (B), PAR-1 (C), or wild-type control (D).

(E) Western blot analysis demonstrating increased 12E8-positive h-tauM (top) or endogenous fly tau (bottom) after PAR-1 expression. Phosphorylation-independent antibody T14 (top) or a nonspecific crossreacting band (asterisk in bottom panel) was used as loading controls. The graph indicates PAR-1-induced increase of 12E8-positive fly tau is significant.

(F) In vitro kinase assay using proteins immunoprecipitated by preimmune serum (Pre) or PAR-1 antibody (PAR-1). After the kinase reaction, the GST-tau fusion proteins were probed with 12E8 antibody.

(G) In vitro kinase assay using PAR-1 or PAR-1 KN. Arrows and asterisk mark GST-tau fusion proteins and IgG, respectively. The GST-tau substrates used in the kinase reactions are underlined.

(H) Estimation of level of phosphorylation in h-tauM only and h-tauM/PAR-1 coexpression flies. 12E8-positive h-tauM was immunoprecipitated from h-tauM or h-tauM/PAR-1 transgenic brain extracts and 50 and 100 arbitrary units of the precipitated material were run together with 25 and 50 arbitrary units of the total brain extracts and probed with 12E8 and T14 antibodies. Asterisks and arrows indicate IgG bands and tau bands, respectively. The ratio of 12E8/T14 signals was used to estimate the degree of phosphorylation. The graph represents quantitative analysis of western results. Asterisk indicates $p < 0.01$ in Student's t test.

was also confirmed by Western blot analysis (Figure 3E). Thus, overexpression of PAR-1 can lead to elevated phosphorylation of both h-tauM and fly tau at the two Ser residues recognized by 12E8.

To test whether PAR-1 directly phosphorylates tau at these sites, we performed in vitro kinase assays using purified PAR-1 and bacterially expressed recombinant glutathione-*S*-transferase (GST) fusion proteins of wild-type h-tau and fly tau. Without preincubation with PAR-1, these GST-tau fusion proteins did not react with 12E8. Immunoreaction with 12E8 was readily detected after incubation with PAR-1 (Figure 3F). When the kinase-negative form of PAR-1 (PAR-1 KN) was used in the assay, 12E8 immunoreactivity was barely detectable (Figure 3G). Together, these experiments demonstrate that PAR-1 directly phosphorylates h-tau and fly tau to generate pS262 and pS356 residues recognized by 12E8.

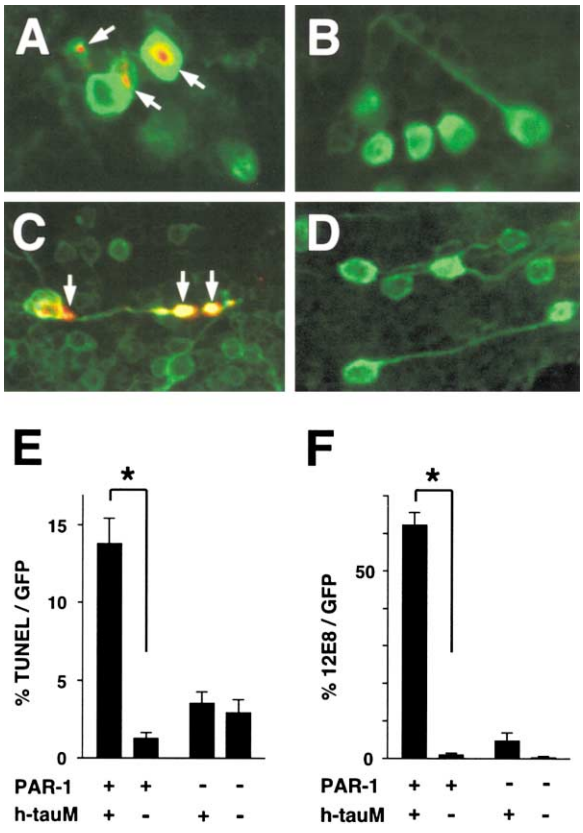

Figure 4. PAR-1 Activity Is Required for h-tauM Phosphorylation and Toxicity

(A and B) Cortical neurons in par-1$^+$/h-tauM$^+$ (A) and par-1$^-$/h-tauM$^+$ (B) MARCM clones were double-labeled for GFP (green) and TUNEL (red). Arrows point to TUNEL-positive nuclei.

(C and D) Neurons in par-1$^+$/h-tauM$^+$ (C) and par-1$^-$/h-tauM$^+$ (D) MARCM clones in the optic lamina were double-labeled for GFP (green) and 12E8 (red). Arrows point to 12E8-positive swellings in neuronal processes.

(E and F) Quantification of TUNEL-positive (E) and 12E8-positive (F) neurons within corresponding MARCM clones. Asterisks indicate $p < 0.01$ in Student's t test.

PAR-1 Function Is Required for Tau Phosphorylation and Cytotoxicity

We next sought to address whether PAR-1 is normally required for the phosphorylation and toxic effects of h-tau under physiological conditions. Since null mutations in *par-1* are embryonic lethal, which prevented us from assessing the effects of loss of PAR-1 function on tau toxicity in homozygous adult animals, we made tissue clones of homozygous mutant cells in heterozygous adult animals using mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999). This technique allowed us to generate *par-1* mutant clones expressing h-tauM under *elav-GAL4* control and at the same time positively mark these clones with a green fluorescent protein (GFP) reporter (par-1$^-$/h-tauM$^+$ clones). In parallel, we generated GFP-marked par-1$^+$/h-tauM$^+$, par-1$^-$/h-tauM$^-$, and par-1$^+$/h-tauM$^-$ clones.

