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Functional genomic screen and network analysis reveal novel modifiers of tauopathy dissociated from tau phosphorylation

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A functional genetic screen using loss-of-function and gain-of-function alleles was performed to identify modifiers of tau-induced neurotoxicity using the 2N/4R (full-length) isoform of wild-type human tau expressed in the fly retina. We previously reported eye pigment mutations, which create dysfunctional lysosomes, as potent modifiers; here, we report 37 additional genes identified from ~1900 genes screened, including the kinases *shaggy*/GSK-3beta, *par-1*/MARK, *CamKI* and *Mekk1*. Tau acts synergistically with *Mekk1* and p38 to down-regulate extracellular regulated kinase activity, with a corresponding decrease in AT8 immunoreactivity (pS202/T205), suggesting that tau can participate in signaling pathways to regulate its own kinases. Modifiers showed poor correlation with tau phosphorylation (using the AT8, 12E8 and AT270 epitopes); moreover, tested suppressors of wild-type tau were equally effective in suppressing toxicity of a phosphorylation-resistant S11A tau construct, demonstrating that changes in tau phosphorylation state are not required to suppress or enhance its toxicity. Genes related to autophagy, the cell cycle, RNA-associated proteins and chromatin-binding proteins constitute a large percentage of identified modifiers. Other functional categories identified include mitochondrial proteins, lipid trafficking, Golgi proteins, kinesins and dynein and the Hsp70/Hsp90-organizing protein (*Hop*). Network analysis uncovered several other genes highly associated with the functional modifiers, including genes related to the PI3K, Notch, BMP/TGF-β and Hedgehog pathways, and nuclear trafficking. Activity of GSK-3β is strongly upregulated due to TDP-43 expression, and reduced GSK-3β dosage is also a common suppressor of Aβ42 and TDP-43 toxicity. These findings suggest therapeutic targets other than mitigation of tau phosphorylation.

INTRODUCTION

Tau is a microtubule-associated protein that is a major component of paired helical filaments (PHFs), insoluble intracellular aggregates, the presence of which defines a class of neurodegenerative diseases termed tauopathies. There are several neurodegenerative diseases in which PHFs are the sole or primary neuropathological hallmark, including frontotemporal lobar degeneration (FTLD), corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP). Some tauopathies feature co-pathology with other protein aggregates, such as β-amyloid in Alzheimer's disease (AD) (1) or α-synuclein in Lewy body dementia (2). Pedigree studies in hereditary cases of frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (3,4) identified mutations in the *MAPT* gene, demonstrating that dysregulated or dysfunctional tau can be a causative factor in inducing neurodegeneration. However, most tauopathies are idiopathic and lack causative mutations in *MAPT*, suggesting that other factors that regulate or modify wild-type tau function must be involved.

Tau stabilizes microtubules and is thought to provide structural integrity to axons, although it is also present in dendrites

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and glia (5,6). The *MAPT* gene can be alternatively spliced into six isoforms that vary in the number of C-terminal microtubule binding domain repeats (3R or 4R) and N-terminal exons included (0N, 1N, or 2N) (7). Several post-translational modifications have also been reported, including phosphorylation, acetylation (8,9), glycosylation and nitration (10–13). Tau clearance may involve several processes, as it has been reported that tau can be targeted to lysosomes or cathepsin-mediated degradation (14,15), can be ubiquitinated and targeted to the proteasome (16,17) or can be digested by proteases such as puromycin-sensitive aminopeptidase (PSA) (18,19).

Tau is found in a hyperphosphorylated state in PHFs (20,21), and there are 79 putative serine/threonine sites for phosphorylation of which at least 45 are known to be phosphorylated (22,23). Several tau kinases have been identified, including glycogen synthase kinase-3β (GSK-3β; 24,25), microtubule-associated protein/microtubule affinity-regulating kinase (MARK; 26), cyclin-dependent kinase 5 (Cdk5; 27,28), extracellular regulated kinase (ERK; 29,30), protein kinase A (31,32), p38 (33) and c-Jun amino-terminal kinase (34). Protein phosphatases 1, 2A and 2B (PP1, PP2A, PP2B) have been shown to dephosphorylate tau (22). Hyperphosphorylated tau dissociates from microtubules (35–37), and synaptic impairment and neurodegeneration have been hypothesized to be due to destabilized microtubules in axons. However, despite the presence of hyperphosphorylated tau in PHFs, the role of tau phosphorylation in neurodegeneration is complex. As an example, phosphorylation at the Ser235, Ser404 and Thr50 sites may in fact promote microtubule binding, formation and stability at specific sites (38,39). Furthermore, tau phosphorylation at specific sites has been shown to ameliorate tau-induced deficits and toxicity (40), and *in vivo* fly models of tau toxicity using phosphorylation-resistant tau constructs show equivalent or even increased toxicity when compared with wild-type tau (41,42).

Several invertebrate and vertebrate transgenic animal models have shown that tau can induce neurodegeneration and apoptosis (43–46). Our laboratory generated a model of tauopathy in *Drosophila melanogaster* by expressing human wild-type full-length (2N/4R) tau in the eye using a direct fusion construct of the human tau cDNA to the eye-specific *glass* (*gl*) promoter (*gl*-tau fly). This misexpression causes a rough eye phenotype associated with abnormalities of photoreceptor neurons and other cell types in the underlying retina (47). The degree of roughness is intermediate and more pronounced in the anterior eye, making it useful for enhancer–suppressor modifier screens. A 'suppressor' of the phenotype will produce a larger and more wild-type-like eye, whereas an 'enhancer' of roughness will exacerbate the tau-induced toxicity and produce a smaller and more rough eye. This approach was used to validate the *in vivo* protective effects of PSA, with genetic loss-of-function (LOF) mutations in *Drosophila* PSA (dPSA) enhancing the tau phenotype and overexpression of dPSA suppressing the roughness (48).

We report here the results of two genetic enhancer–suppressor screens conducted with the *gl*-tau fly using published collections of LOF and gain-of-function (GOF) transposon insertions. The first screen utilized the 'P lethal' collection, which consists of LOF alleles caused by *LacZ* containing transposable P element insertions in essential genes (49). The second screen utilized the 'EY' collection, which consists of 'empty' UAS elements inserted in the promoters of endogenous genes (50), and can be used to overexpress the downstream gene if the UAS-binding transcription factor, GAL4, is co-expressed. We refer to this as the 'EY' or 'overexpression' screen; however, the UAS insertion can also block transcription of the downstream gene if it is inserted in the opposite orientation. These two collections were chosen for several reasons. Both were generated by random insertion of P elements throughout the fly genome, thus representing a large, unbiased, genome-spanning assortment of genes: ∼1000 P lethal stocks and 900 EY stocks were screened. The P lethal collection represents solely LOF alleles, and no LOF screen with a tauopathic animal model has previously been reported. Additionally, the use of published P element-based mutations allows for the rapid identification of the modifier genes by referencing the FlyBase database. Also, essential genes in the fly are more likely to have a homolog in vertebrates and thus may correspond to relevant human modifier genes in tauopathies. The EY collection was chosen as a GOF screen to complement the P lethal screen. A screen using a similar enhancer-promoter collection—the Rørth EP collection (51)—using a tau eye phenotype has been previously reported (52); however, the EY collection represents affected genes that are largely non-overlapping with those in the EP collection. The screen described here also differs from previously reported screens in that (i) tau expression and the phenotype of *gl*-tau eye are not GAL4-dependent, thus eliminating potential confounds of modifiers of GAL4 function and not tau toxicity, and (ii) the tau toxicity is induced by wild-type (non-mutant) tau.

In total, 37 genes of the ∼1900 lines screened were uncovered as strong modifiers of tau toxicity. From these genes, a computational network of highly associated genes was assembled that encompasses a wide range of functional categories associated with tau toxicity. The modifiers were assayed for tau phosphorylation state; however, no consistent pattern of phosphorylation correlated with suppression or enhancement of tau toxicity. However, we demonstrate a novel synergistic capacity of tau and p38 to regulate ERK activity, providing evidence for tau regulation of its own kinases. We also demonstrate that GSK-3β (shaggy) is a common suppressor of tau, Aβ42 and TDP-43 toxicity, and that GSK-3β activity is strongly upregulated due to mutant TDP-43 expression. These results provide novel associations with genes and cellular processes that widen our understanding of tau function that may represent novel therapeutic targets for tauopathies and other neurodegenerative proteinopathies. Moreover, these data provide further evidence that tau phosphorylation is not critical for tau toxicity.

RESULTS

Suppressors were selected if the eye was larger, less rough and had a more wild-type-like ommatidial organization than control eye phenotypes. Enhancers were identified if the eye was smaller and had increased ommatidial fusion and bristle loss. Additionally, a gene was called an enhancer if a necrotic plaque was present even if the eye was not smaller when compared with controls, as necrotic plaques were never observed in controls.

P lethal screen reveals 23 modifiers and EY screen reveals 14 modifiers

The locations of the P element insertions in both the P lethal and EY collections have been previously mapped and reported in the online database FlyBase.org and can be referenced using the stock number for each P lethal line. Our screen was carried out with blinded phenotypic scoring, as P lethal lines were initially known only by their stock numbers; only after the F_1 phenotypes were scored for modifying effect on the tau eye phenotype was information on P element location and the affected gene obtained. A total of 23 modifiers—11 suppressors and 12 enhancers—were identified from the P lethal screen of 920 stocks, with P element mutagens inserted on the X, 2nd and 3rd chromosomes. Figure 1 depicts scanning electron micrographs of representative eye phenotypes: all panels (except 'wild-type') have a copy of the *gl*-tau transgene in *trans* to one disrupted copy of the gene listed in the panel (genes are listed alphabetically). *Atg6* was included as an enhancer due to large black plaques in the anterior region of the eye that were commonly found in *gl*-tau/*Atg6* progeny (see arrows in Fig. 1) that were not present in controls. We have termed these plaques as 'necrotic' due to the observation of a lack of underlying tissue; however, we note that these plaques may be due to increased melanization rather than necrosis. No modifier stock showed an intrinsic effect on eye morphology when crossed to w^{1118} in order to remove any effects of balancer chromosomes (Supplementary Material, Fig. S1). The modifier stocks were then crossed to a GMR-*hid* line (53) in order to exclude suppressors of general apoptosis. The *hid* gene is proapoptotic and produces a near-complete ablation of the eye. No modifiers from the P lethal screen showed any appreciable suppression of the *hid* phenotype (Fig. 2).

Although the entire EY collection is a continually growing library of over 3000 lines, only 895 of these stocks were associated with named gene function at the time this screen was initiated. To facilitate identification of relevant genes, only these 895 annotated EY stocks were screened. Of these, 19 were putative modifiers. These putative modifiers were crossed to GMR-GAL4 alone in order to determine whether the modified eye phenotype was independent of tau toxicity; of these, 16 modifiers showed no independent effect on eye morphology. Finally, these modifiers were crossed to a GMR-*hid* line expressed in *trans* to GMR-GAL4 on the X chromosome (GMR-GAL4;GMR-hid) in order to exclude modifiers of general apoptosis. Of the 16 lines screened, 12 showed no appreciable suppression of GMR-*hid* phenotype (Fig. 2). Although *Hr39* and *CamKI* demonstrated moderate suppression of the *hid* phenotype, they showed enhancement of tau toxicity; thus, their effect on tau toxicity is unlikely related to any anti-apoptotic effects and they were included as tau modifiers making for a total of 14 EY modifiers. Finally, to ascertain whether the EY element insertion was a GOF or LOF allele, the 14 modifier genes were crossed to the original *gl*-tau fly without GMR-GAL4. If the insertion induced a GOF, then crossing to *gl*-tau without GAL4 will show either no modifier effect when compared with the *gl*-tau control or will show a phenotype opposite to that of the GMR-GAL4;*gl*-tau phenotype. If crossing the modifier line to *gl*-tau alone showed the same phenotype as crossed to *gl*-tau with GAL4, then we classified the insertion as an LOF allele, although it is possible that such a result could also be due to GAL4-dependent RNA antisense effects of the insertion. Figure 3 depicts light micrographs of representative eye phenotypes of the 14 modifiers of the EY screen with the gene symbol to identify the modifier gene; these images were obtained using a Nikon AZ100M microscope using an 'extended depth of focus' (EDF) algorithm to display all focal planes in one compressed image.

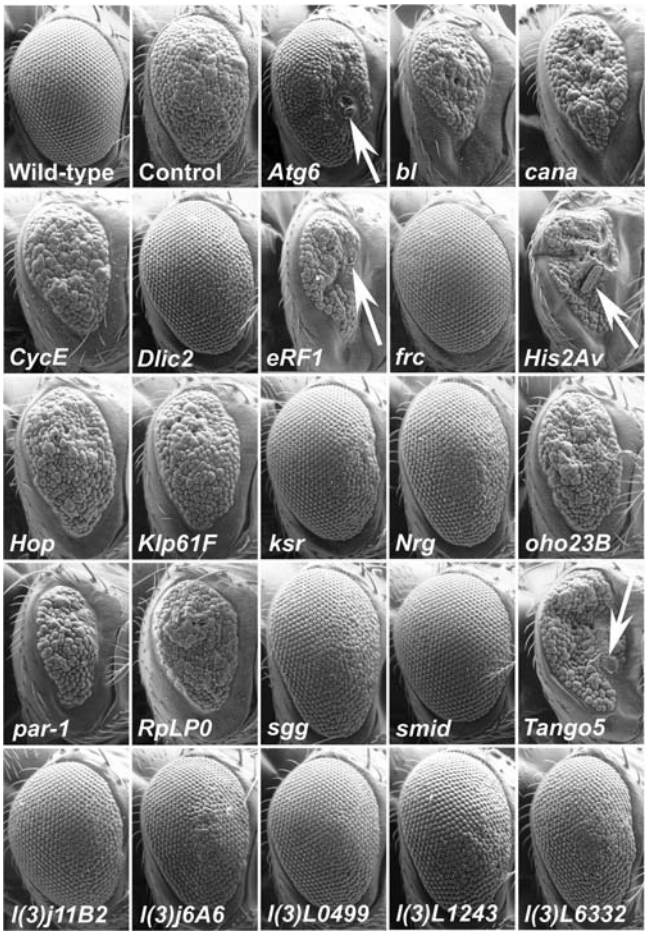

Figure 1. Genetic modifiers of tau-induced neurotoxicity identified from P lethal LOF screen. 'Control': w^{1118}/+;*gl*-tau/+. All other panels, except wild-type, contain one copy of *gl*-tau transgene in *trans* to one disrupted copy of the gene listed in the panel. Genes are listed alphabetically; non-annotated genes comprise the bottom row. Arrows point to necrotic plaques that were initially identified by light microscopy.

