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Microtubule affinity–regulating kinase 4 with an Alzheimer's disease-related mutation promotes tau accumulation and exacerbates neurodegeneration

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Accumulation of the microtubule-associated protein tau is associated with Alzheimer's disease (AD). In AD brain, tau is abnormally phosphorylated at many sites, and phosphorylation at Ser-262 and Ser-356 plays critical roles in tau accumulation and toxicity. Microtubule affinity–regulating kinase 4 (MARK4) phosphorylates tau at those sites, and a double *de novo* mutation in the linker region of MARK4, ΔG316E317D, is associated with an elevated risk of AD. However, it remains unclear how this mutation affects phosphorylation, aggregation, and accumulation of tau and tau-induced neurodegeneration. Here, we report that MARK4$^{\Delta G316E317D}$ increases the abundance of highly phosphorylated, insoluble tau species and exacerbates neurodegeneration via Ser-262/356–dependent and –independent mechanisms. Using transgenic *Drosophila* expressing human MARK4 (MARK4^{wt}) or a mutant version of MARK4 (MARK4$^{\Delta G316E317D}$), we found that coexpression of MARK4^{wt} and MARK4$^{\Delta G316E317D}$ increased total tau levels and enhanced tau-induced neurodegeneration and that MARK4$^{\Delta G316E317D}$ had more potent effects than MARK4^{wt}. Interestingly, the *in vitro* kinase activities of MARK4^{wt} and MARK4$^{\Delta G316E317D}$ were similar. When tau phosphorylation at Ser-262 and Ser-356 was blocked by alanine substitutions, MARK4^{wt} did not promote tau accumulation or exacerbate neurodegeneration, whereas coexpression of MARK4$^{\Delta G316E317D}$ did. Both MARK4^{wt} and MARK4$^{\Delta G316E317D}$ increased the levels of oligomeric forms of tau; however, only MARK4$^{\Delta G316E317D}$ further increased the detergent insolubility of tau *in vivo*. Together, these findings suggest that MARK4$^{\Delta G316E317D}$ increases tau levels and exacerbates tau toxicity via a novel gain-of-function mechanism and that modification in this region of MARK4 may affect disease pathogenesis.

Accumulation of the microtubule-associated protein tau is a pathological hallmark of AD and other neurodegenerative diseases (1–6). Tau is a natively unfolded protein, and its post-translational modifications affect the processes of its oligomerization and aggregation (6). Tau proteins are phosphorylated at multiple sites by a number of kinases (3–5, 7). Among them, kinases that belong to the conserved Par-1/microtubule affinity–regulating kinase (MARK) family phosphorylate tau within the microtubule-binding repeats at Ser-262 and Ser-356. Phosphorylation of tau at these sites regulates its microtubule binding, intracellular localization, and protein–protein interactions (8–15). Tau phosphorylated at these sites are found in preneurofibrillary tangles and associated with higher potency of accumulation seeding, thus are believed to play an initiating role in tau abnormality (8, 11–14, 16–19).

Mammals have four Par-1/MARK family members, MARK1–4 (1). MARK4 dysregulation has been proposed to play a role in the pathogenesis of AD. MARK4 expression is elevated in the brains of AD patients, and its activity colocalizes with early pathological changes (20). MARK4 can potentiate tau aggregation *in vitro* (21), and a significant SNP has been mapped to MARK4 in a regional Bayesian genome-wide association study of AD (22). Importantly, a *de novo* mutation in MARK4, resulting in double amino acid change (ΔGly-316, E317D), has been linked to an elevated risk of early-onset AD (23). The level of tau phosphorylated at Ser-262 is higher when tau is coexpressed with mutant MARK4 than when tau is coexpressed with WT MARK4 (23), suggesting that this mutation increases the risk of AD by promoting the production of abnormally phosphorylated tau. However, it is not fully understood how this mutation alters the effects of MARK4 on tau metabolism and toxicity.

In this study, we used a *Drosophila* model to compare the effects of MARK4 carrying the ΔGly-316, E317D mutation (MARK4$^{\Delta G316E317D}$) on tau accumulation and toxicity with those of WT MARK4 (MARK4^{wt}). The results revealed that coexpression of MARK4$^{\Delta G316E317D}$ increases the abundance of highly phosphorylated and insoluble tau species, resulting in enhanced accumulation and toxicity of tau, via a novel gain-of-function mechanism.

Results

MARK4$^{\Delta G316E317D}$ increases tau accumulation and promotes tau toxicity to a greater extent than MARK4^{wt}

To assess the differences between the effects of MARK4^{wt} and MARK4$^{\Delta G316E317D}$ on metabolism and toxicity of tau *in vivo*, we established transgenic fly lines carrying human MARK4^{wt} or MARK4$^{\Delta G316E317D}$ under the control of a Gal4-responsive upstream activation sequence (UAS) sequence (Fig. 1*A*). MARK4^{wt} or MARK4$^{\Delta G316E317D}$ was coexpressed with human

This article contains supporting information.
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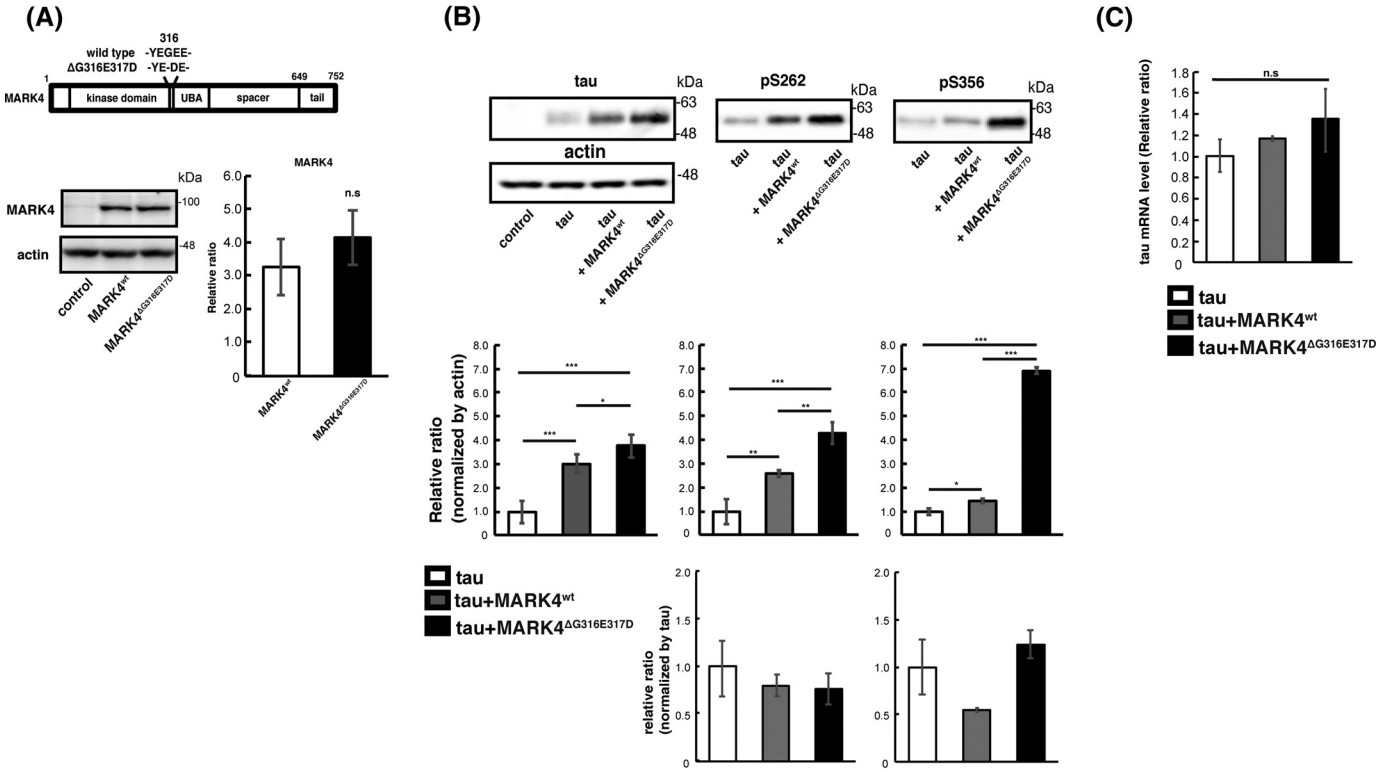