To test the effect of removing PAR-1 function on tau toxicity, we performed TdT-mediated dUTP nick end labeling (TUNEL) assays on the mosaic clones. A significant portion of neurons in par-1$^+$/h-tauM$^+$ clones ($\sim$13%, n = 763, 8 brain samples) stained positive in TUNEL assay (Figure 4A). In contrast, only 4% of neurons in par-1$^-$/h-tauM$^+$ clones (n = 936, 8 brain samples) stained positive, a level similar to that observed in par-1$^-$/h-tauM$^-$ (n = 476, 5 brain samples) and par-1$^+$/h-tauM$^-$ (n = 1028, 5 brain samples) clones (Figures 4B and 4E). The latter may represent the endogenous level of TUNEL staining in fly brain under our experimental conditions.

We next analyzed the effect of removing PAR-1 activity on the phosphorylation state of h-tauM at the 12E8 sites. The majority of neurons in par-1$^+$/h-tauM$^+$ clones (63%, n = 631, 6 brain samples) were 12E8 positive (Figure 4C). The lack of detectable 12E8 staining in the remaining neurons may reflect cell type-specific variations in PAR-1 kinase activity or an unidentified phosphatase activity. In contrast, only a small fraction of neurons in par-1$^-$/h-tauM$^+$ clones were 12E8 positive ($\sim$5%, n = 818, 6 brain samples) (Figures 4D and 4F). We interpret the residual 12E8 staining in par-1$^-$/h-tauM$^+$ clones as a result of the perdurance of PAR-1 protein or phosphorylation of h-tauM at S262 and S356 by other kinases. Together, these results show that PAR-1 is the major in vivo kinase that phosphorylates h-tauM at the 12E8 sites and this phosphorylation event is important for conferring h-tauM toxicity.

Mutating PAR-1-Dependent Phosphorylation Sites Abolishes h-tauM Toxicity

To further demonstrate the key effect of phosphorylation by PAR-1 in conferring h-tauM toxicity, we made Ala substitutions in PAR-1 phosphorylation sites (S262A and S356A) and assessed the effects of these mutations. Overexpression of h-tauMS262A/S356A (referred to as S2A) in photoreceptor neurons had little effect on eye morphology or photoreceptor number (Figures 5C and 5D), although Western blot analysis showed that comparable amounts of tau protein were produced between S2A and h-tauM transgenic flies (Figure 5E). Since S2A could still associate with neuronal microtubules in vivo (Figures 5F and 5G), it is unlikely that the elimination of h-tauM toxicity by the two point mutations is caused by a drastic change in protein conformation. Instead, these results argue that the presence of the two phospho-Ser residues in the MT binding domain is critical for h-tauM toxicity.

Phosphorylation by PAR-1 Is Necessary for a Subsequent Multisite Phosphorylation Event

We further characterized the overall in vivo phosphorylation profile of S2A (Figure 5H). As expected, S2A could no longer be recognized by 12E8. Surprisingly, Western blot analysis indicated that phosphorylation of many other sites was also greatly reduced in S2A. Phosphorylation at several sites recognized by AD-relevant antibodies was virtually eliminated. These include AT100 (pT212 and pS214) and AT8 (pS202 and pT205) sites. In parallel, there was an increase in the level of tau recognized by Tau-1 antibody, which detects unphosphorylated S199 and S202 residues (data not shown). The reduction of phosphorylation at S202 was also independently confirmed by the pS202-specific CP13 anti-

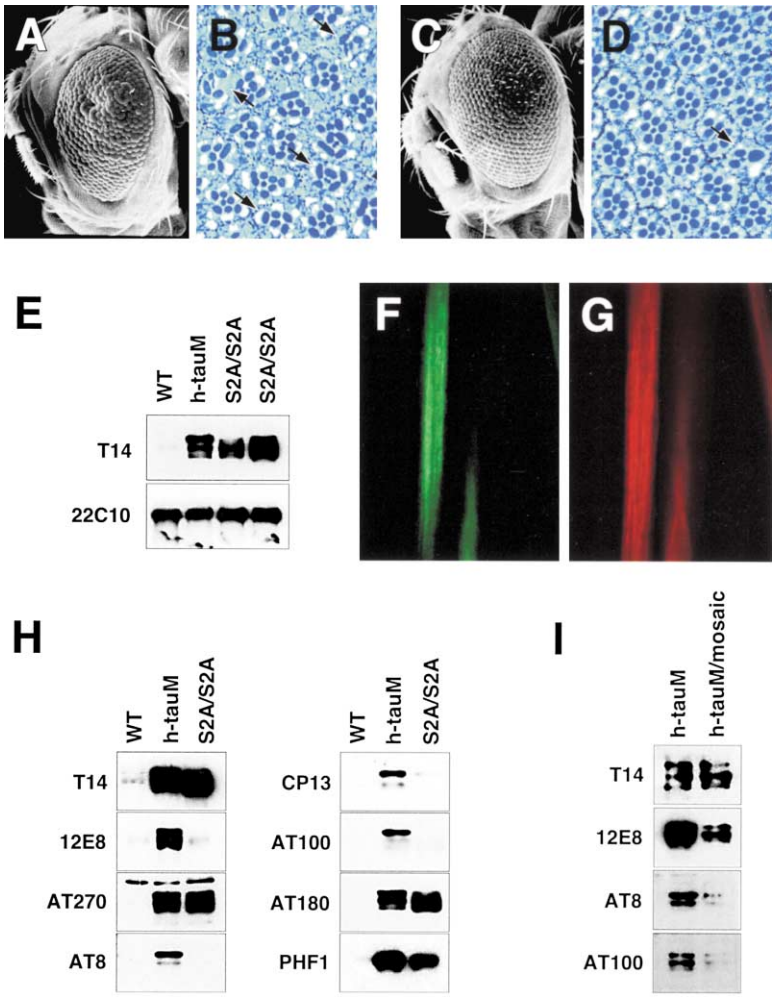

Figure 5. Blocking PAR-1-Dependent Phosphorylation at 12E8 Sites Leads to h-tauM Hypophosphorylation and Diminished Toxicity

(A and C) SEM of transgenic eyes expressing h-tauM (A) or S2A (B).