Suppressors and enhancers of the *gl*-tau eye phenotype show strong changes in morphological eye volume, which can be used as a quantitative metric of modification. Using the EDF algorithm, three-dimensional reconstructions of the eye can be rendered, which allows greater visualization and measurement of eye volumes. Figure 4A shows the representative images of these 3D reconstructions, from both a 'top-down' view and rotated for a 'side' view, depicting a typical wild-type eye and a *gl*-tau control eye with a volume approximately half of a wild-type eye. Also shown is a representative enhancer, *Hop*, which shows even further decrease in

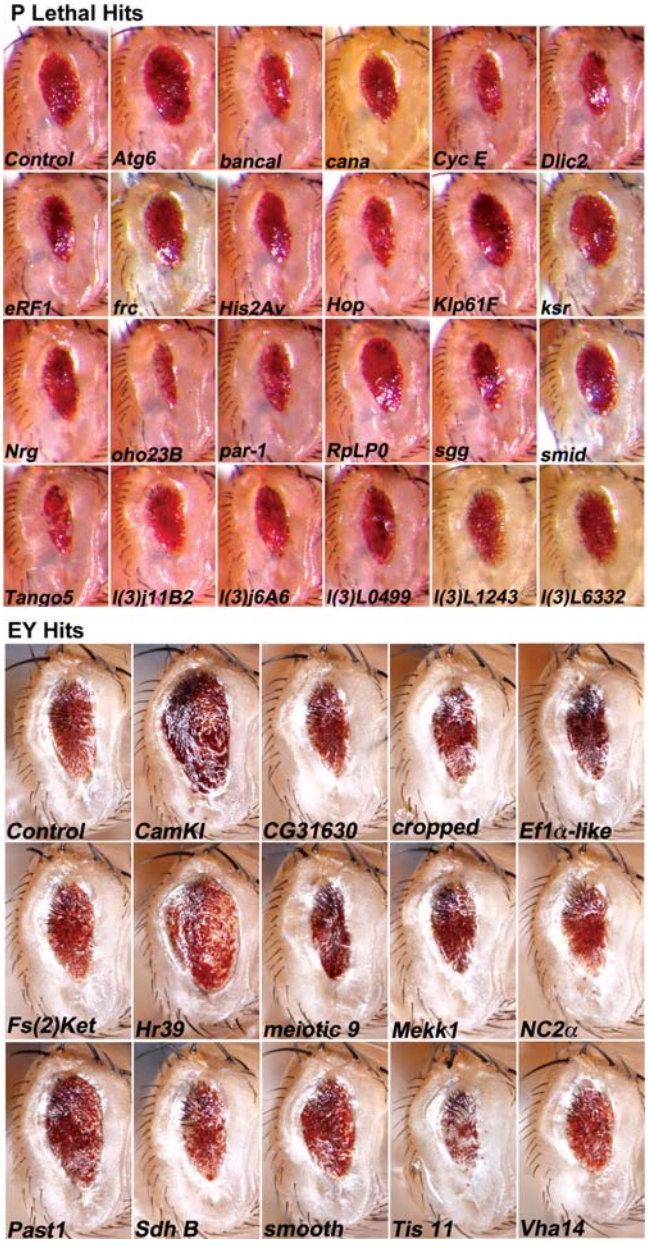

Figure 2. Modifiers of tau-induced neurotoxicity show little effect on apoptosis. The 37 modifiers identified from P lethal and EY functional screens were tested for their ability to suppress the effects of the proapoptotic gene *hid*. P lethal control: w^{1118};GMR-*hid*/+. All other P lethal genotypes have one copy of GMR-*hid* in *trans* to one disrupted copy of the listed gene. EY control: GMR-GAL4/w^{1118};GMR-*hid*/+. All other EY genotypes have one copy of GMR-GAL4 on the first chromosome and one copy of GMR-*hid* in *trans* to the affected listed gene. Genes are listed alphabetically. Nearly all modifiers failed to show an effect on apoptosis when compared with controls. *CamKI* and *Hr39* showed a moderate suppression of apoptosis but enhanced tau toxicity. Modifiers *oho23B*, *Tango5* and *Tis11* showed mildly increased phenotypes when compared with *hid*.

volume, and a representative suppressor, *sgg*, which shows increased volume over control, approaching near wild-type levels. Figure 4B shows the scatter plots of actual volumes from samples of all modifiers and demonstrates that suppressors show consistently higher volumes than control samples, whereas enhancers show consistently lower volumes.

Additional alleles of each identified gene were also screened where possible to validate the modifier gene. The complete list and details of modifier genes from both screens are summarized in Table 1. All the Bloomington stock numbers originally screened along with all additional alleles tested are listed in Supplementary Material, Table S1. In most of the cases, at least two alleles were tested and confirmed to have the same effect on tau toxicity, either through an LOF or a GOF allele. However, for the following genes, only a single allele was obtainable for screening through donated lines or lines obtained from stock centers: *Atg6*, *CamKI*, *Fs*(*2*)*Ket*, *Hr39*, *mei-9*, *Mi-2*/*l(3)L1243*, *NC2α*, *par-1*, *Past1*, *smooth*, *smid*, *Tango5*; and the five non-annotated genes, *l(3)j11B2*, *l(3)j6A6*, *l(3)L0499*, *l(3)L6332* and *CG31630*. Thus, out of a total of 40 modifier genes, 17 do not meet the more rigorous criteria as applied to the other modifiers; identification of *par-1* confirms prior reports.

Modifiers show little to no effect on polyglutamine toxicity

The modifiers identified were further tested to determine their specificity to tau toxicity by examining their ability to modify another model of neurodegeneration—polyglutamine-induced toxicity. We used a UAS-Q108 model of polyglutamine-induced toxicity that encodes a polypeptide containing an 108 repeat glutamine tract in the eye, in combination with a GMR-GAL4 driver (54). In this model, the eye is slightly reduced with increased ommatidial fusion and loss of interommatidial bristles when compared with wild-type. The tau modifier stocks were crossed to the Q108 stock, and the F_1 progeny were examined for modification of the polyglutamine eye phenotype. No hits from the P lethal screened showed any robust effect on the Q108 phenotype; similarly, almost none of the modifiers from the EY screen was appreciably different from the control (Fig. 2). However, *NC2α* and *SdhB* did show moderate suppression of the Q108 phenotype. Mitochondrial dysfunction may contribute to a number of neurodegenerative diseases, including AD and Huntington's disease (HD; 55–57); thus, it is not surprising that increased expression of *Succinate dehydrogenase B* (*SdhB*) would ameliorate both tau- and polyglutamine-induced toxicity in the fly retina. Indeed, reduction of succinate dehydrogenase (SDH), a component of mitochondrial complex II, has been described in HD brain; moreover, inhibition of SDH using 3-nitropropionic acid has been used to model HD (58,59). *NC2α* is a transcriptional regulator of the common 'downstream-promoter element' containing promoters (60), and thus it is not surprising that altered expression of *NC2α* would affect the expression of many other genes that may act on tau toxicity and some polyglutamine toxicity. Further work will be needed to identify those genes modulated by *NC2α* that regulate tau and polyglutamine phenotypes.

Computational network demonstrates wide range of functional categories

Although the collection of genes screened was large and spanned the genome, our screen was not saturating: less than 20% of the ~12 000 fly genes were screened. Our screen was selective for dominant effects/haploinsufficiency

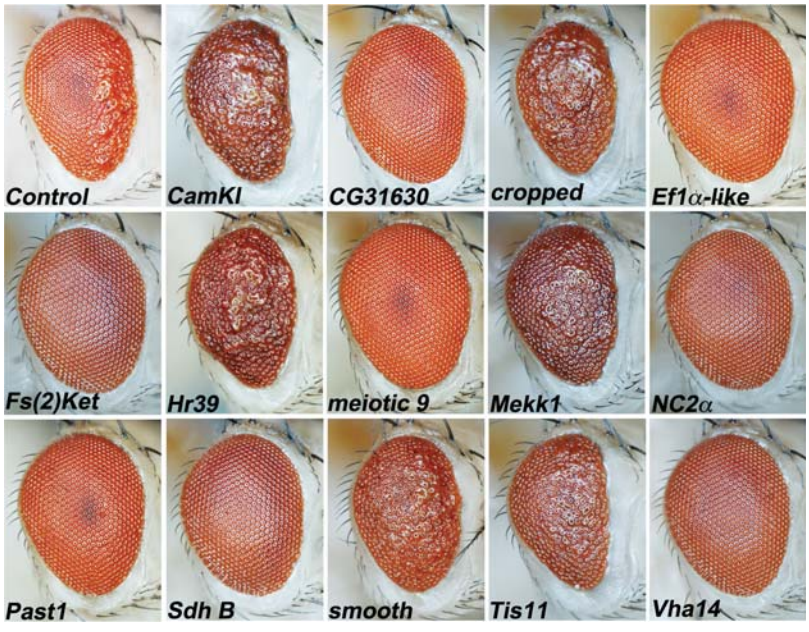

Figure 3. Genetic modifiers of tau-induced neurotoxicity identified from EY collection screen. 'Control': GMR-GAL4/+;*gl*-tau/+. All other panels contain one copy of GMR-GAL4 on the X chromosome and one copy of *gl*-tau transgene in *trans* to the gene listed in the panel affected by the EY element. Genes are listed alphabetically.

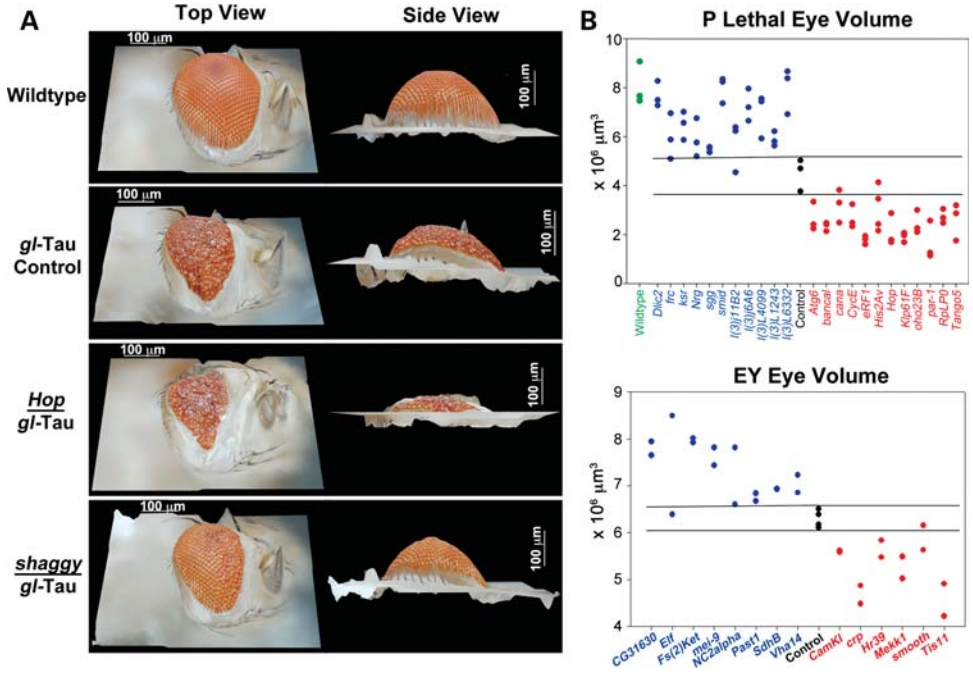

Figure 4. Quantification of eye volumes of modifiers. (**A**) Three-dimensional reconstructions of representative eyes of wild-type, control *gl*-tau (+/*gl*-tau), enhancer (*Hop*) and suppressor (*shaggy*) phenotypes. (**B**) Estimated eye volumes of modifier phenotypes from P lethal and EY screens indicated in scatter plots. Blue, suppressors; red, enhancers; black, control; green, wild-type. Black horizontal lines delineate range of control eye volumes; suppressors have larger eye volumes, whereas enhancers have smaller eye volumes when compared with control.

and for essential genes in the case of the P lethal screen. Also, some modifiers may induce subtle changes in tau pathology, which would have been excluded based on our criterion of robust suppression or enhancement of the *gl*-tau eye. Thus, important modifiers might not have been detected by our functional screen design. However, we constructed a computational network of genes highly associated with modifiers in order to extrapolate cellular processes, pathways and other genes that may also have a role in modifying tau-induced toxicity. Thirty-two of the 37 hits were annotated with a described function. We incorporated genes *white*, *brown* and *rosy* into the network, which we identified as

Table 1. Gene functions of hits from functional genetic screen

Gene symbol	*Drosophila* ID	En/Su; LOF/OE	Collection	Human ortholog	Human ID	Functional category
Atg6	Autophagy-specific gene 6	En; LOF	PZ	Beclin-1-like protein 1	BECN1	Autophagy/lysosomal
bl / hnRNP K	bancal	En; LOF	LacW	hnRNP K	HNRNPK	RNA processing
bw	brown	En; LOF	n/a	ATP-binding cassette transporter		Autophagy/lysosomal
CamKI	Calcium/calmodulin-dependent protein kinase I	En; LOF	EY	Calcium/calmodulin-dependent protein kinase type 1D	CAMK1D	Kinases or phosphatases
cana	CENP-ana	En; LOF	LacW	Centromeric protein E	CENPE	Motor and cytoskeletal proteins
crp	cropped/AP4	En; OE	EY	Transcription factor AP-4	TFAP4	Transcription factors
CycE	Cyclin E	En; LOF	LacW	G1/S-specific cyclin-E2	CCNE2	Cell cycle/nuclear
Dlic2	Dynein light intermediate chain	Su; LOF	LacW	Cytoplasmic dynein 1 light intermediate chain 1	DYNC1LI1	Motor and cytoskeletal proteins
Elf	Elongation1α-like factor	Su; LOF	EY	Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	GSPT1	Ribosomal
eRF1	eukaryotic release factor 1	En; LOF	PZ	Eukaryotic peptide chain release factor subunit 1	ETF1	Ribosomal
frc	fringe connection	Su; LOF	LacW	UDP-*N*-acetylglucosamine/UDP-glucose/GDP-mannose transporter	SLC35D2	Glycosylation
Fs(2)Ket	Female sterile (2) Ketel	Su; LOF	EY	Importin subunit beta-1	KPNB1	Nuclear trafficking
His2Av	Histone H2A variant	En; LOF	PZ	Histone H2A.Z	H2AFZ	Chromatin regulation
Hop	Hsp70/Hsp90 organizing protein homolog	En; LOF	LacW	Stress-induced phosphoprotein 1	STIP1	Proteases and chaperones
Hr39	Hormone receptor-like in 39	En; OE	EY	Steroidogenic factor 1	NR5A1	Autophagy/lysosomal
Klp61F	Kinesin-like protein at 61F	En; LOF	PZ	Kinesin-like protein KIF11	KIF11	Motor and cytoskeletal proteins
ksr	Kinase suppressor of Ras	En; LOF	LacW	Kinase suppressor of Ras 2	KSR1	ERK pathway
l(3)L1243/Mi-2	Mi-2	Su; LOF	LacW	Chromodomain-helicase-DNA-binding protein 3	CHD3	Chromatin regulation
mei-9	meiotic 9/XPF	Su; OE	EY	DNA repair endonuclease XPF	ERCC4	Chromatin regulation
Mekk1	Mekk1	En; OE	EY	Mitogen-activated protein kinase kinase kinase 1	MAP3K1	Kinases or phosphatases
NC2α	NC2α	Su; LOF	EY	Dr1-associated corepressor	DRAP1	Transcription factors
Nrg	Neuroglian	Su; LOF	LacW	Neural cell adhesion molecule L1	L1CAM	ERK pathway
oho23B	Overgrown hematopoietic organs at 23B	En; LOF	LacW	40S ribosomal protein S21	RPS21	Ribosomal
par-1	par-1	En; LOF	LacW	MARK1	MARK1	Kinases or phosphatases
Past1	Putative Achaete Scute Target 1	Su; OE	EY	EH domain-containing protein 4	EHD4	Endosomal
RpLP0	Ribosomal protein LP0	En; LOF	PZ	60S acidic ribosomal protein P0	RpLP0	Ribosomal
ry	rosy	Su; LOF	n/a	Xanthine dehydrogenase/oxireductase; aldehyde oxidaseysosomal		
SdhB	Succinate dehydrogenase B	Su; OE	EY	Succinate dehydrogenase	SDHB	Mitochondrial
sgg	shaggy	Su; LOF	LacW	GSK-3βeta	GSK3B	Kinases or phosphatases
sm/hnRNP L	smooth	En; OE	EY	hnRNP L-like	HNRPLL	RNA processing
smid	smallminded	Su; LOF	LacW	Nuclear valosin-containing protein-like	NVL	Cell cycle/nuclear
Tango5	Transport and Golgi organization 5	En; LOF	LacW	Vacuole membrane protein 1	TMEM49	Endosomal
Tis11	Tis11 homolog	En; LOF	EY	Tristetraproline	ZFP36	RNA processing
Vha14	Vacuolar H+ ATPase 14 kDa subunit	Su; LOF	EY	V-type proton ATPase subunit F	ATP6V1F	Autophagy/lysosomal
w	white	En; LOF	n/a	ATP-binding cassette sub-family G member 1	ABCG1	Autophagy/lysosomal
l(3)j11B2	Not annotated	Su; LOF	LacW			
l(3)j6A6	Not annotated	Su; LOF	LacW			
l(3)L0499	Not annotated	Su; LOF	LacW			
l(3)L6332	Not annotated	Su; LOF	LacW			
CG31630	Not annotated	Su; LOF	EY			

Drosophila gene symbol and full names are listed along with human gene ortholog and symbol, if applicable. En, enhancer; Su, suppressor; LOF, loss-of-function allele; OE, overexpression allele. Human orthologs were identified using the PANTHER classification system (www.pantherdb.org).