Figure 1. MARK4$^{\Delta G316E317D}$ increases the level of tau to a greater extent than MARK4^{wt}. *A*, (*top*) schematic representation of MARK4 and ΔG316E317D mutation. (*bottom*) MARK4^{wt} and MARK4$^{\Delta G316E317D}$ are expressed at similar levels in fly eyes. MARK4 expression was driven by the pan-retinal driver GMR-Gal4. Levels of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ in fly head lysates were examined by Western blotting. Actin was used as a loading control. Representative blots (*left panels*) and quantification (*right panels*) are shown. *B*, coexpression of MARK4^{wt} increased the levels of phosphorylated tau and total tau, and coexpression of MARK4$^{\Delta G316E317D}$ increased the levels of total tau, pSer-262–tau, and pSer-356–tau in the eyes to a significantly greater extent than MARK4^{wt}. Western blotting was performed with anti-tau antibody T46 (tau), anti-phospho–Ser-262 tau antibody (pSer-262), or anti-phospho–Ser-356 antibody (pSer-356). Representative blots (*top*) and quantification normalized to actin, or to total tau (*bottom panels*), are shown. *C*, coexpression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ does not affect tau mRNA levels. mRNA levels of tau expressed alone (*tau*), tau coexpressed with MARK4^{wt} (*tau+MARK4^{wt}*), and tau coexpressed with MARK4$^{\Delta G316E317D}$ (*tau+MARK4$^{\Delta G316E317D}$*) were measured by quantitative PCR. Means ± S.D. (*error bars*); $n = 4$. *n.s.* (not significant), $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ (one-way ANOVA and Tukey's post-hoc test).

tau in the retina using the pan-retinal GMR-Gal4 driver. Western blotting revealed that these flies expressed MARK4^{wt} and MARK4$^{\Delta G316E317D}$ at similar levels (Fig. 1*A*). MARK4 phosphorylates tau at Ser-262 and Ser-356 (24), and we previously reported that tau phosphorylation at Ser-262 and Ser-356 by Par-1, a member of the PAR-1/MARK family, stabilizes tau and increases total tau levels (11, 12). Both MARK4^{wt} and MARK4$^{\Delta G316E317D}$ increased the levels of total tau and tau phosphorylated at Ser-262 and Ser-356; MARK4$^{\Delta G316E317D}$ had stronger effects on both (Fig. 1*B*). The anti-phospho Ser-262 tau antibody and anti-phospho–Ser-356 tau antibody do not detect tau species that are not phosphorylated at corresponding sites ((11, 12) and Fig. S1). The prominent increase in tau phosphorylated at Ser-356 by MARK4$^{\Delta G316E317D}$ is noteworthy because tau phosphorylation at Ser-356 in this model reflects abnormally elevated PAR-1/MARK activity (12). Coexpression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ did not affect the mRNA levels of tau (Fig. 1*C*), indicating that the increase in tau levels was not due to elevated transcription of the tau transgene.

Next, we analyzed the effect of coexpression of MARK4 on tau toxicity. Expression of human tau in the eyes causes age-dependent and progressive neurodegeneration in the lamina, the first synaptic neuropil of the optic lobe containing photoreceptor axons (17). We found that flies coexpressing tau and MARK4^{wt} or MARK4$^{\Delta G316E317D}$ exhibited more neurodegeneration in the lamina than those expressing tau alone. Moreover, coexpression of MARK4$^{\Delta G316E317D}$ caused more prominent neurodegeneration than the coexpression of MARK4^{wt} (Fig. 2, *A* and *B*). By contrast, expression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ alone, without tau, did not cause neurodegeneration at the same age (Fig. 2, *A* and *B*). Together, these results suggest that MARK4$^{\Delta G316E317D}$ promotes tau accumulation and exacerbates tau toxicity to a greater extent than MARK4^{wt}.

MARK4^{wt} and MARK4$^{\Delta G316E317D}$ have similar kinase activities in vitro

A previous study reported that, when HEK293 cells were cotransfected with tau and either MARK4^{wt} or MARK4$^{\Delta G316E317D}$, cells transfected with MARK4$^{\Delta G316E317D}$ exhibited a significant increase in tau Ser-262 phosphorylation relative to those transfected with MARK4^{wt} (23). It was interpreted as evidence that the *de novo* mutation in MARK4 increases the ability of MARK4 to phosphorylate tau on Ser-262 more efficiently (23). However, it has not yet been tested whether MARK4$^{\Delta G316E317D}$ has higher kinase activity than MARK4^{wt}. To explore this