(B and D) Toluidine blue staining of photoreceptor neurons in transgenics expressing h-tauM (B) or S2A (D). Arrows point to ommatidia with missing photoreceptors.

(E) Western blot analysis showing comparable expression of S2A and h-tauM in transgenic brain extracts. Reprobing with 22C10 antibody serves as loading control.

(F and G) Larval motor neuron axons were immunostained for S2A (F) and tubulin (G) to show their colocalization.

(H) Western blot analysis of wild-type or h-tauM and S2A transgenic head extracts using T14 antibody and a series of phosphorylation-dependent tau antibodies.

(I) Western blot analysis of fly heads with *par-1*$^{+/+}$ or *par-1*$^{-/-}$ mosaic eyes expressing h-tauM.

Sevenless-GAL4 driver was used in all panels except (F) and (G), where *elav-GAL4* was used.

body. Phosphorylation at PHF-1 (pS396 and pS404) sites showed a moderate reduction. It should be noted that not all phosphorylation sites in tau are affected by the S262A/S356A mutations. For example, phosphorylation at T181 recognized by AT270 antibody was not affected.

The results described above suggest that phosphorylation by PAR-1 at S262 and S356 is required for the subsequent phosphorylation events. To test this possibility further, we examined the phosphorylation state of h-tauM when PAR-1 function is removed. For this purpose, we used a modified FLP/FRT technique to generate mosaic eyes enriched for *par-1* mutant clones (Newsome et al., 2000). The h-tauM transgene was uniformly expressed in these eyes under *sevenless-GAL4* control. On average, more than half of the mosaic eye were composed of homozygous *par-1* mutant clones. Western blot analysis on single fly heads indicated that h-tauM phosphorylation at 12E8, AT8, and AT100 epitope sites was significantly reduced in *par-1*$^{-/-}$ mosaic eyes compared to *par-1*$^{+/+}$eyes (Figure 5I). Together with the Western blot analysis of S2A transgenic flies, these data suggest that phosphorylation by PAR-1 at S262 and S356 is a prerequisite for the subsequent phosphorylation by other kinases in a temporally ordered phosphorylation process.

GSK-3 and Cdk5 Act Downstream of PAR-1 in the Multistep Phosphorylation Process

Recent transgenic animal studies have implicated two kinases, GSK-3 and Cdk5, in the phosphorylation of tau in vivo. Analyses of tau phosphorylation status in transgenic mice overexpressing GSK-3 or Cdk5 have detected increased phosphorylation at certain sites previously identified as their in vitro phosphorylation sites. For example, S202 and PHF-1 sites (S396 and S404) have been shown to be prominent Cdk5 and GSK-3 phosphorylation sites, respectively, and the two kinases may have overlapping specificity at these sites (Liu et al., 2002; Lovestone et al., 1994; Lucas et al., 2001; Michel et al., 1998; Noble et al., 2003; Patrick et al., 1999). We tested whether these sites in h-tauM were also phosphorylated by the corresponding fly kinases. The activity of Cdk5 is regulated by its binding with neuron-specific activators. Overexpression of *Drosophila* P35 activator has been shown to elevate endogenous Cdk5 activity (Connell-Crowley et al., 2000). In P35 and h-tauM coexpression flies, we observed that the level of phosphorylation at S202 recognized by CP13 antibody is elevated. In addition, phosphorylation at AT270 sites was also significantly increased. Phosphorylation at AT100, AT180, and PHF-1 sites was relatively unchanged (Figure 6D). Thus, phosphorylation at S202 and T181