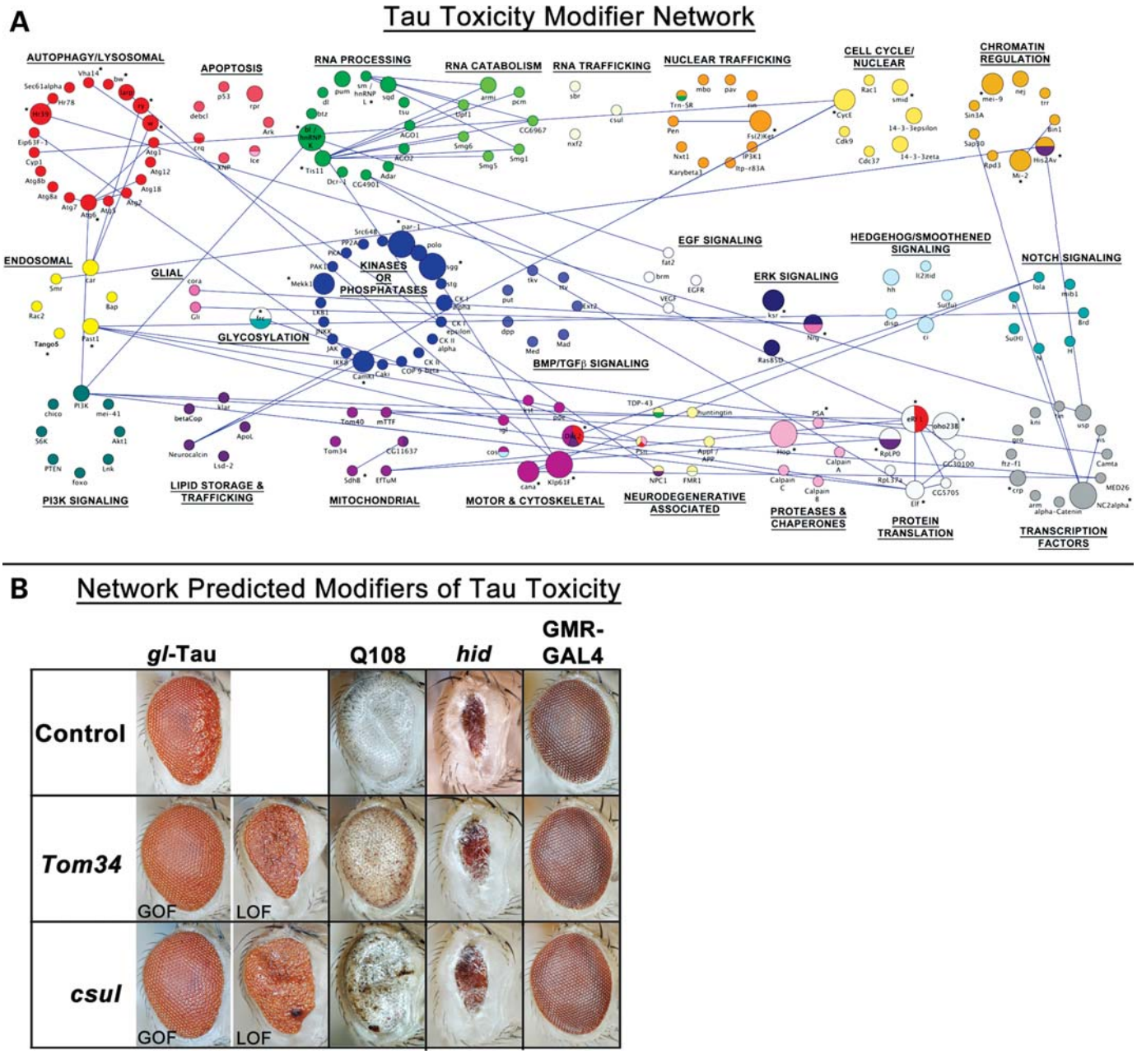

Figure 5. Computational tau toxicity modifier network can predict novel tau modifiers. (**A**) Computational predictions of tau interactors based on high association with hits from the functional genetic screen. Hits from the functional screen are marked with asterisks. Size of the nodes is proportional to degree of connectivity with other genes in the network. Although all genes selected for inclusion have high connectivity to many genes in the network (see Materials and Methods for parameters of inclusion), for clarity only the strongest interactions with a quantified interaction (P-value <0.001) are indicated in blue lines. Each functional group is coded with a unique color; a node was color-divided to indicate whether multiple functions are attributed to that gene. (**B**) Network association genes—mitochondrial gene, *Tom34*, and RNA trafficking gene, *csul*—both strongly modify tau toxicity, showing strong suppression when overexpressed (GOF) and enhanced toxicity when expression is reduced (LOF). *Tom34* and *csul* expression do not modify apoptosis (as assayed using the GMR-GAL4;GMR-*hid* phenotype), nor show any intrinsic eye phenotypes (as assayed with GMR-GAL4 alone). However, both show effects on the GMR-GAL4::UAS-Q108 phenotype—*Tom34* suppresses Q108 toxicity, whereas *csul* enhances toxicity.

modifiers independent of the collections we screened (61). These 35 annotated modifiers were individually run through the Endeavor-HighFly software analysis (62), a novel analytical program that assigns P-values to all other annotated genes in the *Drosophila* genome (~10 000 genes) based on ontology, creating individual network profiles for all 35 hits. All 35 network profiles were then cross-referenced to one another in order to determine the most significant predicted genetic interactions with tau (see Materials and Methods for criteria of network inclusion). A network diagram (Fig. 5A) was created, showing a simplified interaction map and grouped by known functions that are color-coded. It should be noted that certain genes are ascribed multiple functions, which we attempted to show by color-

dividing the relevant representative circle, but their placement in the diagram represents a model felt to be most relevant in discussing tauopathy. Genes from the functional screens and from the computational analysis are depicted in the diagram, with validated hits from the functional screens marked with an asterisk. A comprehensive list of all genes in the network is shown in Supplementary Material, Table S2. The size of the circle representing the gene is proportional to its connectivity in the network: the larger the circle, the more the connections to other genes in the network. Note that although only connections with the most significant associations ($P < 0.001$) are shown in blue lines, many other significant associations ($0.05 > P > 0.001$) are *not* depicted with a line for the sake of simplicity. For all genetic associations with their computational *P*-values, see Supplementary Material, Table S3.

As expected, kinases and phosphatases occupy the largest functional group, and motor or cytoskeletal genes show a high degree of connectivity to many genes in the network. However, autophagy/lysosomal genes and genes related to RNA processing were also surprisingly highly represented, along with protein translation or ribosomal-associated genes. Additionally, the ERK signaling pathway appears to be relevant, with *ksr* and *Nrg* identified in the screen, but other signaling pathways were also predicted to be relevant, including the BMP/TGF-β, epidermal growth factor (EGF) and Hedgehog/Smoothened pathways, which are not commonly discussed in relation to tau pathology. The PI3K is the largest represented and the most highly connected signaling pathway in the network. The kinase *shaggy*/GSK-3β is one of the most highly connected genes in the network and is regulated by several pathways, including PI3K and Wnt, although no other Wnt pathway components were identified functionally or computationally. Several cell-cycle or nuclear-associated genes also have a high representation, which supports hypotheses of cell-cycle-mediated neurodegeneration (discussed further below).

To determine whether this network could be used to predict tau toxicity interactions, a few genes identified through network association were empirically tested with the *gl*-tau line. *Tom34*, identified in the Mitochondrial gene cluster, and *csul*, identified in the RNA Trafficking cluster, showed very strong effects on the *gl*-tau phenotype (Fig. 5B). Using both GOF and LOF approaches, we observed that both *Tom 34* and *csul* suppress tau toxicity when overexpressed and enhance toxicity when expression is reduced. These alleles neither appear to mitigate general apoptosis (assayed with GMR-*hid*) nor have any intrinsic effects on eye morphology (assayed with GMR-GAL4 alone). However, both genes also modify polyglutamine toxicity. Overxpression of *Tom34* suppresses Q108 toxicity, as evidenced by reduced ommatidial fusion and increased pigment retention; however, *csul* overexpression increases Q108 toxicity, as evidenced by a smaller and more collapsed eye and increased pigmentation abnormalities.

Several genes encoding subunits to the *Translocase of outer mitochondrial membrane* (*Tom* or *Tomm*) protein have recently been linked to neurodegenerative diseases. Most notably, *Tomm40* has been reported in several publications to be a risk factor in AD (63). Additionally, *Tom20* and *Tom70* have been associated to Parkinson's disease-linked parkin and ALS-linked SOD1 (64,65). Although associated with mitochondria, Tom34 is found more predominantly in the cytosol and binds to the chaperone proteins Hsp90 and Hsp70, as does Tom70 (66–68). This suggests that Tom34 and Tom70 function more as chaperone proteins which would help to explain why increased expression of Tom34 suppresses tau toxicity. Indeed, mature protein substrates of Tom70 were observed to be aggregate-prone in the absence of Tom70, suggesting a crucial role of Tom70 in preventing aggregation (69). The gene *csul* interacts with histones, splicesosomal proteins and other small ribonucleoproteins and can affect protein–RNA affinity and intracellular localization of certain mRNAs (70–72). A recent report observed that mRNAs also associate with Tom70 (73), further establishing a link between RNA and RNA-associated proteins and tau toxicity. RNA regulation may also play a significant role in polyQ toxicity as well, as the CAG repeats in polyglutamine-encoding RNAs can induce toxicity (74,75), and polyglutamine proteins can also bind RNA with an affinity dependent on the polyQ expansion length (76). Other genetic screens of polyQ toxicity have also uncovered RNA-binding proteins (77–79), although those genes identified do not overlap with the modifiers presented in this study. Given this, however, it is peculiar that most other RNA-associated genes uncovered in the screen did not also modify Q108 phenotype. However, no other genes that are directly involved with RNA and protein localization or transport were screened, which suggests a specific role of RNA trafficking or localization in polyQ toxicity.

We also confirmed that the RNA Catabolism gene, *armi*, modified tau toxicity, enhancing toxicity with both LOF and GOF approaches. Alleles tested for RNA Catabolism gene, *Upf1*, and the mitochondrial gene, *Tom20*, did not show significant changes in the *gl*-tau phenotype (data not shown). This may simply indicate that the eye and retina may not be the ideal tissue to induce or observe effects of these genes on tau toxicity. However, the ability to empirically validate modification of toxicity of at least two genes identified by network association, from two different gene categories, suggests the potential of this network to identify future modifiers. Although we emphasize that this network neither represents a full genomic network nor includes all demonstrated or putative modifiers, we believe this network does bring assistance in discerning how the uncovered modifiers may interact, and yields novel associations that may prove fruitful.

Phosphorylation of tau does not correlate with enhancement or suppression of tau toxicity

PHFs are primarily composed of 'hyperphosphorylated' tau; thus, increased tau phosphorylation is regarded as a key component in tau-related pathogenesis. Tau phosphorylation at S202/T205 as detected by the AT8 antibody (80) is commonly found in tauopathies (38,81–84). On the basis of this hypothesis, we predicted that suppressor genes would decrease tau phosphorylation, whereas enhancer genes would increase tau phosphorylation. Phospho-tau levels from protein extracts of flies expressing *gl*-tau in *trans* to modifier genes were analyzed by immunoblot (Fig. 6). Unexpectedly, no significant differences in AT8 levels were found for nearly all modifiers from the P lethal screen when compared with controls. Six