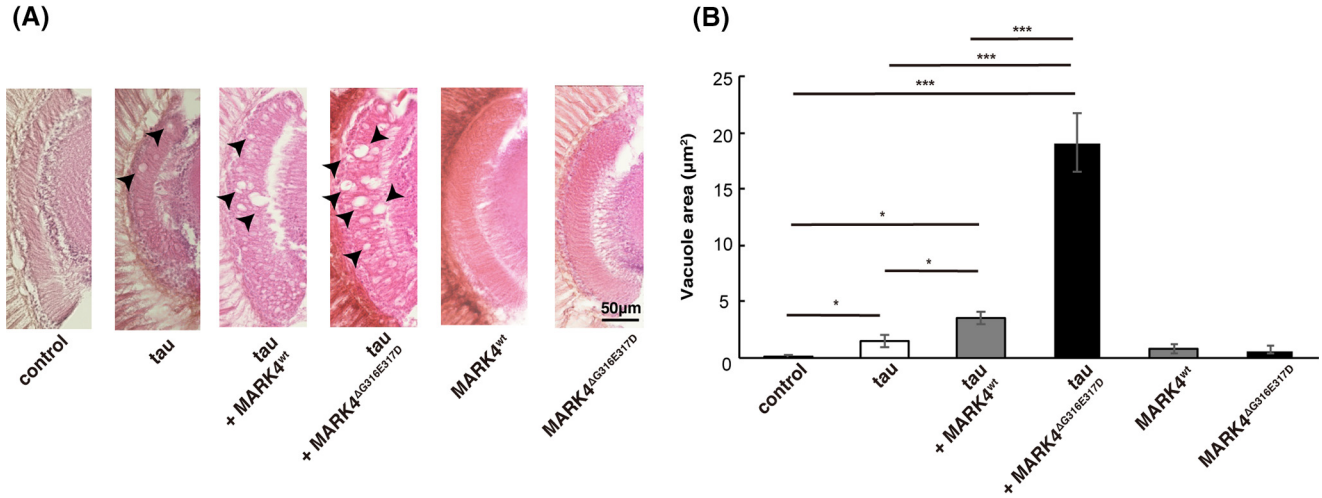

Figure 2. MARK4$^{\Delta G316E317D}$ increases tau toxicity to a greater extent than MARK4^{wt}. *A*, coexpression of MARK4 increased tau-induced neurodegeneration, and MARK4$^{\Delta G316E317D}$ exerted a stronger effect than MARK4^{wt}. Shown are lamina of flies carrying GMR-Gal4 driver alone (*control*), flies expressing tau (*tau*), flies coexpressing tau and MARK4^{wt} (*tau+MARK4^{wt}*), or flies coexpressing tau and MARK4$^{\Delta G316E317D}$ (*tau+MARK4$^{\Delta G316E317D}$*). Expression of MARK4^{wt} (*MARK4^{wt}*) or MARK4$^{\Delta G316E317D}$ (*MARK4$^{\Delta G316E317D}$*) alone did not cause neurodegeneration. Neurodegeneration is observed as vacuoles, indicated by *arrows*. *B*, quantification of the vacuole area. Means ± S.E. (*error bars*). $n = 5$. *n.s.* (not significant), $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ (one-way ANOVA and Tukey's post-hoc test).

possibility, we carried out an *in vitro* kinase assay. Specifically, we expressed MARK4^{wt} or MARK4$^{\Delta G316E317D}$ in HEK293 cells, immunoprecipitated MARK4 proteins from cell lysates, and measured their kinase activities using recombinant tau as a substrate. MARK4^{wt} or MARK4$^{\Delta G316E317D}$ had similar kinase activities (Fig. 3), indicating that the higher level of tau accumulation in cells expressing MARK4$^{\Delta G316E317D}$ was not due to a difference in kinase activity.

MARK4$^{\Delta G316E317D}$, but not MARK4^{wt}, increases tau levels and exacerbates tau toxicity in a Ser-262/356–independent manner

We previously reported that Par-1 overexpression causes tau accumulation via its phosphorylation at Ser-262 and Ser-356 and thus enhances neurodegeneration (11, 12). To determine whether the exacerbation of tau accumulation and toxicity by MARK4^{wt} or MARK4$^{\Delta G316E317D}$ is also mediated by tau phosphorylation at Ser-262 and Ser-356, we used a tau mutant in which both of those sites are replaced by alanines (S2A) (25). Similar to Par-1 (11, 12), the expression of MARK4^{wt} did not increase the level of S2A tau (Fig. 4*A*). We also found that MARK4^{wt} did not exacerbate neurodegeneration caused by S2A tau (Fig. 4*B*). These results suggest that MARK4^{wt} increases tau levels and tau toxicity through tau phosphorylation at Ser-262 and Ser-356.

By contrast, MARK4$^{\Delta G316E317D}$ significantly increased the levels of S2A tau (Fig. 4*A*), and coexpression of MARK4$^{\Delta G316E317D}$ with S2A tau exacerbated neurodegeneration (Fig. 4*B*). These results suggest that coexpression of MARK4$^{\Delta G316E317D}$ increases tau levels and tau toxicity via a novel gain-of-function mechanism that does not involve phosphorylation at Ser-262 and Ser-356.

Expression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ does not disrupt overall protein degradation

We found that MARK4$^{\Delta G316E317D}$ increased tau protein levels without affecting the mRNA levels of tau (Fig. 1*C*). To investigate the mechanisms underlying the accumulation of tau caused by MARK4$^{\Delta G316E317D}$, we sought to determine whether this effect is specific to tau protein. When coexpressed with firefly luciferase, MARK4$^{\Delta G316E317D}$ did not increase luciferase levels, indicating that the mutant protein does not cause nonspecific accumulation of exogenously expressed proteins (Fig. 5*A*).

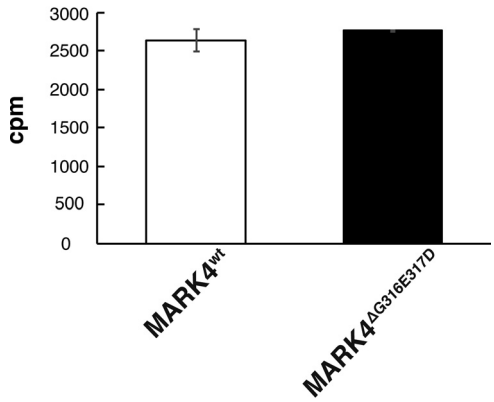

Figure 3. MARK4^{wt} or MARK4$^{\Delta G316E317D}$ have similar kinase activity *in vitro*. MARK4^{wt} and MARK4$^{\Delta G316E317D}$ expressed in HEK293 cells were immunoprecipitated and subjected to *in vitro* kinase assays. Incorporation of ^{32}P in recombinant tau protein is expressed as means ± S.D. (*error bars*). ($n = 3$, *n.s.* (not significant), $p > 0.05$ (Student's *t* test)).

We next examined whether MARK4$^{\Delta G316E317D}$ inhibits autophagic activity. MARK4 is known to negatively regulate the mTOR complex 1 (mTORC1) (26). mTORC1 is a conserved regulator of autophagy, which mediates the degradation of a variety of proteins, including tau (27). To test whether MARK4^{wt} and MARK4$^{\Delta G316E317D}$ had distinct effects on mTOR signaling, we first investigated whether expression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ in the fly retina inhibits TOR signaling. We found that neither MARK4^{wt} nor MARK4$^{\Delta G316E317D}$ affected phosphorylation of S6K, a target of TOR (Fig. 5*B*). In addition, neither MARK4^{wt} nor MARK4$^{\Delta G316E317D}$ affected LC3-II/LC3-I