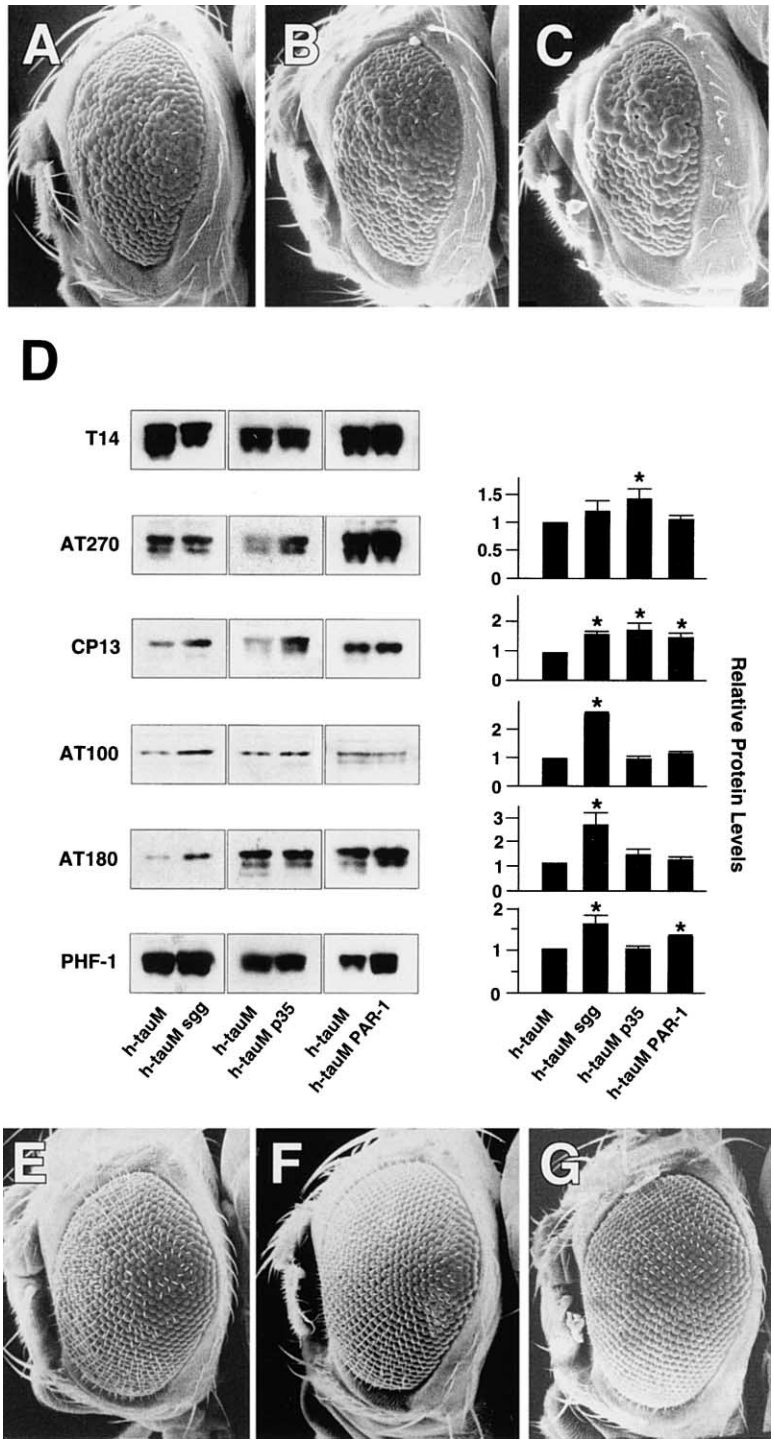

Figure 6. GSK-3 and Cdk5 Act Downstream of PAR-1 in a Sequential Phosphorylation Process

(A–C) SEM of transgenic eyes expressing h-tauM alone (A), coexpressing h-tauM and P35 (B), or coexpressing h-tauM and Shaggy (C).

(D) Western blot analyses comparing tau phosphorylation between h-tauM and h-tauM/PAR-1, h-tauM/P35, or h-tauM/Shaggy transgenics. The bar graphs show quantitative analysis of relative phosphorylation level changes after PAR-1, P35, or Shaggy coexpression.

(E–G) SEM of S2A transgenic eyes coexpressing PAR-1 (E), P35 (F), or Shaggy (G).

Sevenless-GAL4 driver was used in all panels.

responds to changes in Cdk5 levels. The eye morphology of P35 and h-tauM coexpressing flies appeared similar to that of flies expressing h-tauM alone (Figures 6A and 6B), suggesting that elevated Cdk5 activity does not significantly enhance tau toxicity. We next analyzed Shaggy and h-tauM coexpression flies. As reported previously (Jackson et al., 2002), coexpression of Shaggy and h-tau resulted in enhanced eye degeneration phenotypes (Figure 6C). In the coexpression flies, we observed significantly increased tau phosphorylation at PHF-1, CP13, AT180, and AT100 sites (Figure 6D). We conclude that these phospho-epitopes contain GSK-3 phosphorylation sites and that elevated phosphorylation at these sites enhances tau toxicity.

The fact that many of the above-tested phosphorylation sites for GSK-3 and Cdk5 kinases are affected in S2A suggests that phosphorylation by the two kinases is regulated by prior PAR-1 action. To test this idea further, we analyzed the phosphorylation status of GSK-3 and Cdk5 phosphorylation sites in PAR-1 and

h-tauM coexpression flies. In addition to 12E8 sites, significant increase of phosphorylation was observed at CP13 and PHF-1 sites in these flies (Figure 6D). In contrast, phosphorylation at other sites such as AT100 sites was little changed, suggesting that PAR-1 is not a rate-limiting factor for these phosphorylation events. Since in vitro kinase assays showed that PAR-1 is incapable of directly phosphorylating the CP13 and PHF-1 sites (data not shown), the elevated phosphorylation at these sites in PAR-1 coexpressing flies are likely mediated by downstream kinases such as Cdk5 and GSK-3.

We further tested whether coexpression of PAR-1, GSK-3, or Cdk5 has any modulating effect on S2A toxicity in vivo. PAR-1 and S2A coexpression flies showed a mild rough eye phenotype similar to PAR-1 overexpression alone (Figure 6E, compare to Figure 1B), indicating that PAR-1 overexpression does not confer additional toxicity to S2A. Cooverexpression of GSK-3 or Cdk-5 also did not change S2A toxicity (Figures 6F and 6G). These results further support the notion that phosphorylation by PAR-1 at S262 and S356 is a prerequisite for the subsequent phosphorylation by downstream kinases such as GSK-3 and Cdk5 to generate toxic tau species.

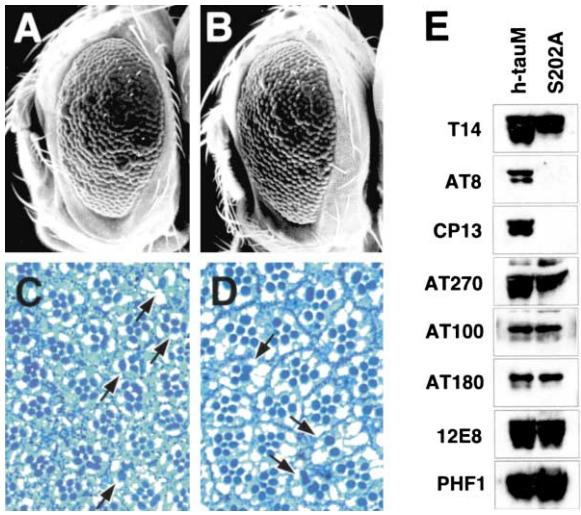

Figure 7. Effects of S202A Mutation on h-tauM Phosphorylation and Toxicity

(A and B) SEM of transgenic eyes expressing h-tauM (A) or S202A (B).