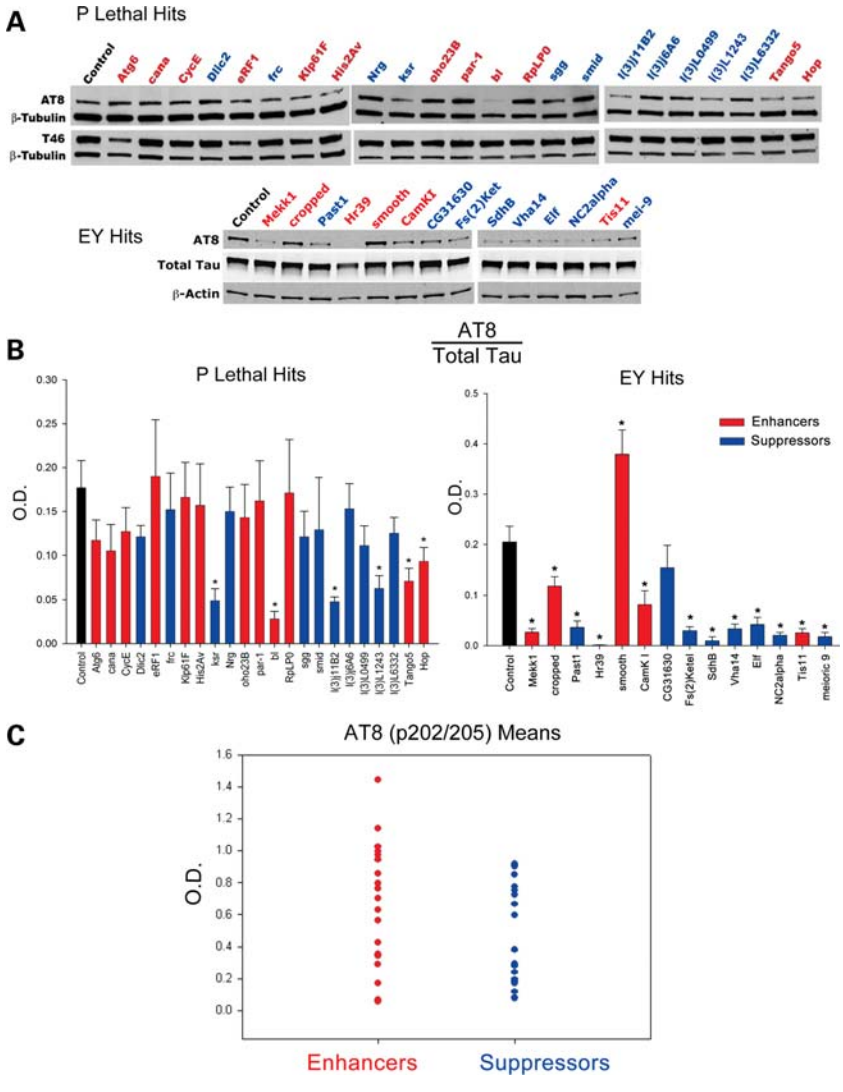

Figure 6. Modifying tau toxicity does not require altering tau phosphorylation state. (**A**) Immunoblots from tau toxicity modifiers probed for total tau (T46 or E178) and tau phosphorylation at S202/T205 (AT8). β-Tubulin or β-actin shown as loading control. P lethal control: w^{1118}/+;*gl*-tau/+. EY control: GMR-GAL4/+;-*gl*-tau/+. Genes labeled in red designate enhancers, whereas genes labeled in blue are suppressors. Blots for EY hits were visualized by two-color immunoblots with fluorescent secondaries and imaged with the Odyssey Near-IR Scanner (Li-Cor), but are shown in grayscale. Two-color immunoblots allowed for visualization and measurement of total tau (E178, rabbit IgG) and AT8 (mouse IgG) simultaneously. (**B**) Quantification of phosphorylated tau. Modifiers did not significantly alter total tau expression. AT8 levels were normalized to total tau; significant differences ($P < 0.05$) when compared with control are marked with asterisks. Approximately half (18 of 40) of the modifiers showed no significant differences in phospho-tau when compared with control. The other half (22 of 40) showed a significant difference in AT8; however, no pattern of phosphorylation correlated with enhancement or suppression of the tau phenotype. (**C**) Scatterplot of the mean values for AT8 signal of the 40 modifiers identified shows little difference in phosphorylation state between enhancers and suppressors.

modifiers showed a significant decrease in phosphorylation: three were enhancers (*bl*, *Hop* and *Tango5*) and three were suppressors (*ksr*, *l*(*3*)*j11B2* and *l*(*3*)*L1243*). On the other hand, nearly all modifiers from the EY screen—suppressors and enhancers alike—showed robust decreases in phosphorylation. Suppressor *CG31630* did not show a change in phosphorylation, and the enhancer *smooth* was the only gene from either screen that had a significant increase in its AT8 level. Thus, no consistent pattern of S202/T205 phosphorylation can be attributed to a suppressive or enhancing effect of tau-induced toxicity. This result is reminiscent of that obtained with the genetic modifiers *white*, *brown* and *rosy* (61). The distribution of phosphorylation is depicted as a scatter plot of the mean AT8 levels for all 40 modifiers classified as either enhancer or suppressor (Fig. 6C). Modifiers did not show a statistically significant difference in total tau levels relative to controls (Supplementary Material, Fig. S3), indicating that altered toxicity was not due to changing tau expression. Only *l*(*3*)*L6332* showed a significant increase in tau expression but suppressed toxicity; thus, its modifying effect is not attributable to any effect on tau expression. Although at first surprising, this finding supports other recent data from our laboratory that also demonstrates dissociation of tau phosphorylation from neurodegeneration (42). We also evaluated two other phospho-tau antibodies—12E8, which detects pS262 and pS356 (85) and AT270, which detects pT181 (86) with modifiers from the EY screen. No significant differences in phospho-tau levels relative to

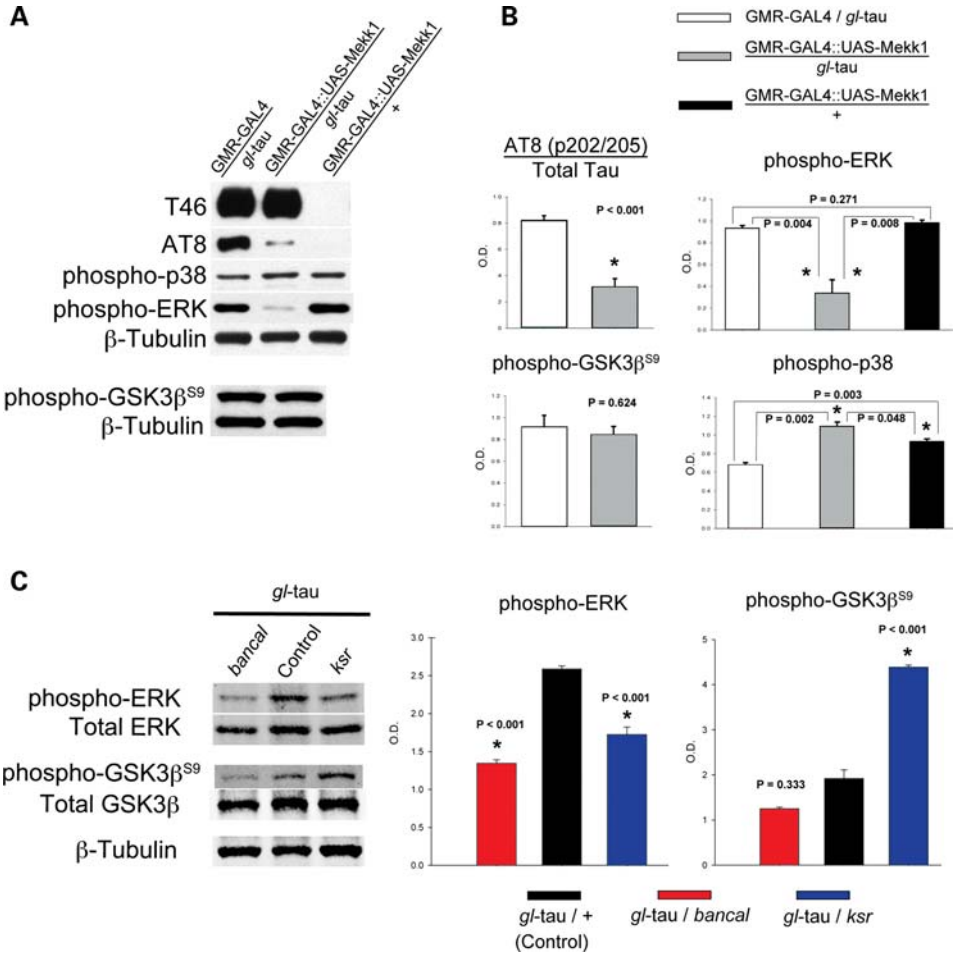

Figure 7. p38 interacts synergistically with tau to regulate ERK activity and *ksr* decreases GSK-3β and ERK activities. (**A**) Immunoblots and (**B**) quantification of kinase activity induced by *Mekk1* overexpression, which enhanced tau toxicity. *Mekk1* overexpression induced a modest significant increase in p38 activity (phospho-p38) independent of human tau expression. ERK activity (phospho-ERK) was equivalent for tau expression alone (first lane) and *Mekk1* expression alone (third lane); however, tau and *Mekk1* co-overexpression strongly reduced phospho-ERK levels (second lane). Overexpression of *Mekk1* also strongly reduced S202/T205 tau phosphorylation levels (AT8), but did not alter total tau levels (T46). No significant difference was observed for GSK-3β activity (phospho-GSK-3β-Ser9). (**C**) Both enhancer *bancal* and suppressor *ksr* decrease ERK activity, but *ksr* also strongly reduced GSK-3β activity, while *bancal* trended to increase GSK-3β activity.

controls were observed (Supplementary Material, Fig. S4). Taken together, these results show that modifying the severity of tau-induced toxicity does not require altering the phosphorylation state of tau.

Tau demonstrates synergy with Mekk1 and p38 to decrease ERK activity

In total, four kinases were identified in the functional screen—*sgg*/GSK-3β, *par-1*/MARK1, *CamKI* and *Mekk1*. However, *sgg* and *par-1* mutations did not show any significant effect on AT8 levels in *trans* to *gl*-tau, whereas *CamKI* and *Mekk1*, both enhancers, showed significant decreases in AT8 levels. To probe these unexpected results, we further investigated the downstream effects of increased *Mekk1* activity. Mekk1 is a stress-induced MAP3K that leads to phosphorylation and activation of the stress-activated kinase, p38 MAPK (87). Using the GMR-GAL4 driver with the *Mekk1* EY line, immunoblots showed modest but statistically significant increases in activated p38 in GMR-GAL4/+;$Mekk1^{EY}$/*gl*-tau fly heads when compared with GMR-GAL4/+;*gl*-tau/+ flies (Fig. 7A). This increase in p38 activity was comparable to overexpression of *Mekk1* alone (GMR-GAL4/+;$Mekk1^{EY}$/+), indicating that p38 activity is not tau-dependent. The p38 pathway has been reported to interfere with ERK activity during apoptosis (see 88 for review); thus, ERK activity was assayed in these genotypes by measuring phospho-ERK levels. Overexpression of *Mekk1* alone (GMR-GAL4/+;$Mekk1^{EY}$/+) did not produce any difference in phospho-ERK levels when compared with *gl*-tau-only flies (GMR-GAL4/+;*gl*-tau/+). However, when tau was co-expressed with *Mekk1*, a profound reduction (∼60%) in phospho-ERK was observed (Fig. 7A). No change in GSK-3β activity was observed with *Mekk1* overexpression. These data demonstrate a synergistic effect between Mekk1/p38 and tau in down-regulating ERK activity and suggest that tau has signaling properties to regulate the kinases or phosphatases that in turn regulate tau itself.

GSK-3β phosphorylation state correlates with toxicity in reduced ERK activity conditions. We were intrigued by the

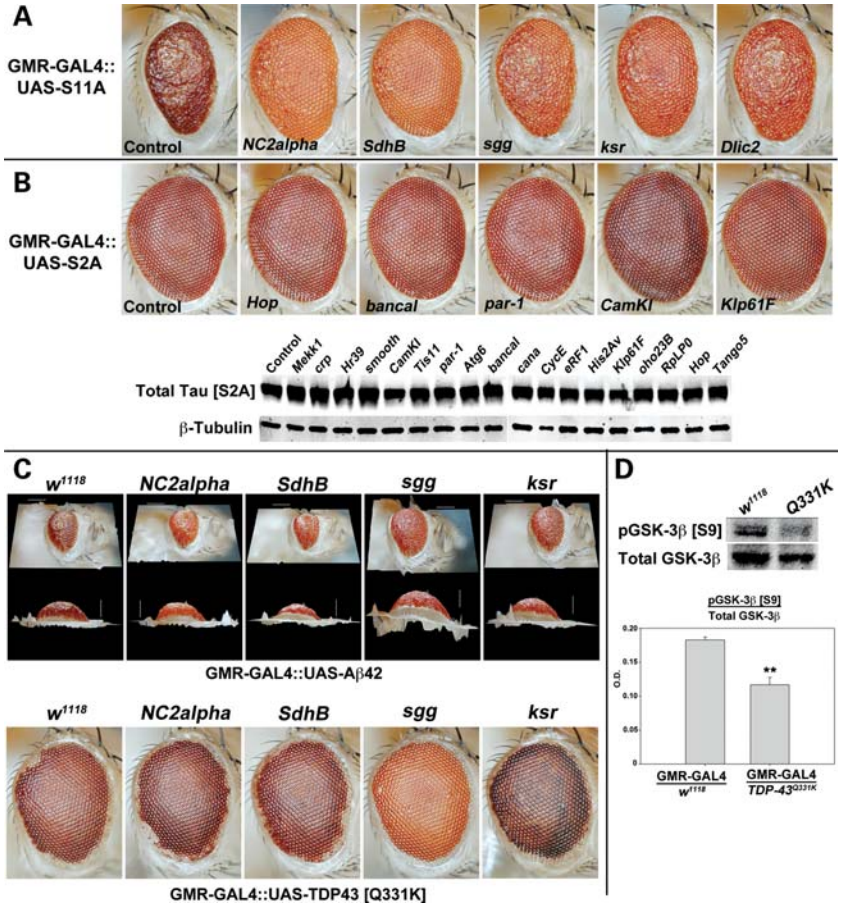

Figure 8. GSK-3β/*shaggy* is a common suppressor of S11A, Aβ42 and TDP-43 toxicity, whereas S2A is resistant to all enhancers. (**A**) Expression of the phosphorylation-resistant Tau^{S11A} isoform shows strong toxicity, stronger than Tau^{WT} expression. However, select suppressors of *gl*-Tau^{WT} were also able to suppress S11A toxicity. (**B**) Expression of the phosphorylation-resistant Tau^{S2A} isoforms shows no toxicity. All enhancers identified in the screen were tested against S2A phenotype; five representative enhancers are depicted. No enhancer could induce a rough eye phenotype with Tau^{S2A}. Western blots confirm robust expression of Tau^{S2A} protein with all enhancers assayed. (**C**) *NC2α* and *SdhB*, which suppressed both tau and polyglutamine toxicity, were tested for their effects on Aβ42 and $TDP\text{-}43^{Q331K}$ toxicity. Neither gene showed any significant effect on the GMR-GAL4::UAS-Aβ42 phenotype, nor on the GMR-GAL4::UAS-$hTDP\text{-}43^{Q331K}$ phenotype when compared with controls. However, *shaggy*/GSK-3β showed suppression of toxicity, as indicated by increased size and eye volume of the Aβ42 eye, and by increased color and pigment retention in the TDP-43 eye. Modifier *ksr* shows a mild suppression of Aβ42 and TDP-43 toxicity, although not as robust as *sgg* suppression. Scale bar, 100 μm. (**D**) Expression of $hTDP\text{-}43^{Q331K}$ shows a robust increase in GSK-3β activity, as indicated by western blot of reduced Ser9 phosphorylation of GSK-3β ($P = 0.004$).

result that both *ksr* and *bancal* showed significant reductions in pS202/T205 (AT8) levels, although *ksr* is a strong suppressor, whereas *bancal* is a strong enhancer. As both genes have reported interactions in the MAPK/ERK pathway, we examined the levels of activated ERK in *gl*-tau flies with *ksr* or *bancal* alleles. As expected, both *ksr* and *bancal* showed significantly decreased phospho-ERK levels, indicating reduced ERK activity (Fig. 7B). Owing to ongoing experiments from our laboratory and from the literature, we hypothesized that *bancal* mutants would show increased GSK-3β activity. When assayed for pGSK-3β[Ser9] (inactivated GSK-3β), we did indeed see a reduction of inactivated GSK-3β in *bancal* mutants when compared with control, although the result was not statistically significant (Fig. 7B). However, surprisingly, we observed a strong and statistically significant increase in inactivated GSK-3β in *ksr* mutants. Given this result, we assayed all the modifiers for GSK-3β phosphorylation state, as an indicator of GSK-3β activity, to determine whether GSK-3β was a common final pathway for toxicity, but no consistent pattern between suppressors or enhancers was observed (data not shown). Together, we conclude from these results that (1) ERK activity correlates with S202/T205 phosphorylation, but not toxicity; (2) *ksr* not only participates in the MAPK/ERK pathway, but can also interact in the GSK-3β pathway; and (3) in conditions of reduced ERK activity, GSK-3β activity correlates with toxicity.