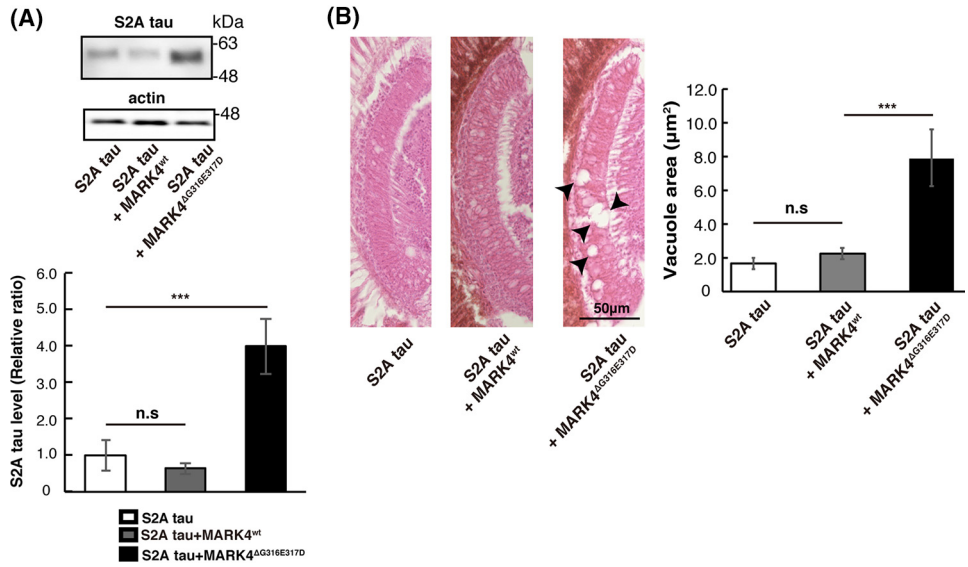

Figure 4. MARK4$^{\Delta G316E317D}$ increases tau levels and tau toxicity in a Ser-262/356–independent manner. *A*, MARK4$^{\Delta G316E317D}$ positively regulated tau levels in a Ser-262/356–independent manner. Western blots of fly heads expressing S2A tau alone or coexpressing S2A tau with either MARK4^{wt} (*S2A tau+MARK4^{wt}*) or MARK4$^{\Delta G316E317D}$ (*S2A tau+MARK4$^{\Delta G316E317D}$*). Means ± S.D. (*error bars*), $n = 4$. *n.s.* (*not significant*), $p > 0.05$; ***$p < 0.005$ (one-way ANOVA and Tukey's post-hoc test). *B*, coexpression of S2A tau with MARK4$^{\Delta G316E317D}$, but not with MARK4^{wt}, enhanced neurodegeneration. Shown are lamina of flies harboring expressing S2A tau (*S2A tau*), coexpressing S2A tau and MARK4^{wt} (*S2A tau+MARK4^{wt}*), or coexpressing S2A tau and MARK4$^{\Delta G316E317D}$ (*S2A tau+MARK4$^{\Delta G316E317D}$*). Means ± S.E. (*error bars*); $n = 5$. *n.s.* (not significant), $p > 0.05$; ***$p < 0.005$ (one-way ANOVA and Tukey's post-hoc test).

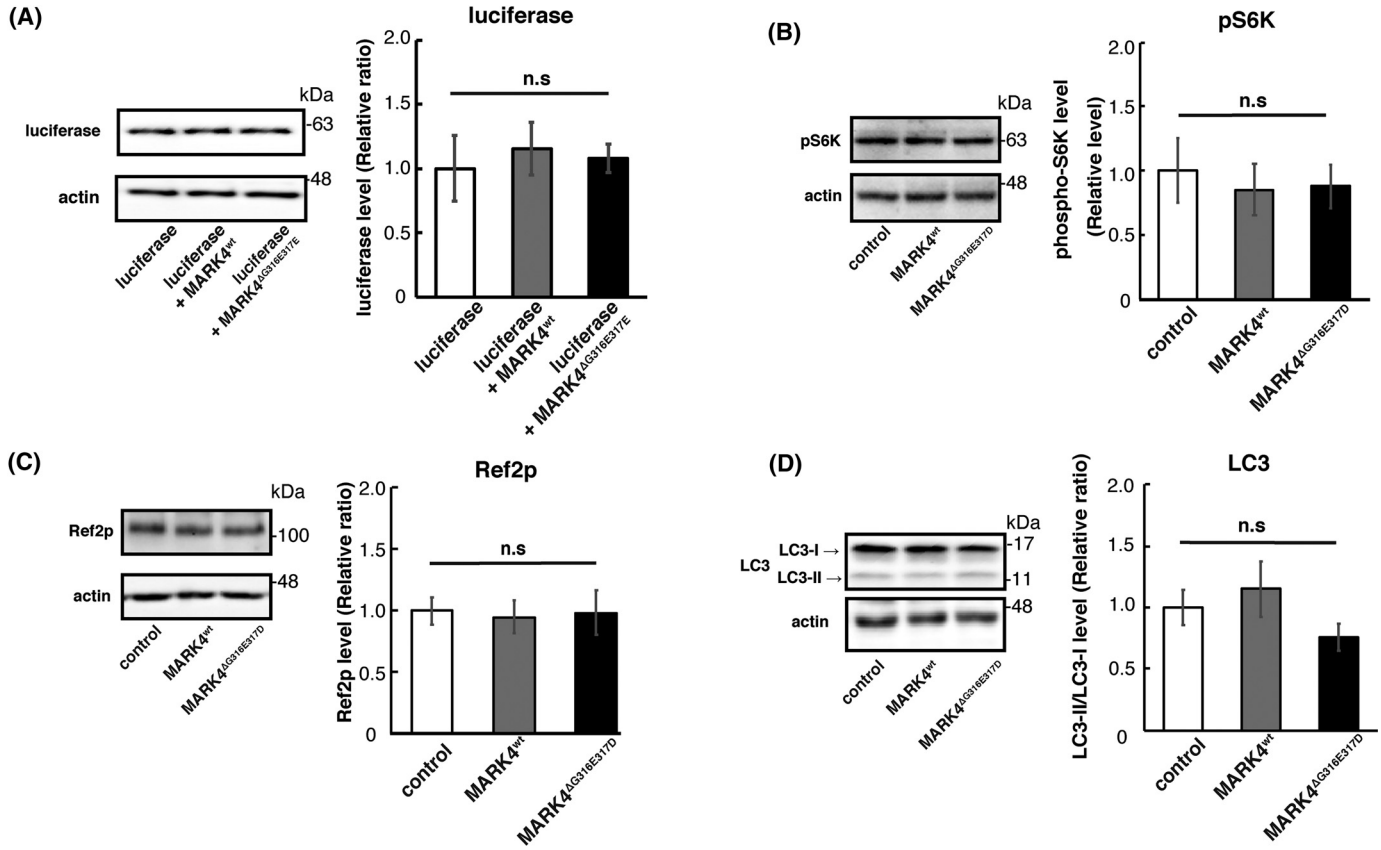

Figure 5. Neither MARK4^{wt} nor MARK4$^{\Delta G316E317D}$ inhibit the TOR pathway or increase autophagic activity. *A*, MARK4$^{\Delta G316E317D}$ did not increase the levels of luciferase coexpressed in fly eyes. Western blots were performed on fly heads expressing luciferase alone (*luciferase*), coexpressing luciferase and MARK4^{wt} (*luciferase+MARK4^{wt}*), or coexpressing luciferase and MARK4$^{\Delta G316E317D}$ (*luciferase+MARK4$^{\Delta G316E317D}$*). Actin was used as a loading control. Representative blots and quantitation are shown. Means ± S.D. (*error bars*); $n = 4$. *n.s.* (not significant), $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ (one-way ANOVA and Tukey's post-hoc test). *B–D*, expression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ did not promote autophagy. Western blots were performed on fly heads expressing control, MARK4^{wt}, or MARK4$^{\Delta G316E317D}$. Blots were performed with anti-pS6K (*pS6K*) (*B*), anti-Ref2P (*Ref2P*) (*C*), and LC3 antibody (*LC3*) (*D*). Representative blots and quantitation are shown. Means ± S.D. (*error bars*); $n = 3$. *n.s.* (not significant), $p > 0.05$ (one-way ANOVA and Tukey post-hoc test). Actin was used as a loading control.