(C and D) Toluidine blue staining of photoreceptors in transgenics expressing h-tauM (C) or S202A (D).

(E) Western blot analysis of transgenic head extracts expressing h-tauM or S202A.

Sevenless-GAL4 driver was used in all panels.

Mutating a Downstream Cdk5/GSK-3 Phosphorylation Site (S202A) Has Much Smaller Effects on Overall tau Phosphorylation and Toxicity than S2A Mutations

Since the S2A mutation disrupts tau phosphorylation at multiple downstream sites, it does not allow us to distinguish the contribution of individual phosphorylation sites to tau toxicity. We wished to address this issue by making point mutations in the downstream phosphorylation sites. We focused on the S202 site because it is phosphorylated by Cdk5 and GSK-3 in vivo and because AT8 antibody, which is sensitive to phosphorylation at this site, was considered an Alzheimer-diagnostic antibody. We generated transgenic flies that express h-tauM containing an Ala substitution at S202 (S202A). Western blot analysis demonstrated that, as predicted, S202A protein was no longer recognized by CP13 or AT8 antibodies. Significantly, phosphorylation at 12E8, AT100, PHF-1, AT180, and AT270 sites was unaffected by S202A mutation (Figure 7E). This suggests that unlike S262 and S356 sites, the phosphorylation state of S202 does not influence that of other sites. Examination of external eye morphology by SEM and photoreceptor staining of eye sections showed that, unlike S2A, S202A is as toxic as h-tauM (Figures 7B and 7D). This suggests that phosphorylation by GSK-3 and Cdk5 at S202 site plays a rather limited role in conferring tau toxicity. This result supports the notion that PAR-1 plays an initiator role in the pathogenic phosphorylation process and further suggests that phosphorylation at downstream sites other than S202 or a combination of those downstream phosphorylation events makes a major contribution to tau toxicity.

Discussion

The molecular mediators and cellular events that contribute to the pathogenesis of most human neurodegenerative diseases are still poorly defined. Studying these diseases in model organisms offers the power of genetic analysis to dissect the disease processes. In this study, we have shown that PAR-1, the fly homolog of mammalian MARK kinase, plays a central role in conferring tau toxicity in vivo. Our study reveals PAR-1 function in triggering a temporally ordered phosphorylation process that is responsible for generating toxic forms of tau. This multisite phosphorylation process involves downstream kinases such as Cdk5 and GSK-3, whose action depends on prior phosphorylation of h-tau by PAR-1. A nonphosphorylatable mutation at S202, one of the downstream GSK-3/Cdk5 target sites whose phosphorylation depends on prior PAR-1 action, has a much smaller impact on overall tau phosphorylation and toxicity than mutations at PAR-1 phosphorylating sites. This strongly supports the initiator role of PAR-1 in generating toxic species of tau and further implies that the toxic form of tau may be phosphorylated at a subset or all of the other downstream sites.

Our results indicate that tau phosphorylation by PAR-1/MARK represents one of the earliest events in the pathological process. Consistent with this notion, the 12E8 epitope sites are phosphorylated in all the isoforms of h-tau found in AD brain (Augustinack et al., 2002; Seubert et al., 1995). Relatively little is known about the upstream events that act through PAR-1/MARK to regulate h-tau phosphorylation in the disease process. Understanding how PAR-1/MARK kinases are regulated during normal development may provide some clues. PAR-1 belongs to a group of evolutionarily conserved PAR proteins that control cell polarity. Genetics studies have shown that PAR-1 is regulated by a PAR-3/PAR-6/aPKC protein complex (Pellettieri and

Seydoux, 2002). Recently, the mammalian PAR-3 and PAR-6 proteins have been shown to regulate hippocampal neuronal polarity through interaction with the phosphatidylinositol 3-kinase signaling pathway (Shi et al., 2003). Furthermore, recent biochemical studies have identified a protein kinase capable of activating MARK/PAR-1 (Timm et al., 2003). It would be interesting to test whether these factors may regulate h-tau toxicity through PAR-1.

It was previously shown that PAR-1 regulates the Wingless/Wnt pathway in *Drosophila* and *Xenopus* by phosphorylating the core component Dishevelled (Sun et al., 2001). It is thus interesting that GSK-3, another core component of Wingless pathway, acts downstream of PAR-1 to phosphorylate h-tau. These results are consistent with the notion that the Wingless pathway may be involved in regulating tau phosphorylation (Jackson et al., 2002). It has been proposed that the pathway components are utilized differently in tau phosphorylation than in canonical Wnt signaling. Our data indicate that PAR-1 and GSK-3 directly phosphorylate tau in an ordered fashion, with PAR-1 action preceding that of GSK-3. One parsimonious explanation for the requirement of prior phosphorylation by PAR-1 is that PAR-1 phosphorylation reduces the affinity of tau for MT and releases it from the MT network, therefore allowing easy access by other kinases. If that is the case, the mechanism may operate in a region-specific manner since certain phosphorylation sites do not depend on prior PAR-1 action. Our data are also consistent with the idea that PAR-1 phosphorylation at 12E8 sites provides docking sites for intermediary kinase(s) and/or adaptor molecule(s), which facilitate subsequent phosphorylation by GSK-3 and Cdk5. It appears that the phosphorylation at certain downstream sites is achieved through a complex process. For example, phosphorylation at AT100 sites depends on prior PAR-1 action, but PAR-1 co-overexpression does not increase phosphorylation at these sites. Instead, co-overexpression of GSK-3 can lead to increased phosphorylation at AT100 sites. Previous in vitro studies have shown that the generation of AT100 epitope requires a PHF-like conformation of tau and the sequential phosphorylation by GSK-3 and PKA (Zheng-Fischhofer et al., 1998). It remains to be determined whether GSK-3 and PKA act downstream of PAR-1 to phosphorylate AT100 sites in flies.