Suppressors of wild-type Tau toxicity also suppress S11A toxicity, while S2A is resistant to enhancers. To further explore the role of tau phosphorylation and the modifiers identified, we assayed two different phosphorylation-resistant isoforms of tau that have opposing phenotypes. The Tau^{S11A} (or simply S11A) isoform has 11 serines or threonines mutated to alanines to prevent phosphorylation. The sites mutated are ones commonly observed as hyperphosphorylated in tauopathic tissue and are known to be phosphorylated by key kinases such as GSK-3β and CDK-5. Instead of alleviating toxicity, S11A demonstrates severe toxicity when expressed

in the eye, producing an even smaller and rougher eye than wild-type tau (42). We chose select suppressors to test for suppression of this stronger form of tau toxicity (Fig. 8A). All suppressors tested were able to suppress S11A toxicity. Importantly, a *sgg*/GSK-3β LOF allele still showed suppression of the phosphorylation-resistant S11A construct, indicating that the role of GSK-3β in tau toxicity extends beyond direct tau phosphorylation. The increased toxicity of S11A may be due to increased microtubule binding, as suggested by Chatterjee *et al.* (42). Alleles of *Dynein light chain 2* (*Dlic2*) suppressed S11A toxicity, lending further credence to the hypothesis that microtubule-binding protein dynamics play a significant role in S11A and wild-type tau toxicity.

Conversely, the Tau^{S2A} (or S2A) isoform has only two serines mutated to alanines (S262, S356), yet shows no toxicity in the eye. All enhancers identified in the screen were assayed with the S2A line to determine whether these enhancers could induce toxicity with S2A. Surprisingly, no enhancer showed any effect with S2A; robust expression of S2A with the enhancer alleles was confirmed by western blot (Fig. 8B). We conclude from these results that these two select serines mutated to alanines effectively make the tau protein inert in effecting toxicity, even in conditions promoting degeneration. These results also further validate the specificity of the enhancers in acting on the toxic effects of tau and not simply on the effect of misexpression of human tau protein.

GSK-3β suppresses Aβ42 and TDP-43 toxicity, and mutant TDP-43 induces increased GSK-3β phosphorylation activation state. Given that *NC2α* and *SdhB* suppressed both tau and polyglutamine toxicity, we assayed the abilities of these genes to suppress other neurodegenerative models: expression of the 42 amino acid isoform of β-amyloid (Aβ42) and an amyotrophic lateral sclerosis-causing mutation in TAR DNA Binding Protein-43, $TDP\text{-}43^{Q331K}$. Similar to tau and to polyglutamine proteins, expression of Aβ42 and $TDP\text{-}43^{Q331K}$ in the fly eye driven by GMR-GAL4 leads to eye phenotypes. The Aβ42 eye produces a very rough and small eye, with increased roughness and ommatidial fusion toward the posterior of the eye (Fig. 8C; 89). The TDP-43 phenotype is more subtle, with no obvious ommatidial fusion or roughness, but with characteristic discoloration to a darker brown-like color, and loss of pigmentation, starting in the periphery of the eye and leading to a mosaic-like pattern with aging (Fig. 8C). Although *NC2α* and *SdhB* suppressed tau and polyglutamine toxicity, neither gene had a significant effect on the Aβ42 or the $TDP\text{-}43^{Q331K}$ phenotype. We also tested other identified suppressors of wild-type tau against Aβ42 toxicity, including *Dlic2*, *Elf*, *Fs(2)Ket*, *mei-9*, *ksr*, *Past1*, *Nrg*, *smid*, *sgg* and *Vha14*. Of these suppressors, only *sgg* showed a robust suppression of the phenotype, with increased size and volume of the eye, best appreciated with 3D views of the eye (Fig. 8C). The suppressor *ksr* also showed moderate suppression of the Aβ42 phenotype, but not as robust as seen with *sgg*. Given the ability to suppress Aβ42 toxicity, we also assayed *sgg* and *ksr* against $TDP\text{-}43^{Q331K}$ toxicity and found strong suppression with *sgg*. Pigment loss was highly reduced, and the eye maintained a wild-type-like red color with *sgg*. With *ksr*, there was reduced pigment loss around the periphery of the eye, but discoloration was still observed, from which we conclude that *ksr* partially suppressed the $TDP\text{-}43^{Q331K}$ phenotype.

As the suppressive effects of GSK-3β/*sgg* on TDP-43 toxicity have not previously been reported, we examined whether GSK-3β activity had indeed been altered due to $TDP\text{-}43^{Q331K}$ expression. A very strong increase in GSK-3β activity induced by $TDP\text{-}43^{Q331K}$ was observed, as indicated by reduced levels of pGSK-3β [Ser9] (Fig. 8D). This result helps to explain that the *sgg* mutation might suppress toxicity by reducing levels of Sgg/GSK-3β and that *ksr* might exert its suppressive effects by increasing inactivated GSK-3β, as observed in *gl*-tau flies (Fig. 7B).

DISCUSSION

Here, two parallel functional genetic screens were performed to identify modifiers of wild-type human tau-induced neurotoxicity, using a collection of LOF alleles of essential genes, as well as a collection of enhancer-promoter elements to drive expression of endogenous genes (EY screen). These collections allowed for an unbiased, genome-spanning, blinded genetic screen. In total, 37 hits were identified from ∼1900 lines screened (Figs 1 and 3). Additionally, in the process of conducting this screen, the background genes *white*, *brown* and *rosy* were also identified as modifiers (61), yielding a total of 40 modifier genes. These modifiers showed a high degree of specificity for tau-induced toxicity, as they (a) did not have independent effects on eye morphology, (b) did not have anti-apoptotic effects (as assessed by their ability to suppress the proapoptotic effects of *hid*; see Fig. 4) and (c) 38 of the 40 showed no effect on polyglutamine toxicity, with *NC2α* and *SdhB* being the only two exceptions (Fig. 9). To better understand the relationship of these genes to each other and to find highly associated genes that may also be relevant modifiers, a computational network was constructed based on annotated gene ontology using the Endeavor-HighFly software (Fig. 5A, Supplementary Material, Tables S2 and S3). The genes identified cover a broad range of functions that are consistent with known aspects of tau function and regulation but also reveal many novel or underappreciated associations.

Kinases and dissociation of tau phosphorylation from tau toxicity

Tau phosphorylation is complex: numerous kinases and phosphatases target tau, there are numerous sites of tau phosphorylation, and cross-regulation is thought to occur between tau kinases directly or indirectly. Two modifiers identified in an unbiased manner from these screens, *par*-1 and *shaggy*, are known tau kinases (24,25,90–92), providing a proof of principle that our approach can identify bona fide tau modifiers. However, the opposing effects on tau toxicity exerted by reductions in *par-1*, an enhancer, and *sgg*, a suppressor, emphasize the multifaceted relationship between kinase activity and tau toxicity. This is further demonstrated by the modifiers *Mekk1* and *CamKI*, both kinases that enhance tau toxicity despite producing reductions in AT8 levels (phospho-tau at S202/T205). Moreover, neither *sgg* nor *par-1* loss of one copy produced any change in AT8 signal (Fig. 6). GSK-3β, the mammalian

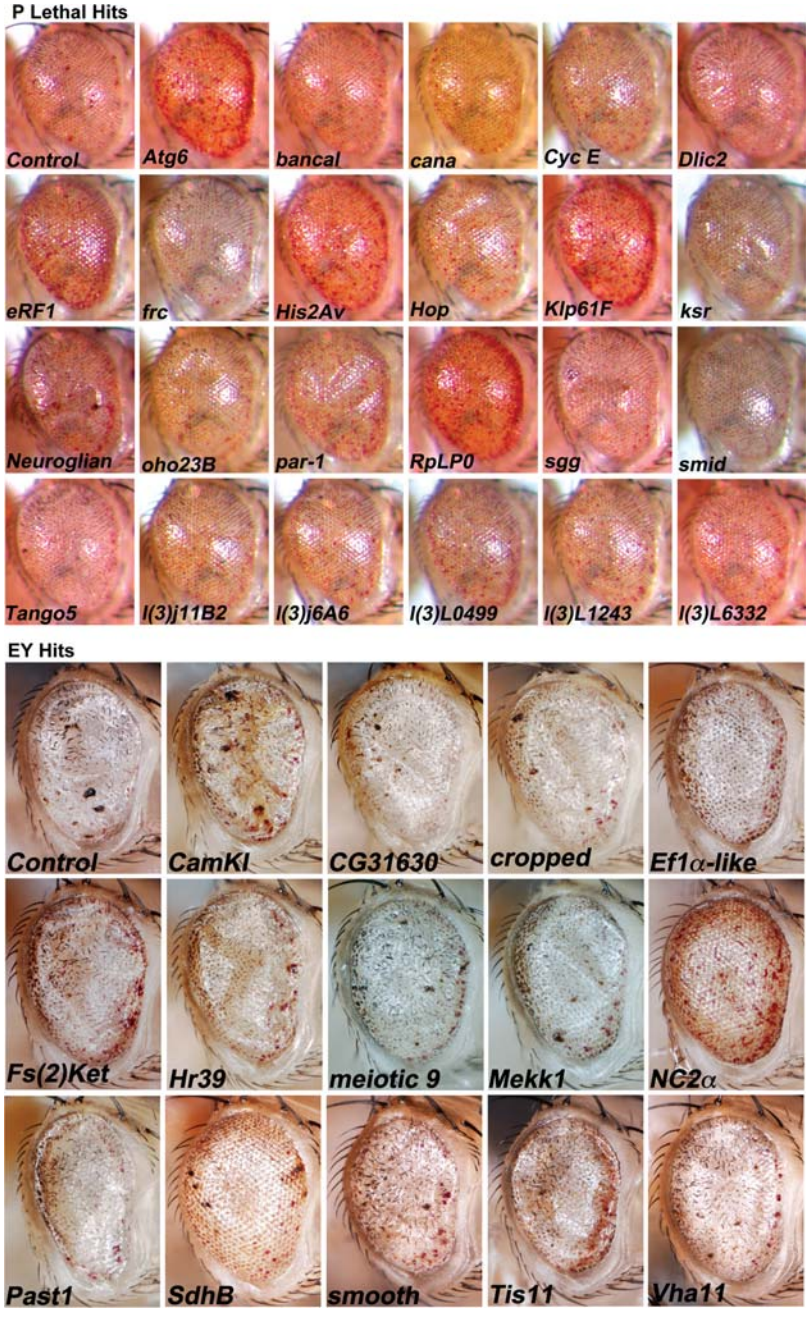

Figure 9. Modifiers of tau-induced neurotoxicity do not modify polyglutamine toxicity. The 37 modifiers identified from P lethal and EY screens were crossed to w^{1118};GMR-GAL4, UAS-Q108/CyO. Controls: w^{1118};GMR-GAL4, UAS-Q108/+. All other genotypes have one copy of GMR-GAL4, UAS-Q108. The gene listed refers to the allele affected by P element or EY element; genes are listed alphabetically. Most tau modifiers showed no effect on polyglutamine toxicity; only *NC2α* and *SdhB* showed suppression.

homolog of *sgg*, is strongly implicated in AD (93), and many investigators have shown that either inhibition or genetic reduction of GSK-3β ameliorates tau-related deficits (94–99). The data presented here agree with these previous reports and replicate a previous report from our laboratory that LOF of one copy of *shaggy* suppresses tau toxicity (47). Furthermore, reduction of *shaggy* was still able to suppress toxicity of the phosphorylation-resistant S11A (Fig. 8A), strongly implicating tau phosphorylation-independent effects of GSK-3β. This suggests that reduced GSK-3β activity correlates with reduced degeneration, but through mechanisms other than direct tau phosphorylation at S202/T205.

The ability of *shaggy* to also suppress Aβ42- and TDP-43-induced toxicity (Fig. 8C) has further implications in understanding neurodegenerative proteinopathies. Suppression of Aβ42 toxicity supports previous reports in cell, mouse and fly models in which Aβ42 induces GSK-3β activity and inhibition or reduction of GSK-3β ameliorates Aβ42-induced degeneration, either independent of or in concert with tau toxicity (99–101). However, to our knowledge, this report is

the first to demonstrate that mutant TDP-43 expression strongly induces GSK-3β activity (Fig. 8D) and that reduction in GSK-3β/*shaggy* can suppress TDP-43 toxicity (Fig. 8C). TDP-43 can be phosphorylated, which is hypothesized to play a role in its toxicity (102,103). However, GSK-3β has not been identified as a kinase of TDP-43, even when specifically tested for TDP-43 phosphorylation (102); given our data suggesting a tau phosphorylation-independent role of GSK-3β in tau toxicity, it is likely that the effects of GSK-3β in TDP-43 toxicity observed here also are TDP-43 phosphorylation-independent.

GSK-3β has several downstream targets and is a regulator in many pathways, including Wnt, PI3K and Hedgehog signaling (104–106). GSK-3β phosphorylates the transcription factor Armadillo/β-catenin, which targets it for degradation (107); thus a reduction in *sgg* may suppress tau toxicity by allowing increased Armadillo to activate transcription of target genes. Changes in expression of *armadillo* and Armadillo's transcriptional binding partner, dTCF, or increasing Armadillo stability have previously been suggested to modify tau-induced neurodegeneration (47). Our computational approach found the Insulin/PI3K and Hedgehog/Smoothened pathways to be highly associated with the modifiers identified in the screen. The PI3K pathway is activated by insulin, which leads to the inhibition of GSK-3β activity via phosphorylation of its serine-9 residue by AKT/PKB (108,109). Dysregulated insulin signaling is strongly implicated in AD (110–112); thus, GSK-3β may regulate tau toxicity through its role in metabolic signaling and activation of the FoxO class of transcription factors (113,114). Although the Hedgehog/Smoothened pathway has not been previously implicated in tauopathy, there is much cross-talk with the Wnt pathway through GSK-3β and casein kinase I (115–118); thus, it may be expected that Hedgehog is linked computationally to the tau modifier network.