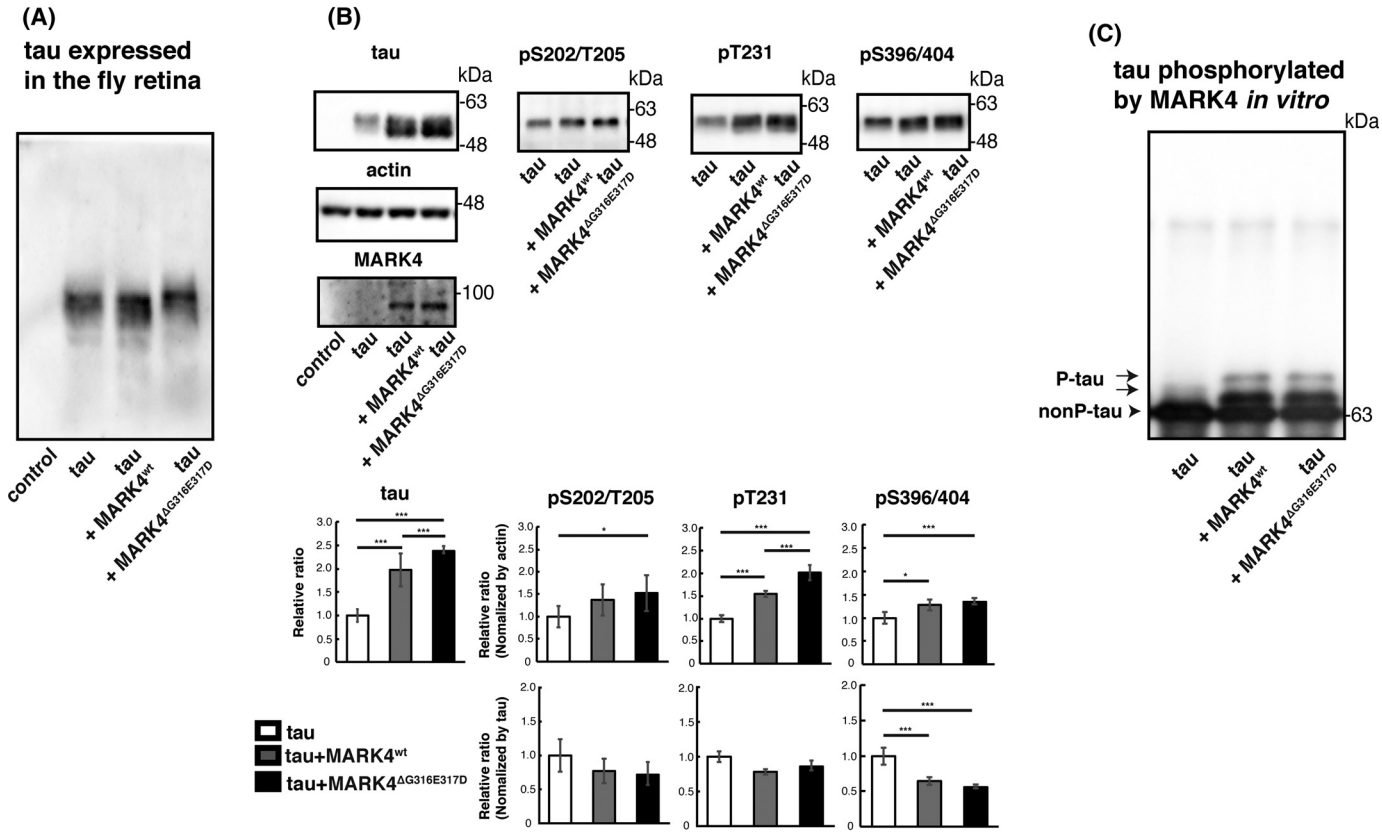

Figure 6. Coexpression of MARK4$^{\Delta G316E317D}$ causes accumulation of total tau and tau-phosphorylated at sites other than Ser-262/356 *in vivo*. *A*, phosphorylation profile of tau expressed alone or coexpressed with MARK4^{wt} or MARK4$^{\Delta G316E317D}$ *in vivo*. Fly head extracts were separated by Phos-tag SDS-PAGE, followed by Western blotting with anti-tau antibody. Coexpression of MARK4^{wt} increased the abundance of faster-migrating tau. By contrast, coexpression of MARK4$^{\Delta G316E317D}$ increased the abundance of slower-migrating tau. *B*, Western blotting was performed using phospho-specific antibodies for SP/TP sites such as AT8 (*pS202/T205*), anti-pThr231 (*pT231*), and PHF1 (*pS396/404*), as well as pan-tau antibody T46 (*tau*). Actin was used as a loading control. Representative blots (*top*) and quantification (*bottom panels*, normalized either to actin or to total tau) are shown. Means ± S.D. (*error bars*); $n = 4$. *n.s.* (not significant), $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$ (one-way ANOVA and Tukey's post-hoc test). *C*, MARK4^{wt} and MARK4$^{\Delta G316E317D}$ phosphorylated tau in the same pattern *in vitro*. MARK4^{wt} and MARK4$^{\Delta G316E317D}$ expressed in HEK293 cells were immunoprecipitated and incubated with recombinant tau. Tau proteins were separated by Phos-tag SDS-PAGE, followed by Western blotting with anti-tau antibody T46.

ratio or the levels of the autophagic substrate Ref2P (Fig. 5, *C* and *D*), suggesting that autophagic activity was not dampened. These results suggest that MARK4$^{\Delta G316E317D}$ causes accumulation of tau via a mechanism other than overall suppression of protein degradation.

MARK4$^{\Delta G316E317D}$ increases the levels of total and phosphorylated tau in vivo, although it does not phosphorylate tau directly at additional sites

Because hyperphosphorylation of tau is correlated with tau pathology (28, 29), we asked whether MARK4$^{\Delta G316E317D}$ increases overall tau phosphorylation levels. We used Phos-tag analysis for comprehensive quantitative profiling of the phosphorylation status of tau (30). *Drosophila* expresses a single member of the Par-1/MARK family, Par-1; we previously reported that Par-1 phosphorylates human tau proteins at Ser-262 and Ser-356, stabilizing them and promoting their subsequent phosphorylation at other sites (13, 17). To highlight the difference between the effects of MARK4^{wt} and MARK4$^{\Delta G316E317D}$ on tau phosphorylation in this model, we carried out this experiment in a Par-1–knockdown background. Tau protein expressed in the fly retina separated into several bands, indicating that it was phosphorylated in multiple patterns (Fig. 6*A*). Interestingly, the phosphorylation forms of tau whose abundances increased differed depending on whether MARK4^{wt} or MARK4$^{\Delta G316E317D}$ was coexpressed. MARK4^{wt} increased the intensity of a faster-migrating band, and coexpression of MARK4$^{\Delta G316E317D}$ further increased the intensity of the slower-migrating band, indicating that MARK4$^{\Delta G316E317D}$ increased tau species with additional phosphorylation *in vivo* (Fig. 6*A*).