Recent studies suggest that synaptic dysfunction may be one of the earliest events in the pathogenesis of AD (Selkoe, 2002). It is reasonable to speculate that abnormally phosphorylated tau may contribute to synaptic dysfunction in AD. MT is known to be essential in synaptic vesicle transport during neurotransmission and in the formation and maintenance of synaptic structures. Given the known function of tau in MT binding, it is conceivable that phosphorylation may induce the detachment of tau from MT, thereby affecting MT dynamics and disrupting synaptic functions. Alternatively, phospho-tau could acquire certain new activities unrelated to MT binding, such as gaining affinity for some unidentified factors at the synapse and interfering with their function. While our estimates of the levels of tau phosphorylation at S262/S356 sites in h-tauM only and h-tauM and PAR-1 coexpression transgenic flies do not distinguish between these two possibilities, they nevertheless indicate that phospho-tau constitutes a substantial fraction of the total protein. Future genetic studies in *Drosopila* could help reveal the exact mechanisms of phospho-tau toxicity.

Rapid progresses in our understanding of the biogenesis of amyloid plaques have led to efforts to develop AD therapy based on inhibition of the processing and aggregation of Aβ or clearance of mature amyloid plaques. In view of the role of tau in the disease process, it might be more rational to target both amyloid and tau pathologies in therapeutic approaches. This study is beginning to dissect the relative contribution by individual phosphorylation events to tau toxicity. Further analysis will identify the kinases acting downstream of PAR-1 and playing dominant roles in conferring tau toxicity. Future studies will also test whether deregulation of the PAR-1-initiated multisite phosphorylation process may underlie certain idiopathic tauopathies and whether PAR-1 and the downstream kinases could serve as therapeutic targets.

Experimental Procedures

Fly Stocks and Genetics

All general fly stocks and *GAL4* lines were obtained from the Bloomington *Drosophila* stock center. The *UAS-tau(R406W)* and the *Cha-GAL4* lines were kindly provided by Drs. Mel Feany and Paul Salvaterra, respectively. SEM analysis of eye morphology was performed essentially as described (Jackson et al., 2002). In most cases, transheterozygous flies from a cross between a *UAS* line and a *GAL4* line were used.

Histology and Immunohistochemistry

Immunohistochemical analyses of adult fly brain was performed as described (Yang et al., 2003). The primary antibodies used were 3E6 monoclonal antibody against GFP (1:1,000), rabbit polyclonal antibody against nonphosphorylated form of h-tau (1:2,500), 12E8 monoclonal antibody against h-tau (1:4,000), AT100 monoclonal antibody against PHF tau (1:1000).

For MARCM analysis, appropriate stocks were used to set up crosses as described (Lee and Luo, 1999). Whole brain tissues were dissected out and immunostained. The primary antibodies used were rabbit anti-GFP and 12E8. Nuclear DNA fragmentation was analyzed by TUNEL staining. Following TUNEL reaction, samples were immunostained for GFP as described above and visualized with Cy2-conjugated secondary antibody. Images were taken with a Zeiss confocal microscope. For quantitative analysis, whole-mount brain samples were scanned with confocal microscope, and TUNEL- or 12E8-positive cells among GFP-positive clones were counted in the merged images.

In Vitro Phosphorylation

DNA sequences corresponding to full-length h-tau and fly tau, or the MT binding domains of fly tau (d-tau MTBD), were amplified by polymerase chain reaction and inserted directionally into *pGEX 5X1* to produce in-frame GST fusion constructs. Bacterially expressed proteins were affinity purified using glutathione agarose. To purify active PAR-1 kinase, 0–16 hr mixed stage w^- embryonic extract was subject to immunoprecipitation with PAR-1 antibody or preimmune antiserum. Immunocomplexes were washed first with lysis buffer and then with kinase buffer. The kinase reactions were carried out essentially as described (Riechmann et al., 2002). Similarly, to analyze the effect of PAR-1 (KN) on tau phosphorylation, extracts of fly heads expressing Myc-tagged PAR-1 KN or wild-type PAR-1 were subject to immunoprecipitation with Myc antibody. Immunoprecipitates were used in kinase reactions.

Site-Direct Mutagenesis and Transgenics

A cDNA clone encoding h-tau(R406W) was subject to site-directed mutagenesis to change codons 262 and 356 from TCCs to GCC

and GCT and codon 202 from TCC to GCC to produce S2A and S202A, respectively. Resulting S2A and S202A cDNAs were fully sequenced and subcloned into *pUAST* vector for germline transformation. Multiple transgenic lines were obtained. Protein expression levels were examined by Western blotting with monoclonal phosphorylation-independent antibody T14 (1:500) as described below, and representative lines expressing the highest level of S2A and S202A were used in subsequent studies.

Western Blotting

To analyze the phosphorylation status of tau, dissected adult fly heads or larval brain and ventral nerve cords were homogenized in a lysis buffer (10 mM Tris/Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10% glycerol, 50 mM NaF, 1 mM Na_3VOF_3, 5 mM NaPPi, 5 mM DTT, 4 M urea, and a cocktail of protease inhibitors). Extracts were mixed with SDS sample buffer, heated at 60°C, centrifuged at 8,000 g for 5 min, and separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes and probed with primary antibodies: T14 (1:1000), 12E8 (1:4000), AT270 (1:1000), AT8 (1:500), AT100 (1:500), CP13 (1:500), PHF-1 (1:500), Tau1 (1:1000), and 22C10 (1:200). The membranes were incubated with peroxidase-labeled anti-mouse IgG, and signals were detected with chemiluminescence reagents.