GSK-3β also may regulate tau toxicity through regulation of MARK-2/PAR-1. GSK-3β has recently been shown to phosphorylate a key regulatory Ser in PAR-1; however, it remains unclear whether this activates (119) or inactivates PAR-1 (120,121). In the data presented here, reductions in *sgg* and *par*-1 gene dosage have opposite effects on the tau phenotype, supporting a GSK-3β-mediated inactivation of PAR-1 model, i.e. reduced *sgg* leads to increased active *par-1*. There is additional evidence of shared regulatory pathways between GSK-3β and PAR-1. LKB-1 phosphorylates and activates PAR-1 (120,122,123), but also phosphorylates the inactivating Ser-9 on GSK-3β and may be the elusive GSK-3β kinase activated by Wnt signaling (124). AKT phosphorylates and enhances PAR-1 activity (125). Together, these data suggest a model of GSK-3β and PAR-1 antagonism, in which kinases that inactivate GSK-3β also enhance PAR-1 activity. Conversely, it has been suggested that *par-1* may regulate GSK-3β activity by 'priming' the tau protein, whereby tau phosphorylation by PAR-1 is required before GSK-3β or Cdk5 can phosphorylate tau (92). However, our laboratory observed that GSK-3β phosphorylation of tau was independent of phosphorylation by PAR-1 (42).

Hyperphosphorylated tau dissociates from microtubules (35–37), leading to the hypothesis that synaptic impairments and neurodegeneration are due to destabilized microtubules in axons. PAR-1/MARK-2 (mammalian homolog microtubule-associated protein/microtubule affinity-regulating kinase 2) has been shown to phosphorylate tau at the S262/S356 sites, causing a significant reduction in binding affinity of tau for microtubules (26). This finding would support the hyperphosphorylation/microtubule instability hypothesis. However, studies of the role of PAR-1 in tau toxicity have yielded, in part, contradictory results.

Both Nishimura *et al*. (92) and Chatterjee *et al*. (42) reported that increased *par-1* expression increases tau toxicity, whereas Shulman and Feany (52) found that increased *par-1* expression suppressed mutant tau-induced toxicity; the latter finding agrees with the results from the P lethal screen reported here. Furthermore, MARK-2 overexpression in primary neurons reduces tau-induced deficits by alleviating organelle and vesicle transport blockade (40,126).

It is possible that other phosphorylation sites on tau may be more relevant to GSK-3β-related toxicity; however, when either 5 (41) or 11 (42) putative GSK-3β phosphorylation sites were mutated to alanine, tau toxicity either comparable to or more robust than the toxicity induced by wild-type tau was still observed. To further examine potential phospho-site dependence, we examined phosphorylation at S262/S356 and T181 (using 12E8 and AT270, respectively; 85,86) for the EY screen-derived modifiers. There were no significant differences between suppressors and enhancers at either 12E8 or AT270 epitopes (Supplementary Material, Fig. S4).

The presence of hyperphosphorylated tau in PHFs underscores that phosphorylation is an important regulatory feature of tau function. Indeed, the S2A mutant tau construct, with only two residues (S262 and S356) made resistant to phosphorylation, exhibits no toxicity at all in the eye and furthermore cannot be rendered toxic by genetic enhancers of wild-type tau toxicity (Fig. 8B). This result underscores the role phosphorylation can play in tau toxicity. However, the difference between lack of toxicity with the S2A mutant and the severe toxicity observed with S11A highlight that it is the functional consequences of phosphorylation that determine toxicity and not simply increased levels of phosphorylation. Furthermore, the lack of correlation we observed between tau modifiers and their effects on phosphorylation suggest that this post-translational modification is not the only determinant of tau toxicity. Apart from its effects on microtubule binding, phosphorylation can alter tau subcellular localization to dendritic spines (127) or can alter its affinity for the plasma membrane (128), thereby causing alterations in synaptic function (127,129). The results presented here support a plethora of data that show phosphorylation *can* affect tau toxicity but demonstrate that alteration of tau phosphorylation state is *not required* to modify tau toxicity.

Signal transduction and tau signaling properties

In addition to the PI3K and Wnt signaling pathways discussed above, our screen implicates other key pathways in tauopathy, including the ERK/MAPK and p38/MAPK pathways. ERK can phosphorylate tau (29), and elevated activity of MAPK/ERK has been reported in several tauopathies and may play a role in early stages of neurofibriliary tangle (NFT) formation (82,130,131). However, reduced ERK activity has also been

shown to enhance tau-induced toxicity (132); thus, the role of ERK in neurodegeneration remains incompletely understood. LOF alleles of *ksr* and *Neuroglian* (*Nrg*), both of which promote ERK signaling (133–136), suppress tau toxicity, suggesting that reducing ERK activity is beneficial.

The effects of ERK activity on toxicity, however, may be secondary to effects on GSK-3β activity. Two genes—*bancal* and *ksr*—intrigued us as they showed similar reductions in AT8 levels (Fig. 6) but had opposite effects on toxicity. Both are involved in the ERK pathway and, as expected, both *bancal* and *ksr* showed significantly reduced levels of pERK (Fig. 7B), which correlates well with the reduced AT8 levels seen with both genes. However, *ksr* showed a significantly higher level of inactivated GSK-3β, whereas *bancal* trended to lower levels of inactivated GSK-3β (Fig. 7B). To our knowledge, this is the first report that *kinase suppressor of Ras* (*ksr*), classically identified in the Ras-Raf-MAPK/ERK pathway, can also influence the GSK-3β pathway. This difference in GSK-3β activity correlates well with the difference in toxicity modification, with reduced GSK-3β activity induced by *ksr* suppressing toxicity and marginal increased GSK-3β activity induced by *bancal* enhancing toxicity. However, when we assayed for GSK-3β activity among all other modifiers, we could not observe a consistent pattern between suppressors or enhancers (data not shown). This may indicate that the role of GSK-3β in tau toxicity may be more prominent in conditions where ERK activity is reduced. We conclude that the suppressive effect of *ksr* on Aβ42 and TDP-43 is due to reduced GSK-3β activity, however, the effect of *ksr* may not be as robust as *sgg* suppression due to decreased ERK activity also found with *ksr*. This also indicates that the modifiers identified in this screen operate at different levels in tau toxicity, providing a diverse range of therapeutic targets.

It is interesting to note that both KSR and NRG (137–139) are involved in scaffolding membrane-associated proteins; thus their effects on tau toxicity may not be directly related to their role in ERK signaling. LOF of *Nrg* causes significant reduction in microtubules at synaptic terminals (140,141), and previous efforts have suggested that tau toxicity may be partially due to 'hyperstabilization' of microtubules (42); therefore, an increase in cytoskeletal instability caused by reduced *Nrg* in a tau-induced hyperstabilized environment may result in alleviation of tau toxicity.

Mekk1 is a MAP3K that leads to the phosphorylation and activation of p38/MAPK (87), which is activated in response to cell stress (142–144). Overexpression of *Mekk1* was found to strongly enhance tau toxicity in this screen (Fig. 3) and increases p38 activity in our model (Fig. 6). Elevated p38 activity is found in brains from patients with tauopathies (145–147) and in transgenic AD mice models expressing human tau (148–150) or mutant APP (151), both of which correlate with tau aggregation. Furthermore, there is abundant evidence linking p38 to tau pathology. Phospho-p38 is found exclusively in cells with NFT or tau aggregates in AD cases (152,153), co-localizes with tau aggregates in tauopathic brains (145,154) and co-precipitates with insoluble tau in both human cases and transgenic AD mouse models (145,148). Furthermore, tau can be phosphorylated by p38, which can induce microtubule assembly *in vitro* (33,39).

We report novel synergy between tau and *Mekk1* through p38 that has potent effects on ERK activity. Neither *Mekk1* expression nor tau expression alone affects ERK activity, whereas co-expression leads to marked reduction of phospho-ERK levels (Fig. 6). Phospho-p38 is increased relative to phospho-ERK in AD, CBD and PSP (155), in AD mouse models (151) and tauopathy mouse models (156). It has also been reported that the extracellular domain of L1-CAM, the mammalian homolog of *Neuroglian* (*Nrg*), reduces p38 activity (157). These data indicate that an antagonistic cross-talk takes place between ERK and p38 activities, with tau playing a modulatory role. The function of the N-terminus of the tau protein has long been elusive; however, a recent report observed that the N-terminus can activate protein phosphatase 1 (PP1) (158–175). In combination with our data, we hypothesize that phosphorylation of tau by p38 causes a specific conformational change that allows for increased exposure of the N-terminus of tau, which activates PP1, thereby reducing phospho-ERK levels. Furthermore, if tau has the ability to regulate its own kinases, and if this regulation is phosphorylation-dependent and potentially conformationally dependent, this would broaden our understanding of the role of tau phosphorylation, which to date has been primarily associated with reduced microtubule-binding affinity. This may also give insights into functions of specific phosphorylation sites in regulating kinases, as well as the reasons that certain sites are more responsible for causing toxicity.

The general transcription factors *NC2α*/DRAP-1 and *cropped*/AP4 were identified in this screen. *NC2α* binds to and represses TATA-driven promoters and activates downstream promoter element containing promoters (60), both of which are very common genomic elements. Thus, it is not trivial to pinpoint the genes regulated by *NC2α* that modifies tau toxicity; however, it has been shown that cellular stress induced by hypoxia upregulates *NC2α* activity (159). The cellular stress induced by tau overexpression may activate a similar response. *NC2α* did not affect apoptosis or general eye morphology, but did suppress Q108-induced toxicity, which can also be considered a model of cellular stress. The transcription factor AP4 is activated by *myc* and may be activated by Notch signaling. Several other transcription factors were network-associated, including β-catenin/*armadillo* (*arm*). Armadillo is cytosolic but translocates to the nucleus and binds to the co-activator T-cell factor/lymphoid enhancer factor, inducing transcription of target genes (160–162). Two of these target genes are *c-myc* and *cyclin D1*, both of which converge to positively regulate the Cyclin E/CdK2 complex (163), complementing the observation that *Cyclin E* reduction enhances tau toxicity.

Autophagy/lysosomal pathways, proteases and chaperones

Autophagy (technically macroautophagy) is a pathway for molecular degradation in which autophagosomes engulf organelles or large quantities of protein and later merge with lysosomal bodies to form autolysosomes, wherein contents are degraded due to conditions such as reduced pH and activated cathepsins. Nearly all neurodegenerative diseases characterized by protein aggregates show increased number and abnormal autophagic vacuoles (164–167). There are several points

at which autophagy may become impaired: autophagosome induction and formation, protein entry into autophagosomes, trafficking and fusion of autophagosomes to lysosomes and improper lysosomal activity due to imbalanced pH or non-functional cathepsins (see 168 for a review). A growing body of evidence suggests that autophagy is protective in neurodegeneration. Genetic deletions of autophagic genes are sufficient to induce neurodegeneration with ubiquitin-positive neuronal inclusions (169–171). Transgenic mice expressing human mutant tau isoforms exhibit an increased number of autophagic vacuoles and increased number of lysosomes that show aberrant AD-like morphology (172–179). Tau has been shown to be degraded by lysosomes and autophagic vacuoles (173,174), and inhibition of lysosomal activity leads to increased levels of tau and aggregation of tau fragments (15,175,176). PHFs are seen in certain lysosomal storage disorders, such as Niemann–Pick disease type C and Sanfillipo syndrome type B (177–180). Abnormally enlarged lysosomes associated with LOF mutations of *benchwarmer*/*spinster* (181) or *cathepsin D* (182) are associated with enhanced tau toxicity in fly models of tauopathy.

We uncovered an LOF allele of *Autophagy-specific gene 6* (*Atg6*), the *Drosophila* homolog of mammalian Beclin-1, that increases tau toxicity, further implicating autophagy as a protective process. Several other reports have also found Atg6/Beclin-1 to be protective in degenerative conditions. Pickford *et al*. (171) found reduced levels of Beclin-1 in brains of early-stage AD and demonstrated that Beclin-1-deficient mice have enhanced APP-mediated neurodegeneration and reduced clearance of APP and APP cleavage products; these deficiencies were rescued by the expression of Beclin-1. Our screen also identified *Vha14*, a subunit of the V_1 head group of the vacuolar ATPase (v-ATPase) complex that is required for lysosomal acidification (183). Recent reports have demonstrated that disruption of the v-ATPase complex, through LOF of the V_0 transmembrane domain, leads to increased number of lysosomes that are impaired in proteolysis (184) and causes progressive neurodegeneration (185) and enhanced sensitivity to Aβ or tau (186). Furthermore, we previously identified mutations in the pigment-associated genes *white*, *brown* and *rosy* that enhance tau toxicity (61). Mutations in *white* and *brown* lead to abnormally large pigment granules that become abnormal autolysosomes (187,188), and we observe a decrease in phospho-S6K in *white* homozygotes when compared with *white* hetereozygotes, suggesting that target of rapamycin-regulated autophagy is activated dose-dependently by *white* mutations (189).

Interestingly, we also observe a strong decrease in GSK-3β activity in *white* and *brown* mutants and a strong increased activity in a *rosy* mutant (61). These observations suggest that dysregulation of autophagy-lysosomal pathway and GSK-3β activity is related; however, the nature of this relation appears to be very complex. Our results suggest that disruption of lysosomal function leads to increased GSK-3β activity. However, other reports claim GSK-3β activity is upstream of autophagy activity, and if so, it is unclear whether GSK-3β inhibits (190,191) or initiates autophagy (192). Although unclear now, the relationship between GSK-3β and autophagy may prove to be very relevant in understanding the role of GSK-3β in tauopathies and other neurodegenerative diseases. GSK-3β may be a checkpoint enzyme where both apoptotic and autophagy pathways converge, and either allows for continued autophagy to maintain cell survival, or initiate apoptosis if autophagy has failed to suppress cellular toxicity.

Two modifiers of tau toxicity—*Atg6* and *Dlic2* (*dynein light chain 2*)—were also recently identified as modifiers of lysosomal/autophagic vacuole trafficking (193), and dynein appears to be the motor protein most responsible for this trafficking (194,195). Autophagosomes are trafficked through the cell along microtubules for fusion with lysosomes (196). Blocking microtubule trafficking slows down this fusion and allows for the accumulation of autophagic vacuoles similar to those seen in AD brain (197). Tau impedes kinesin and dynein motors on microtubules via competition for microtubule binding (198); thus tau may also block proper trafficking and fusion of autophagosomes to lysosomes, causing impaired autophagy. Neurensin-1 (or Neuro-p24) is a lysosomal membrane protein that is enriched in neurons, specifically in neuritic processes, and has a cytosolic tail that contains a microtubule-binding domain homologous to binding repeats found in tau (199). Hence, tau may block neurensin-1 from microtubule binding, leading to impaired lysosomal trafficking when tau is over-abundant.

In other animal models of neurodegeneration, co-expression of chaperone proteins with disease-associated proteins significantly reduces degeneration (99,200). The loss of chaperones may lead to protein misfolding and therefore increased activity of the ubiquitin-proteasome system (UPS) or autophagy to degrade the dysfunctional proteins. *Hsp70/Hsp90 organizing protein homolog* (*Hop*) is a chaperone-binding protein that binds to both Hsp70 and Hsp90, brings them together to form a large complex and regulates the activity of both (201–203). In our screen, LOF of *Hop* enhances tau toxicity, providing further evidence for the role of chaperones in tauopathy. The E3 ubiquitin ligase CHIP (carboxyl terminus of the hsp70-interacting protein) ubiquitinates phosphorylated tau and mediates its degradation (16,17,204). CHIP, Hsp70, Hsp90 and tau have been identified as binding partners (205), which strongly positions *Hop* as a regulator to facilitate clearance of tau via the UPS. *Hop* may also facilitate clearance through autophagy. Autophagy can be induced as a compensatory degradation system when the UPS is impaired (206,207), and hsp70 chaperones tau to lysosomes via chaperone-mediated autophagy (208).