Tau has many serine and threonine sites followed by prolines, which are phosphorylated by proline-directed Ser/Thr kinases (SP/TP kinases) such as GSK3β, MAPK, and cyclin-dependent kinase 5 (Cdk5) (31–34). Although Par-1 does not directly phosphorylate those sites, Par-1 overexpression causes an overall increase in tau levels and proportional increases in tau phosphorylated at SP/TP sites (11). To determine whether MARK4$^{\Delta G316E317D}$ promotes accumulation of tau phosphorylated at SP/TP sites to a greater extent than MARK4^{wt}, we carried out Western blotting using phospho-specific antibodies against tau phosphorylated at the following SP/TP sites: AT8 (Ser-202, Thr-205), AT180 (Thr-231), and PHF1 (Ser-396, Ser-404). Coexpression of tau and MARK4^{wt} increased the levels of tau phosphorylated at these sites (Fig. 6*B*), and coexpression of tau and MARK4$^{\Delta G316E317D}$ further increased them, relative to

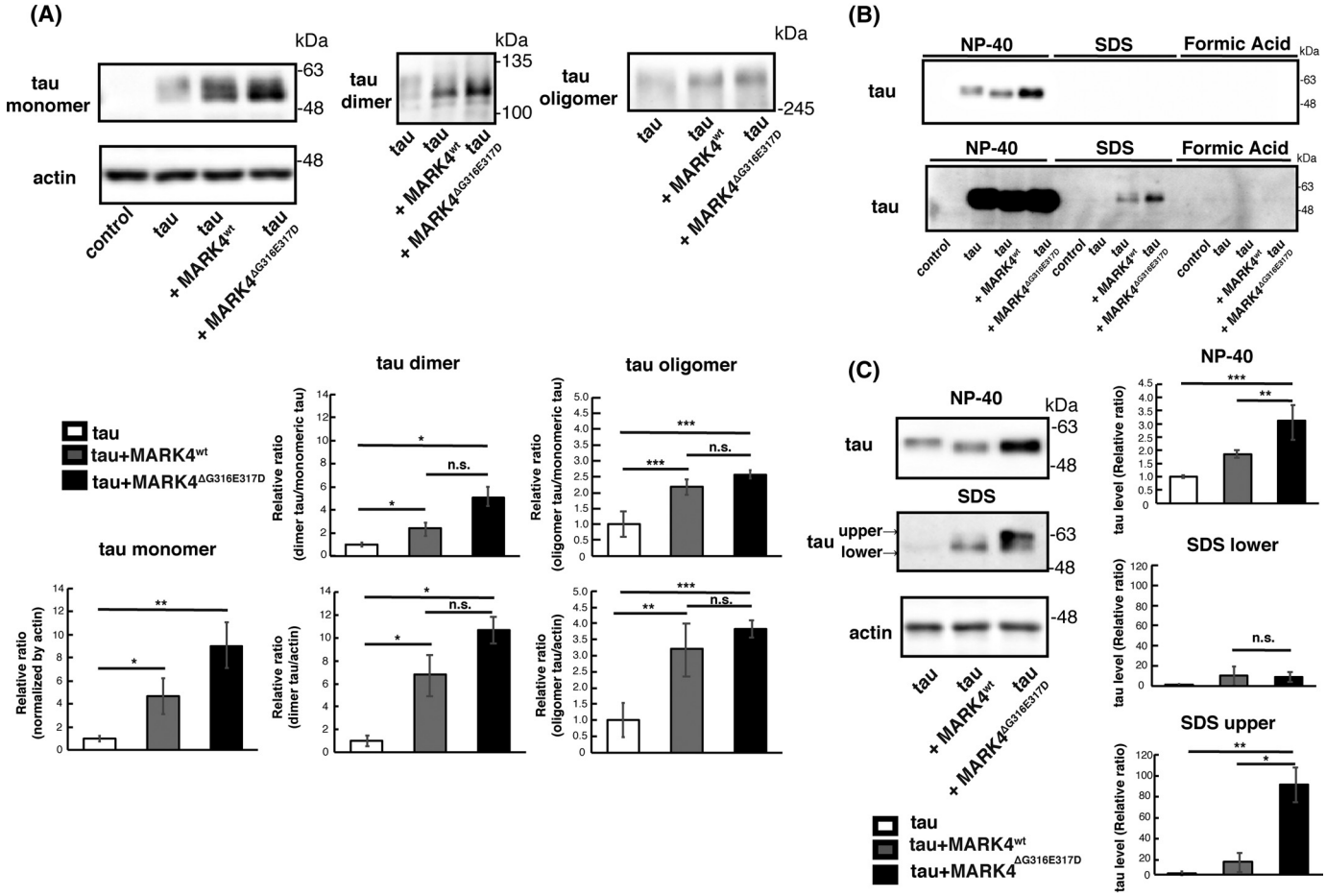

Figure 7. MARK4$^{\Delta G316E317D}$ promotes misfolding of tau. *A*, MARK4^{wt} and MARK4$^{\Delta G316E317D}$ promoted accumulation of tau oligomers and dimers at similar levels. Western blotting was performed on fly head lysates prepared under nonreducing conditions with oligomer-specific antibody T22 (*oligomer*) and pan-tau antibody Tau5 (*dimer*). Representative blots and quantification are shown. Actin was used as a loading control. Means ± S.D. (*error bars*); $n = 3$. *n.s.* (not significant), $p > 0.05$; *$p < 0.05$, **$p < 0.01$ (one-way ANOVA and Tukey's post-hoc test). *B* and *C*, tau in extracts from heads expressing tau alone (*tau*), coexpressing tau and MARK4^{wt} (*tau+MARK4^{wt}*), or coexpressing tau and MARK4$^{\Delta G316E317D}$ (*tau+MARK4$^{\Delta G316E317D}$*) were detected by Western blotting with pan-tau antibody T46. Head homogenates were extracted first with RIPA buffer containing NP-40 (*NP-40*), next with 2% SDS (*SDS*), and finally with 70% formic acid (*FA*). *B*, representative Western blots are shown (shorter exposure (*upper panel*) and longer exposure (*lower panel*)). *C*, Western blotting was performed on fly heads extracted with RIPA buffer containing NP-40 (*NP-40*) and then with 2% SDS (*SDS*). Representative blots (*left*) and quantification (*right*) are shown.

expression of tau alone (Fig. 6*B*). These SP/TP sites are not expected to be direct substrates of MARK4 (13, 35), and the increases in the phospho-SP/TP tau species are proportional to those in total tau levels (Fig. 6*B*), suggesting that the elevated levels of the phospho-SP/TP tau caused by coexpression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ are likely a consequence of the accumulation of total tau.