Acknowledgments

We are grateful to Dr. Kazuaki Yoshikawa for his generous support throughout this study. We thank Drs. Paul Greengard, Hermann Steller, Mel Feany, and Su Guo for reading the manuscript, Drs. Peter Davies, Mel Feany, Mark Fortini, Ed Giniger, Virginia Lee, Paul Salvaterra, Peter Seubert, Pam Silver, Huaxi Xu, Mike Young, and the Bloomington *Drosophila* Stock Center for reagents and fly stocks. We are grateful to Ulrike Gaul for help with fly histology and Nina Lampen at Sloan-Kettering Institute and Angela Klaus at the American Museum of Natural History for help with SEM. This work was supported by JSPS Fellowships (I.N.), The McKnight, Beckman, Sloan Foundations, The Irma T. Hirschl Trust, and The New York City Council Speaker's Fund for Biomedical Research (B.L.).

Received: June 13, 2003
Revised: January 23, 2004
Accepted: January 29, 2004
Published: March 4, 2004

References

Augustinack, J.C., Schneider, A., Mandelkow, E.M., and Hyman, B.T. (2002). Specific tau phosphorylation sites correlate with severity of neuronal cytopathology in Alzheimer's disease. Acta Neuropathol. (Berl.) *103*, 26–35.

Biernat, J., Wu, Y.Z., Timm, T., Zheng-Fischhofer, Q., Mandelkow, E., Meijer, L., and Mandelkow, E.M. (2002). Protein kinase MARK/PAR-1 is required for neurite outgrowth and establishment of neuronal polarity. Mol. Biol. Cell *13*, 4013–4028.

Bohm, H., Brinkmann, V., Drab, M., Henske, A., and Kurzchalia, T.V. (1997). Mammalian homologues of C. elegans PAR-1 are asymmetrically localized in epithelial cells and may influence their polarity. Curr. Biol. *7*, 603–606.

Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. (Berl.) *82*, 239–259.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Connell-Crowley, L., Le Gall, M., Vo, D.J., and Giniger, E. (2000). The cyclin-dependent kinase Cdk5 controls multiple aspects of axon patterning in vivo. Curr. Biol. *10*, 599–602.

Cox, D.N., Lu, B., Sun, T.Q., Williams, L.T., and Jan, Y.N. (2001). Drosophila par-1 is required for oocyte differentiation and microtubule organization. Curr. Biol. *11*, 75–87.

Davies, P. (2000). A very incomplete comprehensive theory of Alzheimer's disease. Ann. N Y Acad. Sci. *924*, 8–16.

Drewes, G., Trinczek, B., Illenberger, S., Biernat, J., Schmitt-Ulms, G., Meyer, H.E., Mandelkow, E.M., and Mandelkow, E. (1995). Microtubule-associated protein/microtubule affinity-regulating kinase (p110mark). A novel protein kinase that regulates tau-microtubule interactions and dynamic instability by phosphorylation at the Alzheimer-specific site serine 262. J. Biol. Chem. *270*, 7679–7688.

Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E.M., and Mandelkow, E. (1997). MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. Cell *89*, 297–308.

Feany, M.B., and Dickson, D.W. (1996). Neurodegenerative disorders with extensive tau pathology: a comparative study and review. Ann. Neurol. *40*, 139–148.

Foster, N.L., Wilhelmsen, K., Sima, A.A., Jones, M.Z., D'Amato, C.J., and Gilman, S. (1997). Frontotemporal dementia and parkinsonism linked to chromosome 17: a consensus conference. Conference Participants. Ann. Neurol. *41*, 706–715.

Goedert, M. (1998). Neurofibrillary pathology of Alzheimer's disease and other tauopathies. Prog. Brain Res. *117*, 287–306.

Goedert, M., Spillantini, M.G., Jakes, R., Crowther, R.A., Vanmechelen, E., Probst, A., Gotz, J., Burki, K., and Cohen, P. (1995). Molecular dissection of the paired helical filament. Neurobiol. Aging *16*, 325–334.

Gotz, J., Chen, F., van Dorpe, J., and Nitsch, R.M. (2001). Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. Science *293*, 1491–1495.

Guo, S., and Kemphues, K.J. (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell *81*, 611–620.

Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science *297*, 353–356.

Heidary, G., and Fortini, M.E. (2001). Identification and characterization of the Drosophila tau homolog. Mech. Dev. *108*, 171–178.

Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., et al. (1998). Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. Nature *393*, 702–705.

Ishihara, T., Hong, M., Zhang, B., Nakagawa, Y., Lee, M.K., Trojanowski, J.Q., and Lee, V.M. (1999). Age-dependent emergence and progression of a tauopathy in transgenic mice overexpressing the shortest human tau isoform. Neuron *24*, 751–762.

Jackson, G.R., Wiedau-Pazos, M., Sang, T.K., Wagle, N., Brown, C.A., Massachi, S., and Geschwind, D.H. (2002). Human wild-type tau interacts with wingless pathway components and produces neurofibrillary pathology in Drosophila. Neuron *34*, 509–519.

Jenkins, S.M., and Johnson, G.V. (2000). Microtubule/MAP-affinity regulating kinase (MARK) is activated by phenylarsine oxide in situ and phosphorylates tau within its microtubule-binding domain. J. Neurochem. *74*, 1463–1468.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451–461.

Lee, V.M., Goedert, M., and Trojanowski, J.Q. (2001). Neurodegenerative tauopathies. Annu. Rev. Neurosci. *24*, 1121–1159.

Lewis, J., McGowan, E., Rockwood, J., Melrose, H., Nacharaju, P., Van Slegtenhorst, M., Gwinn-Hardy, K., Paul Murphy, M., Baker, M., Yu, X., et al. (2000). Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. Nat. Genet. *25*, 402–405.

Lewis, J., Dickson, D.W., Lin, W.L., Chisholm, L., Corral, A., Jones, G., Yen, S.H., Sahara, N., Skipper, L., Yager, D., et al. (2001). Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science *293*, 1487–1491.