The computational network independently identified the protease PSA as highly associated with tau modifiers. It has been reported that PSA directly cleaves tau (18), although a recent report has challenged this finding (209), and the protective effects of PSA may instead be mediated through activation of autophagy (210). Whether by cleavage or by autophagy, genetic manipulation of PSA expression in our model was previously shown in a candidate genetic approach to strongly modify tau toxicity (48). The network identification of PSA helps to validate the applicability of the network and suggests that calpains A, B and C are highly relevant proteases. Indeed, calpain A has been shown to cleave tau and is reported to be responsible for producing a 17 kDa fragment that may be a highly toxic tau derivative (211,212).

The gene *Psn*, which encodes the single fly presenilin, was highly associated with tau modifiers in our computational network. Mutations in presenilins are the most common

causes of familial AD (213), and the classical role of presenilin is as a member of the γ-secretase complex, which cleaves amyloid precursor protein along with β-secretase (214) to make β-amyloid peptides. However, recent reports have identified novel functions of presenilin, e.g. as a calcium leak channel (215,216) and a regulator of vacuolar ATPase required for establishing proper lysosomal pH (184). The presenilin/γ-secretase complex cleaves other type I transmembrane proteins, including Notch (217). The Notch signaling pathway was computationally associated with the tau modifiers, and *fringe connection* (*frc*), a gene required for Notch glycosylation, was found as a tau modifier. However, Notch glycosylation is mediated by two proteins: *fringe* (*fng*), an endoplasmic reticulum (ER) glycotransferase that directly attaches sugars onto Notch, and *frc*, an ER membrane-bound protein that transports the sugars used as substrates by *fng* for glycosylation (218,219). A second allele of *frc* (*frc*0073) confirmed its suppressive effects on tau toxicity; however, none of the *fng* alleles tested ($fngr^{G554}$, fng^{13}, fng^{M69}, fng^{L73}, fng^{52}) showed any effect on the tau phenotype, suggesting that altered Notch signaling does not underlie *frc* suppression of tau toxicity. Many other receptors require glycosylation of their extracellular domains for functional activity; thus *frc* suppression of tau toxicity may be mediated through altered glycosylation of such other transmembrane proteins.

Microtubule, endosomal and lipid trafficking proteins

We demonstrate *in vivo* differential effects of kinesins (*cana* and *Klp61F*) and dynein (*Dlic2*) with tau-induced neurotoxicity. Several investigators have reported that increased tau expression results in axonal transport defects (94,158,220–222), specifically kinesin-mediated anterograde transport to synaptic terminals, whereas dynein-mediated retrograde transport to the soma is relatively undisturbed. Tau, kinesin and dynein all compete for the same binding site on β-tubulin (223); however, tau has a 10-fold increased preference over kinesin (198) and binds to the kinesin heavy chain (224–226). Recently, reductions in kinesin *in vivo* have been reported to exacerbate tau-induced axonopathies and cargo accumulation (227,228), consistent with our findings. As kinesin is critical for axonal transport, it may be expected that reducing kinesin would exacerbate tau toxicity. Unexpected is the suppression of tau toxicity due to reduction in dynein. As tau, dynein and kinesin compete for the same tubulin-binding sites, and as tau preferentially outcompetes kinesin, a reduction in dynein may reduce the competition for microtubule binding and increase kinesin availability for microtubule binding in a milieu of abundant tau, which could improve axonal transport and suppress toxicity.

Two modifiers identified from this study, *Transport and Golgi organization 5* (*Tango5*) and *Klp61F*, were also recently identified as key regulators of Golgi apparatus (GA) structure and organization (229). Dynein has also been demonstrated to participate in Golgi organization (230); thus *Dlic2* may also function with *Klp61F* or other kinesins in GA maintenance and endosomal fusion and fission. Tau mediates interactions between microtubules and the GA to maintain structure of the latter (231), and tau may function in regulating transport of Golgi-derived endosomes and vesicular organelles such as peroxisomes and lysosomes (232,233). Golgi fragmentation is observed in several tauopathies (234,235) and in animal models (233,236,237). This fragmentation appears to be an early step in neurodegeneration and may function as a 'trigger' for apoptosis (237,238). *Klp61F* LOF causes the GA to aggregate and swell, whereas *Tango5* LOF fuses the GA with the endoplasmic reticulum (ER; 230). Collapse of the GA with the ER may induce the ER stress response, which is associated with AD and other neurodegenerative diseases (239,240). GA fragmentation is also associated with decreased AKT activity and concomitant increased activity of GSK-3β, which may itself induce GA fragmentation (241).

Three genes identified as tau modifiers were also recently found to be regulators of lipid droplet biogenesis and regulation—*RpLP0*, *Dlic2* and *His2Av* (242,243). In addition, *white* and *rosy*, two modifiers of tau toxicity identified earlier from this study (61), also are important in lipid trafficking. The gene *rosy* regulates lipid droplet coupling to the plasma membrane during lipid secretion (244), and the mammalian homolog of *white*—ABCG1—is a major effector in lipid trafficking (245). Additionally, expression of both ABCG1 and APOE is regulated by PPARγ (246), and both may function as part of a common lipid trafficking pathway (247). Apolipoprotein E (APOE) is necessary for cholesterol transport and plasma membrane metabolism in the brain, and the epsilon 4 variant (APOE4) is the most well-established genetic risk factor for sporadic AD (248,249), although several other genetic association studies for AD have produced several other candidate genes (250). Several association studies have shown a synergistic effect between APOE4 and certain haplotypes or polymorphisms of *MAPT* in increasing susceptibility to AD and frontotemporal dementia (251,252). Transgenic mice that express full or truncated human APOE4 show increased tau phosphorylation and PHF-like filaments (253–255), and APOE knockout mice show tau-dependent neurodegeneration (256). Thus tau and APOE interaction may be necessary for functional cholesterol trafficking; however, the APOE4 variant has a much lower affinity for tau when compared with other APOE isoforms (257,258), which may impair lipid transport. Dysregulation in the metabolism and trafficking of cholesterol and other lipids is strongly implicated in several tauopathies, including AD and Niemann–Pick Type C (NPC; 259), which is caused by mutations in the gene, *NPC1*. In NPC, the inability to transport lipids, including cholesterol, out of late endosomes/lysosomes leads to engorgement of these organelles (260). Our computational network identified *Npc1*, the *Drosophila* homolog of *NPC1*, as highly associated with the modifier network, further validating the network to identify relevant tauopathic genes. The Patched receptor—part of the Hedgehog signaling pathway—recruits lipoproteins to destabilize plasma membrane–protein interactions through a homologous sterol-sensing domain of NPC-1 (261). Direct chemical inhibition of cholesterol transport in neurons itself leads to increased tau phosphorylation (262), providing further evidence of tau involvement in lipid regulation. It is also interesting to note that increased activity of AKT also leads to increased lipid droplet size (263,264).

These observations would suggest dietary cholesterol also influences tauopathies. Reports in both wild-type and in human tau transgenic mammals indicate that diets with

increased cholesterol do indeed increase tau phosphorylation (265–267). However, these reports do not provide any mechanistic insights regarding the means by which increased cholesterol leads to increased tau phosphorylation; nor do they report increased neurodegeneration as a result of increased phosphorylation. It does seem clear that increased cholesterol intake leads to cognitive impairments (268), of which increased tau phosphorylation may contribute. From these reports and our data, it is reasonable to speculate that tau has direct involvement in lipid trafficking and may even bind directly to cholesterol. Indirect evidence for this comes from observations that tangle-bearing neurons contain more free cholesterol than tangle-free neurons in both AD and NPC brains (269), suggesting that when tau is sequestered into tangles it is unable to bind its normal substrates (e.g. cholesterol).

Cell cycle/nuclear/chromatin binding

A surprising number of modifiers identified from the screen play a role in regulating the cell cycle or chromatin binding, lending further credence to the mitotic failure/cell-cycle re-entry hypothesis of neurodegeneration. This hypothesis posits that cell signaling cues required for synaptic plasticity in post-mitotic neurons are erroneously transduced to re-induce proliferation (270,271); this concept is supported by several studies in models of tauopathy (272–275). The modifier *Cyclin E* (*CycE*) is necessary for Cdk2 activity (276,277), and Cyclin E and Cdk2 have been reported to interact directly with tau (278). Cyclin E has been shown previously to modify toxicity in fly models induced by both mutant (241) and wild-type tau (41). Cdk5 is a known tau kinase (28,279), which may indicate a common regulatory mechanism between Cdk5 and Cyclin E/Cdk2. Phosphorylation of tau increases during mitosis (280), which may be dependent on tau phosphorylation by cdc2-like kinase/Cdk5 (281). Klp61F is phosphorylated and activated by Cdc2 (282), which was recently found to associate with Cyclin E (283). *CamKI* is also involved in regulating the cell cycle, particularly the G1 phase (284,285), and in transcriptional regulation (286–288).

Reduction in *smallminded* (*smid*) gene dosage showed a dramatic suppression of tau-induced toxicity. The primary phenotype of homozygous *smid* mutants is a reduction in the number of neurons found in the central nervous system. Smid is a member of the AAA (ATPases associated with diverse cellular activities) superfamily of proteins, which all share a highly conserved nucleotide-binding domain (AAA) (289). Smid is predicted to be a serine-type endopeptidase, although no substrates have been identified, and total tau protein levels and AT8 signal show no differences in *trans* to *smid* when compared with control, indicating that tau is not a substrate. Interestingly, similar to *CycE*, Smid is also required for induction of S phase in neurons, and it contains four potential sites for phosphorylation by cyclin-dependent kinases. These sites also overlap putative bipartite nuclear localization signals (290), further implicating a role in cell division-related processes. Although *smid* has no obvious mammalian homolog, it contains a duplication of the AAA module that places it in the same Cdc48p/VCP/p97 (valosin-containing protein) subfamily of AAA proteins, which also regulate the cell cycle (291). VCP is a chaperone protein involved specifically in extracting misfolded proteins from the ER, but has also been implicated in a wider range of other cellular functions (292). Mutations in VCP have been linked to several neurodegenerative diseases, such as inclusion body myopathy associated with page disease of bone and frontotemporal dementia (IBMPFD), body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) that presents with cytoplasmic PHFs (293), as well as FTLD (294), and most recently to ALS (295). VCP has also been found to be significantly down-regulated in AD brains but co-localizes with NFT-positive neurons (296). VCP also co-localizes with nuclear inclusions in HD brains and with Lewy bodies in patient brains of sporadic Parkinson's disease and Lewy body dementia (297). LOF alleles of VCP/*ter94* in *Drosophila* suppress polyglutamine toxicity (298) and retinal degeneration (299), similar to our finding of LOF of *smid* suppressing tau toxicity. Torsin proteins are also a subfamily of AAA proteins in which disease-causing mutations have been linked to the movement disorder, torsion dystonia and also appear to function as a molecular chaperone (300). The torsin A protein has also been found to co-localize with α-synuclein-positive inclusions in Parkinson's disease brains (301). Overexpression of torsin A in *Caenorhabditis elegans* can suppress cellular toxicity of dopaminergic neurons (302) and polyglutamine protein aggregation (303). Despite similarities in being members of the AAA protein family and both being molecular chaperones, torsin A and VCP have different phenotypes. Reductions in VCP expression appear to suppress neurodegenerative effects, while increased torsin A expression is beneficial, suggesting different functional pathways, of which our data suggest *smid* may be more closely related to VCP functions.

Tau has the capacity to bind DNA (281,304) and may protect it from oxidative damage (305). Tau also has the ability to nick DNA and affect its helicity (306). Microtubule motor proteins and tau modifiers *cana*, *Klp61F* and *Dlic2* all have critical roles in regulating microtubule dynamics, centrosome and spindle organization in the nucleus during mitosis (272,307–309). Several modifiers identified from this screen interact directly with chromatin: *Histone H2A variant* (*His2Av*), *Mi-2*, *mei-9*, *NC2α* and the heterogeneous nuclear ribonucleoproteins *bancal*/hnRNP K and *smooth*/hnRNP L. Bancal/hnRNP K can bind to chromatin and may protect it from damage during stress (310). Homozygous LOF mutations in *bancal* also cause cell proliferation impairments that cause reductions in cell number and size of appendages (311). It is possible that tau binding facilitates the interaction between chromatin and microtubules and may facilitate chromatin remodeling during transcriptional activation or inhibition. It is interesting to note that only the shortest tau isoform (0N/3R) is expressed during fetal development (7), which may prevent tau from disrupting cell division and transcriptional dynamics in the nucleus necessary for proper development.

RNA binding and ribosomal proteins

Another large category of tau modifiers is comprised of RNA-binding proteins or other proteins associated with ribosomes and protein translation. Previous reports have shown that AT8-immunoreactive tau co-localizes predominantly with free

and endoplasmic reticulum-bound ribosomes in PSP and CBD (312), co-localizes with free ribosomes in FTDP-17 mutant tau in pretangle neurons (313) and with ribosomes in aged sheep that present with NFT in dendritic branches (314). Our data further support an interaction between tau and ribosomes. Tau binds preferentially to rRNA gene regions in which the nucleolar-organizing region is situated (315), which may in fact stabilize nucleolar organization (316,317). RNA has long been recognized as a factor that induces tau aggregation (318,319), and RNA is sequestered in PHFs in the brain in many tauopathies, including AD, Pick's disease, CBD and PSP (320,321), as well as PHFs in muscle (322). Interestingly, this RNA sequestration is limited to PHF and senile plaques, and not to Lewy bodies of α-synuclein aggregates nor Hirano bodies containing actin (323), suggesting that RNA sequestration is a not a common feature of all amyloidogenic proteins. In nearly all RNA-associated modifiers identified in this screen, an LOF led to increased toxicity; this may be due to a reduced number of proteins able to sequester RNA away from the tau protein.

Several functions have been attributed to *RpLP0* and *bancal*/hnRNP K. *RpLP0* regulates the translocation of a nuclear laminar protein from the cytosol to the nucleus (323), and *RpLP0* associates tightly with the nuclear matrix and may play a role in DNA repair (325). *RpLP0* was also identified as a regulator of phagocytosis (324), and both *RpLP0* and *Dlic2*—two modifiers from this screen—were also found to regulate lipid droplet formation and distribution (216). Interestingly, *RpLP0* and *Dlic2* regulate lipid-droplet formation in opposing directions, similar to their effects on tau toxicity. *RpLP0* also regulates *white* expression (325), another tau modifier that may also play a role in lipid trafficking through interaction with APOE, further demonstrating genetic connectivity between tau modifiers.