However, we wondered whether the ΔG316E317D mutation might alter the kinases' substrate specificity and whether MARK4$^{\Delta G316E317D}$ phosphorylates SP/TP sites. To determine whether MARK4$^{\Delta G316E317D}$ directly phosphorylates those sites, we carried out an *in vitro* assay. Recombinant tau was incubated with MARK4^{wt} or MARK4$^{\Delta G316E317D}$, and the phosphorylation pattern of tau was analyzed using the Phos-tag method. Tau exhibited the same phosphorylation pattern when it was incubated with MARK4^{wt} or with MARK4$^{\Delta G316E317D}$ (Fig. 6*C*), suggesting that MARK4$^{\Delta G316E317D}$ increases accumulation of tau species phosphorylated at SP/TP sites via an indirect mechanism *in vivo*.

MARK4^{wt} and MARK4$^{\Delta G316E317D}$ both increase tau dimers and oligomers, and MARK4$^{\Delta G316E317D}$ further promotes accumulation of insoluble forms of tau

Tau can aggregate into higher-order structures prone to accumulate, and tau phosphorylation at SP/TP sites promotes this process (29, 36). Hence, we asked whether coexpression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ would affect the formation of small aggregates such as dimers and oligomers. Tau proteins expressed in the *Drosophila* retina exist mostly in the monomeric form (18, 37). We found that both MARK4^{wt} and MARK4$^{\Delta G316E317D}$ increased the levels of dimers and oligomers to similar extents (Fig. 7*A*).

Next, to assess whether coexpression of MARK4^{wt} or MARK4$^{\Delta G316E317D}$ would affect the detergent insolubility of tau, we performed sequential extraction followed by Western blotting with anti-tau antibody (38). As reported previously, tau proteins in the *Drosophila* retina exist mostly in a detergent-soluble form (18, 37) (Fig. 7*B*). When expressed alone or coexpressed with MARK4^{wt} or MARK4$^{\Delta G316E317D}$, tau was

mostly extracted with RIPA buffer containing Nonidet P-40 (NP-40) (Fig. 7*B*, *NP-40*). However, tau proteins were present in the SDS-soluble fraction when tau was coexpressed with MARK4^{wt} or MARK4$^{\Delta G316E317D}$, and this effect was more prominent when MARK4$^{\Delta G316E317D}$ was expressed (Fig. 7*B*, *SDS*). Tau was not detected in the detergent-insoluble fraction when coexpressed with either MARK4^{wt} or MARK4$^{\Delta G316E317D}$ (Fig. 7*B*, *Formic Acid*).

Tau proteins migrate into several bands with the Laemmli SDS-PAGE, and the migration speeds of these bands are related to their phosphorylation levels. We previously reported that tau in the slower-migrating band (tau^{upper}) is more often phosphorylated at SP/TP sites than tau in the faster-migrating band (tau$_{lower}$) (11). Tau species in the NP-40 fraction were mostly tau$_{lower}$ (Fig. 7*C*, *NP-40*), whereas tau proteins accumulating in the SDS fraction contained both tau^{upper} and tau$_{lower}$. Consistent with the accumulation of phospho-SP/TP tau species (Fig. 6*B*), coexpression of MARK4$^{\Delta G316E317D}$, but not MARK4^{wt}, increased the level of tau^{upper} in the SDS-soluble fraction (Fig. 7*C*, *SDS*).

We further analyzed whether coexpression with MARK4^{wt} or MARK4$^{\Delta G316E317D}$ affects the solubility of S2A tau. In contrast to WT tau, dimers and NP-40–insoluble forms of S2A were undetectable with or without coexpression of either MARK4^{wt} or MARK4$^{\Delta G316E317D}$ (Fig. S2). S2A oligomers were detected, although either MARK4^{wt} or MARK4$^{\Delta G316E317D}$ did not increase S2A oligomers (Fig. S2). These results suggest that MARK4$^{\Delta G316E317D}$ promotes the accumulation of S2A tau through mechanisms other than increasing its insolubility.

Taken together, these results indicate that MARK4^{wt} increases the levels of Ser-262/356–phosphorylated tau, dimers, and oligomers. By contrast, MARK4$^{\Delta G316E317D}$ promotes the accumulation of not only those tau species but also insoluble tau species, which may promote neurodegeneration.

Discussion

Accumulating evidence suggests that Par-1/MARK plays an initiating role in tau abnormalities leading to disease pathogenesis (11–14, 16–18). We previously reported that Par-1 overexpression increases, and knockdown decreases, tau phosphorylation at Ser-262 and Ser-356, which leads to accumulation of tau and exacerbation of neurodegeneration in a fly model of tau toxicity (11, 12). In this study, we found that human MARK4^{wt} increased the levels of pTauSer-262, pTauSer-356, and total tau, and increased tau toxicity (Fig. 1 and Fig. 2). MARK4^{wt} did not increase S2A tau levels or affect its toxicity (Fig. 4), suggesting that MARK4^{wt}, similar to Par-1, affects tau metabolism and toxicity through tau phosphorylation at Ser-262 and Ser-356. On the other hand, we found that MARK4$^{\Delta G316E317D}$ promoted tau accumulation via additional mechanisms. MARK4$^{\Delta G316E317D}$ increased S2A tau levels (Fig. 4), indicating that MARK4$^{\Delta G316E317D}$ can increase the abundance of tau that is not phosphorylated at Ser-262 or Ser-356. We also found that MARK4$^{\Delta G316E317D}$, but not MARK4^{wt}, caused the accumulation of the aggregated forms of tau (Fig. 6 and Fig. 7). These results suggest that MARK4^{wt} promotes accumulation of tau phosphorylated at Ser-262 and Ser-356 and that MARK4$^{\Delta G316E317D}$ further increases tau accumulation via additional mechanisms. Although MARK4$^{\Delta G316E317D}$ promotes accumulation of the detergent-insoluble form of tau (Fig. 7), considering that tau in this model exists mostly as a detergent-soluble form, MARK4$^{\Delta G316E317D}$ is likely to increase total tau levels via not only enhancing tau misfolding but also other yet-to-be-determined mechanisms. Coexpression of MARK4$^{\Delta G316E317D}$ increased S2A tau levels without affecting its solubility (Fig. S2), also suggesting that such mechanisms exist.

Rovelet-Lecrux *et al.* (23) reported that when HEK293 cells were cotransfected with tau and either MARK4^{wt} or MARK4$^{\Delta G316E317D}$ constructs, cells transfected with MARK4$^{\Delta G316E317D}$ exhibited a significant increase in tau phosphorylation at Ser-262 relative to MARK4^{wt}. The authors of that study concluded that this *de novo* mutation in MARK4 results in a gain-of-function in the ability of MARK4 to phosphorylate tau on Ser-262 (23). We also found that coexpression of MARK4$^{\Delta G316E317D}$ increased the level of tau phosphorylated at Ser-262 to a greater extent than MARK4^{wt} in fly eyes (Fig. 1). However, *in vitro* kinase assays revealed that MARK4^{wt} and MARK4$^{\Delta G316E317D}$ did not significantly differ in terms of kinase activity (Fig. 3), arguing against the idea that accumulation of tau is the result of more efficient phosphorylation of tau by MARK4$^{\Delta G316E317D}$.