Litersky, J.M., Johnson, G.V., Jakes, R., Goedert, M., Lee, M., and Seubert, P. (1996). 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. *316*, 655–660.

Liu, F., Iqbal, K., Grundke-Iqbal, I., and Gong, C.X. (2002). Involvement of aberrant glycosylation in phosphorylation of tau by cdk5 and GSK-3beta. FEBS Lett. *530*, 209–214.

Lovestone, S., Reynolds, C.H., Latimer, D., Davis, D.R., Anderton, B.H., Gallo, J.M., Hanger, D., Mulot, S., Marquardt, B., Stabel, S., et al. (1994). Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. Curr. Biol. *4*, 1077–1086.

Lucas, J.J., Hernandez, F., Gomez-Ramos, P., Moran, M.A., Hen, R., and Avila, J. (2001). Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. EMBO J. *20*, 27–39.

Mandelkow, E. (1999). Alzheimer's disease. The tangled tale of tau. Nature *402*, 588–589.

Mandelkow, E.M., and Mandelkow, E. (1998). Tau in Alzheimer's disease. Trends Cell Biol. *8*, 425–427.

Michel, G., Mercken, M., Murayama, M., Noguchi, K., Ishiguro, K., Imahori, K., and Takashima, A. (1998). Characterization of tau phosphorylation in glycogen synthase kinase-3beta and cyclin dependent kinase-5 activator (p23) transfected cells. Biochim. Biophys. Acta *1380*, 177–182.

Moreno, F.J., Medina, M., Perez, M., Montejo de Garcini, E., and Avila, J. (1995). Glycogen synthase kinase 3 phosphorylates recombinant human tau protein at serine-262 in the presence of heparin (or tubulin). FEBS Lett. *372*, 65–68.

Newsome, T.P., Asling, B., and Dickson, B.J. (2000). Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. Development *127*, 851–860.

Noble, W., Olm, V., Takata, K., Casey, E., Mary, O., Meyerson, J., Gaynor, K., LaFrancois, J., Wang, L., Kondo, T., et al. (2003). Cdk5 is a key factor in tau aggregation and tangle formation in vivo. Neuron *38*, 555–565.

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., Metherate, R., Mattson, M.P., Akbari, Y., and LaFerla, F.M. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron *39*, 409–421.

Patrick, G.N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L.H. (1999). Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature *402*, 615–622.

Pellettieri, J., and Seydoux, G. (2002). Anterior-posterior polarity in C. elegans and Drosophila–PARallels and differences. Science *298*, 1946–1950.

Poorkaj, P., Bird, T.D., Wijsman, E., Nemens, E., Garruto, R.M., Anderson, L., Andreadis, A., Wiederholt, W.C., Raskind, M., and Schellenberg, G.D. (1998). Tau is a candidate gene for chromosome 17 frontotemporal dementia. Ann. Neurol. *43*, 815–825.

Riechmann, V., Gutierrez, G.J., Filardo, P., Nebreda, A.R., and Ephrussi, A. (2002). Par-1 regulates stability of the posterior determinant Oskar by phosphorylation. Nat. Cell Biol. *4*, 337–342.

Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. Science *298*, 789–791.

Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G.V., Litersky, J.M., Schenk, D., Lieberburg, I., Trojanowski, J.Q., et al. (1995). Detection of phosphorylated Ser262 in fetal tau, adult tau, and paired helical filament tau. J. Biol. Chem. *270*, 18917–18922.

Shi, S.H., Jan, L.Y., and Jan, Y.N. (2003). Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. Cell *112*, 63–75.

Shulman, J.M., Benton, R., and St Johnston, D. (2000). The Drosophila homolog of C. elegans PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. Cell *101*, 377–388.

Spillantini, M.G., Murrell, J.R., Goedert, M., Farlow, M.R., Klug, A., and Ghetti, B. (1998). Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. Proc. Natl. Acad. Sci. USA *95*, 7737–7741.

Spittaels, K., Van den Haute, C., Van Dorpe, J., Geerts, H., Mercken, M., Bruynseels, K., Lasrado, R., Vandezande, K., Laenen, I., Boon, T., et al. (2000). Glycogen synthase kinase-3beta phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice. J. Biol. Chem. *275*, 41340–41349.

Sun, T.Q., Lu, B., Feng, J.J., Reinhard, C., Jan, Y.N., Fantl, W.J., and Williams, L.T. (2001). PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. Nat. Cell Biol. *3*, 628–636.

Timm, T., Li, X.Y., Biernat, J., Jiao, J., Mandelkow, E., Vandekerckhove, J., and Mandelkow, E.M. (2003). MARKK, a Ste20-like kinase, activates the polarity-inducing kinase MARK/PAR-1. EMBO J. *22*, 5090–5101.

Tomancak, P., Piano, F., Riechmann, V., Gunsalus, K.C., Kemphues, K.J., and Ephrussi, A. (2000). A Drosophila melanogaster homologue of Caenorhabditis elegans par-1 acts at an early step in embryonic-axis formation. Nat. Cell Biol. *2*, 458–460.

Wittmann, C.W., Wszolek, M.F., Shulman, J.M., Salvaterra, P.M., Lewis, J., Hutton, M., and Feany, M.B. (2001). Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. Science *293*, 711–714.

Yang, Y., Nishimura, I., Imai, Y., Takahashi, R., and Lu, B. (2003). Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila. Neuron *37*, 911–924.

Zheng-Fischhofer, Q., Biernat, J., Mandelkow, E.M., Illenberger, S., Godemann, R., and Mandelkow, E. (1998). Sequential phosphorylation of Tau by glycogen synthase kinase-3beta and protein kinase A at Thr212 and Ser214 generates the Alzheimer-specific epitope of antibody AT100 and requires a paired-helical-filament-like conformation. Eur. J. Biochem. *252*, 542–552.