Bancal/hnRNP K is involved in nearly all aspects of protein synthesis, including transcription, translation and mRNA stability and splicing, and it can shuttle between the cytosol to nucleus (see 326 for a review). Bancal is also a component of the nuclear matrix (327) and can bind to promoter regions to actively promote or repress transcription (326). hnRNP K is highly expressed in the mammalian nervous system during development and remains high in the peripheral nervous system in adults, but becomes restricted to the hippocampus and retina in the adult central nervous system (328). HnRNP K binds mRNAs of neurofilaments during development (329). Bancal/hnRNP K is also regulated by EGF signaling through ERK phosphorylation (330,331). It also interacts with Fyn kinase, another known tau kinase implicated in AD and FTLD (332–337), and is phosphorylated through the insulin-signaling pathway (338). This appears to be a reciprocal regulation, as knockdown of hnRNP K leads to decreased activity of ERK and ERK kinase (339), perhaps accounting for the decrease in AT8 levels due to a reduction in *bancal* in *trans* with *gl*-tau (Fig. 5).

Intriguingly, an hnRNP K homology domain is found in FMRP (Fragile X mental retardation protein), which is involved in trafficking mRNA from the nucleus to dendrites and axons and regulates their translation (340,341), suggesting similar functions between hnRNP K and FMRP. *Fmr1*, the *Drosophila* homolog of FMRP, and *TBPH*, the fly homology of TDP-43, were identified through the computational network as highly associated and are both RNA-binding proteins causing neurological diseases (342–346). Ribonucleoproteins are also responsible for the trafficking of non-translated RNA species, which are packaged into either processing bodies (P bodies) or stress granules (347). Stress granules are complexes of mRNAs stalled in translation with ribonucleoproteins, and as the name suggests, are induced by several kinds of cellular stress or when translation is inhibited. FMRP, TDP-43 and hnRNP K have all been shown to interact with stress granules (348–351). As FMRP1-bound RNA granules require kinesins and microtubules for trafficking (352,353), it is reasonable to predict that hnRNP K and TDP-43 do, as well. Given the large number of RNA binding and ribosomal modifiers of tau toxicity found in this screen, in conjunction with a known role for tau in microtubule binding, collectively these data suggest that tau may have a significant role in RNA trafficking that has not previously been documented.

Relation to other modifier screens of tau-induced neurodegeneration

Here we report novel modifiers distinct from those identified in two recent reports that also screened for genetic modifiers of tau-induced neurodegeneration in *Drosophila* (52,98). The differences in modifiers obtained may be attributed to several differences in study design, namely (i) the use of mutant tau (tauV337M), (ii) tau overexpression driven by GAL4/UAS constructs and (iii) different collections of P element insertion lines used for screening, i.e. P{EP} and P{Mae-UAS.6.11}. Our modifiers were also largely distinct from those identified in a *C. elegans* model of tauopathy (97). Despite these differences, common results were found with kinases, specifically GSK-3β and PAR-1/MARK-2 or MARKK, and with chaperone proteins and cytoskeletal proteins, demonstrating the significance of these proteins in tau pathology. Our screen was designed to identify only dominant modifiers. However, the haploinsufficient basis of modifiers identified suggests that relatively small changes in key proteins or other gene products can strongly modify tau toxicity, which is encouraging for the development of therapeutic treatments and may assist in identifying biomarkers that are predictive of neurodegenerative tauopathies.

CONCLUSION

The experiments described here were intended to identify novel modifiers of tau-induced neurodegeneration in order to better understand the function of tau and the processes involved in tau-associated pathogenesis in neurodegenerative tauopathies. In sum, 40 genetic modifiers were identified as strong modifiers of tau toxicity (Table 1); of these, *sgg*/GSK-3β and *par-1*/MARK-2 are known tau kinases, validating the design of the screen to identify modifiers of tau or tau toxicity. The functions of the remaining modifiers, in combination with application of a novel computational network approach to extrapolate other highly associated genes, cover a broad range of functional categories. Some of these

categories, such as kinases or phosphatases and motor and cytoskeletal proteins, have been associated with tau previously, whereas other categories emphasize novel or poorly characterized aspects of tau function, including lipid storage and trafficking, Golgi or endosomal, several RNA-related categories, including RNA splicing, metabolism, trafficking or protein translation, and interactions with chromatin and the nucleolus. Categories such as cell-cycle/nuclear, PI3K signaling, and proteases and chaperones contribute to a growing body of evidence that associates these processes with tau toxicity or neurodegeneration. Tau phosphorylation at S202/T205 (AT8 epitope), S262/S356 (12E8 epitope) or T181 (AT270 epitope) did not correlate with toxicity, arguing against tau phosphorylation as an indispensable factor in tauopathy. Additionally, suppressors of wild-type tau are equally able to suppress the phosphorylation-resistant but more toxic S11A tau isoform, from which we conclude that mechanisms independent of tau phosphorylation can alter toxicity. However, the non-toxic S2A tau isoform could not be induced to show toxicity with genetic enhancers, indicating that the S262 and S356 sites are particularly important in producing toxicity. We observe that tau functions synergistically with *Mekk1* and p38 to down-regulate ERK activity, with a corresponding decrease in AT8-positive phosphorylation. This observation suggests that tau can be induced to regulate its kinases, providing evidence for signaling properties of tau. We observe that GSK-3β activity highly correlates with toxicity, but its effects are most pronounced under conditions of low ERK activity. In addition, we demonstrate that *ksr* can signal through the insulin/GSK-3β pathway as well as through the MAPK/ERK pathway, and may function as a link between both signal transduction systems. Finally, we report that mutant TDP-43^{Q331K} strongly increases GSK-3β activity and that reducing GSK-3β (*sgg*) expression strongly suppresses TDP-43 and Aβ42 toxicity. As alternatives to therapeutics that mitigate tau phosphorylation are developed, such as improved microtubule stability (354,355), the data presented here provide further evidence that such approaches may be productive and identify a novel set of targets for such alternatives.

MATERIALS AND METHODS

Stocks and genetics

A direct fusion construct of the human full-length (2N/4R) tau cDNA to the eye-specific *glass* promoter induces a rough eye phenotype (*gl*-tau line), as previously described in Jackson *et al*. (47). In the course of performing the P lethal screen, it was discovered that the common background mutation *white* (*w*) was itself a modifier of tau-induced neurotoxicity and that the w^+ marker gene in the P elements used in the P lethal and EY collections modified the tau phenotype in a w^+ dose-dependent manner (61). The *gl*-tau transgene has one w^+ marker gene in a w^{1118} homozygous background. To match w^+ copy number between control and experimental crosses, the F_1 *gl*-tau control was on a *white* heterozygous background: w^{1118}/+;*gl*-tau/+. For the EY screen, a GMR-GAL4 transgene on the X chromosome (356) was placed in *trans* to *gl*-tau (GMR-GAL4;*gl*-tau/CyO) to provide a source of GAL4 to drive expression of the gene downstream of the EY insertion. The F_1 control genotype for the EY screen was GMR-GAL4/+;*gl*-tau/+. The EY stocks are in a y^1w^{67c23} background, and GMR-GAL4/y^1w^{67c23};*gl*-tau/+ has a suppressed phenotype versus GMR-GAL4/+;*gl*-tau/+ (not shown); thus, a candidate modifier was identified as a suppressor if it suppressed when compared with the GMR-GAL4/y^1w^{67c23};*gl*-tau/+ control. Enhancers were scored when compared with the GMR-GAL4/+;*gl*-tau/+ control, ensuring that only the most robust and reliable modifiers of toxicity were included. The LOF screen utilized the 'P lethal' library of ∼1000 genes and is comprised of the LacW and PZ collections, created as part of the Berkeley Drosophila Gene Disruption Project (49). Each stock contains one P element that disrupts the expression of a gene and causes recessive lethality. These stocks are only viable as heterozygotes and are maintained over a balancer. For P elements on the X chromosome, virgin females were collected from each stock and crossed to *gl*-tau males. The EY screen utilized 'empty UAS' insertion lines from the EY collection (50). The P lethal, EY and the GMR-*hid* stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University. In most cases, the P element database reported a single gene affected; however, three of the hits had two or more affected genes listed: Bloomington stock numbers 10448 (loci *U2af38* and *Hop*), 10151 (loci *Tango5*, *G0145a* and *G0145c*) and 10691 (loci *bancal* and *rig*). Independent alleles for *U2af38*, *Hop*, *Tango5*, *bancal* and *rig* were screened—$U2af38^{06751}$, Hop^{k00616}, $Tango5^{BG02353}$, $bancal^{kG02524}$ and rig^{05056}. *Hop* but not *U2af38* enhanced toxicity, and $Tango5^{BG02353}$ produced an enhanced rough eye phenotype similar to that observed with stock No. 10151; thus, *Tango5* was the causative gene. Allele rig^{05056} had little effect on the tau phenotype, whereas $bancal^{kG02524}$ and bl^{G13574} showed enhanced toxicity often with necrotic plaques; thus, *bancal* was considered to be the causative gene. Stock number 19628 of the EY collection lists two affected genes: *milton* and *CG31630*; EY stock number 22422, which only affects *milton*, was screened and had no effect on the tau phenotype, thus *CG31630* was considered as the modifying gene. The Q108 stock was provided by Thompson (University of California, Irvine, CA, USA) (54) and was placed in *cis* to GMR-GAL4 (357) using mitotic recombination. Crosses for the LOF screen were performed at 22−23°C, whereas crosses for the overexpression screen were performed at 25°C in order to maximize expression under control of the GMR-GAL4 transgene. All crosses were maintained on standard cornmeal/molasses media (Applied Scientific Jazzmix, Fisher Scientific, Pittsburgh, PA, USA).

The UAS-S11A and UAS-S2A lines developed in our laboratory (42) were crossed to GMR-GAL4 on the X to establish the stable GMR-GAL4;UAS-S11A or UAS-S2A lines. The S11A line has the following sites mutated from serine/threonine to alanine: S46, S50, S199, S202, S205, S212, T214, T231, S235, S396 and S404. The S2A has the following sites mutated from serines to alanines: S262 and S356. Female virgins of each line were crossed to candidate modifier males and reared and eclosed at 25°C. Aβ42 toxicity was assayed using the w^{1118};GMR-GAL4, UAS-Aβ42/CyO line generously

provided by Diego Rincon-Limas (University of Florida) (89). Female virgins were collected and crossed to candidate suppressor males and kept at 29°C through eclosion. Suppression was scored 1–3 days after eclosion. For TDP-43^{Q331K} toxicity, the w^{1118};*Sp*/CyO;UAS-TDP-43^{Q331K} donated by Fen-Biao Gao (University of Massachusetts, Worcester, MA, USA) was used (358). The markers on the second chromosome were removed and the transgene was placed in *trans* to GMR-GAL4 on the X to establish the stable line GMR-GAL4;+;UAS-TDP-43^{Q331K}. Female virgins from this line were crossed to candidate suppressor males and reared at 25°C. After eclosion, the F_1 generation was transferred to 29°C and kept there for 2 weeks before scoring for suppression. All crosses were maintained on standard cornmeal/molasses media (Applied Scientific Jazzmix, Fisher Scientific, Pittsburgh, PA, USA).

Microscopy and volume analysis

Scanning electron microscope (SEM) images were taken using a Hitachi S-2460N SEM. Flies were dehydrated in hexamethyldisilazane prior to mounting for SEM as described previously (47). All light photomicrographs of the EY modifiers were taken using a Nikon AZ100M light microscope and Nikon DS-Fi1 digital camera. Z-stack planar images were compiled and compressed using an EDF algorithm to compile a single image from all focal planes (Nikon NIS-Elements AR 3.0 Software). All photomicrographs of the P lethal modifiers were taken with a digital camera equipped Zeiss dissecting microscope without EDF algorithm capability. Volume analysis was performed on Z-stack images of all genotypes using the Nikon NIS_Elements AR 3.0 Software. Scatter plots were constructed using SigmaPlot 9.0 (Systat, San Jose, CA, USA) and modified with Photoshop CS4 (Adobe, San Jose, CA, USA).

Immunoblotting

Protein from fly heads was collected by homogenizing in tris-buffered saline (TBS) buffer with protease cocktail inhibitors (Roche Diagnostics, Manheim, Germany). Pooled samples were run on either 10% or 10–20% sodium dodecyl sulfate—polyacrylamide gel electrophoresis gels (Bio-Rad, San Diego, CA, USA) and transferred to nitrocellulose membranes. Total Tau protein was detected with the mouse monoclonal T46 antibody (1:3000 dilution; Invitrogen, Carlsbad, CA, USA) or rabbit monoclonal E178 (1:2000 dilution; Epitomics, Burlingame, CA, USA). Phosphorylated tau at S202/T205 was detected with the mouse monoclonal AT8 antibody (1:1000 dilution; Pierce/Thermo Scientific, Rockford, IL, USA). Tubulin was detected with β-tubulin antibody from either Accurate Chemical (Westbury, NY, USA) or from the Developmental Studies Hybridoma Bank (DSHB, E7 clone, University of Iowa), whereas actin was detected by anti-β-actin mouse monoclonal (Ambion/Applied Biosystem, Austin, TX, USA). Blots for P lethal hits were visualized with horseradish peroxidase-conjugated secondaries and enhanced chemiluminescence; total tau levels were measured using blots separate from those used for AT8. Blots for EY hits were visualized by two-color western with fluorescent secondaries and imaged with the Odyssey Near-IR Scanner (Li-Cor), which allowed visualization and measurement of total tau (E178, rabbit IgG) and phospho-tau (AT8, 12E8, or AT270, all mouse IgG) on the same blot. The following antibodies were also used: phospho-GSK3β-Ser9 (1:500) (GeneTex, Irvine, CA, USA), phospho-p38 (1:500) (Cell Signaling, Danvers, MA, USA) and phospho-ERK (1:500) (Invitrogen/Biosource, Carlsbad, CA, USA). Optical densities were measured with ImageJ (http://rsb.info.nih.gov/ij). Statistical analysis was performed with SigmaStat 11.0 and graphical representations were performed with SigmaPlot 9.0 (Systat, San Jose, CA, USA) and Excel 12.1.5 (Microsoft, Seattle, WA, USA). Error bars represent $\pm$SEM ($n = 3–5$). One-way analyses of variance (ANOVAs) with Bonferroni analysis compared with control were analyzed for measurements of total tau, AT8, 12E8 and AT270 levels. One-way ANOVAs were used to analyze measurements of phosphorylated p38, ERK and GSK-3β, if three genotypes were compared; Student's *t*-test was used when two genotypes were compared.

Computational network analysis

Of the 40 modifier genes or 'hits' identified, 35 were annotated and individually run through the Endeavor-HighFly software analysis (62), which assigns *P*-values to all other annotated genes in the *Drosophila* genome (~10 000 genes) based on ontology, creating individual network profiles for all the 35 hits. To determine the most significant predicted genetic interactions with tau, all genes with $P < 0.05$ from all 35 networks were compiled together. If a gene met one of the following parameters, it was considered to be highly associated with the tau modifier network and is included in the network diagram: (a) *P*-value of <0.001 in at least two different 'hit' networks; (b) *P*-value of <0.01 in at least four different hit networks; or (c) *P*-value of <0.05 in at least six different hit networks. The network diagram was created using Cytoscape (v. 2.7; 359) and Adobe Photoshop CS4. Only interactions of $P < 0.001$ are depicted using blue lines. Human orthologues were identified using the PANTHER classification system10 (www.pantherdb.org) (360).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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