The double mutation (ΔG316E317D) is located in the short linker that tethers the kinase to substrates or regulators (23). The predicted three-dimensional structure suggests that the double amino acid change (ΔG316E317D) increases the area of the substrate-binding region, facilitating a strong association of MARK4 with interacting proteins (23). MARK4 has been reported to interact with various proteins, including cytoskeletal proteins, kinases and phosphatases, ubiquitin-related enzymes, signaling molecules, mRNA binding proteins, and transcription mediators (26, 39–43). MARK4 mutation may change affinities to interacting proteins, thus creating the conditions that favor the accumulation of tau. The strengthened interaction of MARK4$^{\Delta G316E317D}$ and substrates other than tau, or interaction with novel substrates, may indirectly stabilize tau.

In addition to AD, MARK4 has been suggested to play a critical role in other neurodegenerative diseases, including ischemic axonal injury (21) and synucleinopathies (44). Under pathological conditions, abnormalities in MARK4, such as additional posttranslational modifications in the linker region, might cause effects similar to those of the ΔG316E317D mutation on MARK4 and thus promote misfolding of tau and other aggregation-prone proteins. Identification of ΔG316E317D-induced changes in MARK4$^{\Delta G316E317D}$ that promote tau accumulation may provide insight into the dysregulation of MARK4 in the pathogenesis of sporadic AD.

Experimental procedures

Fly stocks and husbandry

Flies were maintained in standard cornmeal media at 25 °C under light-dark cycles of 12:12 h. The transgenic fly line carrying the human 0N4R tau, which has four tubulin-binding

domains (R) at the C-terminal region and no N-terminal insert (N), is a kind gift from Dr. M. B. Feany (Harvard Medical School) (45). UAS-PAR-1 RNAi is a kind gift from Dr. Bingwei Lu (Stanford University) (13). UAS-S2A tau, human 0N4R tau carrying alanine substitutions at Ser-262 and Ser-356 (S2A), were reported previously (12, 17). To establish transgenic fly lines carrying UAS-MARK4^{wt} and UAS-MARK4$^{\Delta G316E317D}$, cDNA coding human MARK4 was obtained from DNASU Plasmid Repository (clone HsCD00294885, The Biodesign Institute at Arizona State University). ΔG316E317D mutation was introduced by using PCR-based site-directed mutagenesis. cDNA was subcloned into pUASTattB and injected into the oocytes carrying into P{CaryP}attP2. Genotypes of the flies used in the experiments are described in Table S1.

Chemicals and antibodies

An anti-MYC antibody (4A6) (Merck Millipore), anti-tau (T46) (Thermo Fisher Scientific), anti-tau (Tau5) (Thermo Fisher Scientific), anti-phospho–Ser-262 tau antibody (Abcam), anti-phospho–Thr-231 tau antibody (AT180, Thermo Fisher Scientific), anti-phospho–Ser-356 tau antibody (Abcam), anti-oligomer antibody (T22) (Merck Millipore), anti-phospho-S6K (Cell Signaling Technology), anti-Ref2p (Abcam), anti-LC3 (Merck Millipore), and anti-actin antibody (Sigma-Aldrich) were purchased. AT8 antibody and PHF1 antibody are kind gifts from Dr. Peter Davis (Albert Einstein College of Medicine).

Cell culture and transfection

HEK293 cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Plasmids encoding MARK4 were transfected with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol.

In vitro kinase assay of MARK4

MARK4 expressed in HEK293 cells was immunoprecipitated from the cell lysate with monoclonal anti-Myc antibody (4A6) and Dynabeads protein G (Thermo Fisher Scientific). Its kinase activity was measured using human 2N4R tau, which was expressed and purified from *E. coli* and [γ32P]ATP as substrates. Incorporation of 32P into tau was quantified using a liquid scintillation counter (Beckman).

SDS-PAGE, Phos-tag SDS-PAGE, and immunoblotting

SDS-PAGE for Western blotting of tau, MARK4, and actin was performed using 10% or 7.5% (w/v) polyacrylamide gels. Phos-tag SDS-PAGE was performed using 7.5% (w/v) polyacrylamide gels containing 25 μM or 50 μM Phos-tag acrylamide (Wako Chemicals) for tau. Proteins separated in the gel were transferred to a PVDF membrane (Merck Millipore) using a submerged blotting apparatus and then visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). The chemiluminescent signal was detected by Fusion FX (Vilber), and intensity was quantified using ImageJ (National Institutes of Health). Western blots were repeated a minimum of three times with different animals, and representative blots are shown. Flies used for Western blotting were 2 days old after eclosion.

Histological analysis

Neurodegeneration in the fly retina was analyzed as previously reported (32). Fly heads were fixed in Bouin's fixative for 48 h at room temperature, incubated for 24 h in 50 mM Tris/150 mM NaCl, and embedded in paraffin. Serial sections (7-μm thickness) through the entire heads were stained with hematoxylin and eosin and examined by bright-field microscopy. Images of the sections that include the lamina were captured with Keyence microscope BZ-X700 (Keyence), and the vacuole area was measured using ImageJ (National Institutes of Health). Heads from more than three flies (more than five hemispheres) were analyzed for each genotype.

qRT-PCR

qRT-PCR was carried out as previously reported (32). More than 30 flies for each genotype were collected and frozen. Heads were mechanically isolated, and total RNA was extracted using Isogen Reagent (Nippon Gene) according to the manufacturer's protocol with an additional centrifugation step (11,000 × *g* for 5 min) to remove cuticle membranes prior to the addition of chloroform. Total RNA was reverse-transcribed using PrimeScript Master Mix (Takara Bio). qRT-PCR was performed using TOYOBO THUNDERBIRD SYBR qPCR Mix on a Thermal Cycler Dice Real Time System (Takara Bio). The average threshold cycle value (CT) was calculated from at least three replicates per sample. The expression of genes of interest was standardized relative to rp49. Relative expression values were determined by the ΔΔCT method. Primers were designed using Primer-Blast (National Institutes of Health). The following primers were used for RT-qPCR: htau for 5′-CAAGACAGACCACGGGGCGG-3′, htau rev 5′-CTGCTTGGCCAGGGAGGCAG-3′, rp49 for 5′-GCTAAGCTGTCGCACAAATG-3′, and rp49 rev 5′-GTTCGATCCGTAACCGATGT-3′.

Sequential extraction of tau protein

The following sequential series of solubilizing buffer are RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 1% NP-40, and 50 mM NaF), 2% SDS, and 70% formic acid (38). The sample was mixed with the indicated buffer, sonicated briefly, and centrifuged at 45,000 × *g* for 30 min at 4 °C. The pellet was subjected to extraction with the indicated buffer. The supernatant was collected and subjected to immunoblot analysis as described above. The same volume of each extract was loaded per lane.

Statistics

Statistics were done with Microsoft Excel and R (R Foundation for Statistical Computing, Vienna, Austria: RRID: SCR_001905 http://www.R-project.org/). Differences were assessed using the Student's *t* test or one-way analysis of variance (ANOVA) and Tukey's honest significance difference post-hoc test. p values < 0.05 were considered statistically significant.

Data availability

All data are available in this article and in the supporting information.

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Abbreviations—The abbreviations used are: AD, Alzheimer's disease; MARK, microtubule affinity–regulating kinase; SP/TP kinases, proline-directed Ser/Thr kinases; Cdk, cyclin-dependent kinase; NP-40, Nonidet P-40; ANOVA, analysis of variance.

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