TDP-43 Phosphorylation by casein kinase 1ε promotes oligomerization and enhances toxicity in vivo

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Dominant mutations in transactive response DNA-binding protein-43 (TDP-43) cause amyotrophic lateral sclerosis. TDP-43 inclusions occur in neurons, glia and muscle in this disease and in sporadic and inherited forms of frontotemporal lobar degeneration. Cytoplasmic localization, cleavage, aggregation and phosphorylation of TDP-43 at the Ser409/410 epitope have been associated with disease pathogenesis. TDP-43 aggregation is not a common feature of mouse models of TDP-43 proteinopathy, and TDP-43 is generally not thought to acquire an amyloid conformation or form fibrils. A number of putative TDP-43 kinases have been identified, but whether any of these functions to regulate TDP-43 phosphorylation or toxicity in vivo is not known. Here, we demonstrate that human TDP-43Q331K undergoes cytoplasmic localization and aggregates when misexpressed in Drosophila when compared with wild-type and M337V forms. Coexpression of Q331K with doubletime (DBT), the fly homolog of casein kinase 1ε (CKI1ε), enhances toxicity. There is at best modest basal phosphorylation of misexpressed human TDP-43 in Drosophila, but coexpression with DBT increases Ser409/410 phosphorylation of all TDP-43 isoforms tested. Phosphorylation of TDP-43 in the fly is specific for DBT, as it is not observed using the validated tau kinases GSK-3β, PAR-1/MARK2 or CDK5. Coexpression of DBT with TDP-43Q331K enhances the formation of high-molecular weight oligomeric species coincident with enhanced toxicity, and treatment of recombinant oligomeric TDP-43 with rat CKI strongly enhances its toxicity in mammalian cell culture. These data identify CKI1ε as a potent TDP-43 kinase in vivo and implicate oligomeric species as the toxic entities in TDP-43 proteinopathies.

INTRODUCTION

Ubiquitinated inclusions containing transactive response DNA-binding protein-43 (TDP) are a pathological hallmark of some forms of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) (1). Proposed mechanisms of TDP-43 toxicity include both loss of function arising from aberrant forms of TDP-43 or sequestration within aggregates, as well as toxic gain of function with acquisition of unique protein–protein and/or protein–RNA interactions, along with defective clearance mechanisms. The molecular mechanisms underlying TDP-43 proteinopathies remain poorly characterized. Recent studies have shown that TDP-43 binds to >30% of the mouse RNA transcriptome, suggesting a complex network of TDP-43 functions that may aberrantly affect RNA processing and regulation in disease states (2,3). A major challenge in ALS and FTLD research is searching through and validating this list of putative disease effectors. Here, we used a candidate approach to define a protein–protein interaction that regulates TDP-43 phosphorylation, aggregation and toxicity in vivo.

TDP-43 is inherently aggregation-prone; a variety of factors may enhance its aggregation propensity (4). Studies of

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HEK-293 cells have demonstrated that ALS-associated mutations of TDP-43 enhance the formation of aggregates, fibrils and neurotoxicity (5). Furthermore, recent studies have suggested formation of TDP-43 oligomers linked to toxicity (5). However, factors regulating formation of TDP-43 oligomers in vivo are poorly characterized. Furthermore, it has been difficult to ascertain whether TDP-43 aggregates mediate toxicity or are an epiphenomenon related to other cellular processes. TDP-43 is phosphorylated in ALS and FTLD-TDP-43 patients at ≥5 serine residues, including 379, 403, 404, 409 and 410, among which S409/410 is phosphorylated robustly (6). In addition, the S409/410 site is phosphorylated in inclusions including cytoplasmic pre-inclusions in TDP-43 proteinopathies, suggesting that this may be a disease-specific phenomenon (1,6,7). Phosphorylation of site S409/410 leads to oligomerization and fibril formation in vitro (6). Mutation of serines 409 and 410 to aspartic acid reduces aggregation (8). Phosphorylation of site S409/410 may also play a role in increasing the half-life of TDP-43 and inhibiting the ubiquitin–proteasome system-mediated degradation, contributing to the formation of aggregates (9). However, the exact mechanism by which phosphorylation of TDP-43 leads to toxicity in vivo is not well understood. Furthermore, although many candidates have been nominated, the specific kinase responsible for phosphorylation of site S409/410 in vivo is not known.

We utilized Drosophila melanogaster as an animal model to study underlying mechanisms of toxicity by overexpressing wild-type human TDP-43 as well as two ALS-associated mutants, M337V and Q331K. Mutant forms of TDP-43 are more prone to aggregation and cytotoxicity. In particular, Q331K is more prone to form high-molecular weight species, and its toxicity correlates with the presence of high-molecular weight species of TDP-43. Moreover, the Drosophila homolog of casein kinase 1ε (CK1ε), Doubletime (DBT), phosphorylates the disease-specific site Ser409/410, and phosphorylation of S409/410 by DBT produces a severe retinal phenotype for Q331K. Phosphorylation of TDP-43(S331K) by DBT increases the formation of SDS-stable oligomers. Other kinases, such as GSK-3β, CDK5, and PAR-1/MARK2, do not phosphorylate Ser409/410. These data provide the first evidence for phosphorylation-mediated formation of TDP-43 oligomers in vivo and a rationale for targeting CK1ε in therapeutics for TDP-43 proteinopathies.

RESULTS

We used the GMR-GAL4 driver (10) to misexpress wild-type, M337V or Q331K forms of human TDP-43 in all cells of the retina beginning in the third instar larval eye imaginal disc. In this tissue, the expression of the zinc finger transcription factor glass commences posterior to a structure known as the morphogenetic furrow (hatched area in Fig. 1A). Differentiation of photoreceptor neurons occurs in this area, as indicated by staining with the neuronal nuclear protein Elav (Fig. 1B–E), which is seen to advantage in conventional apical views. Both wild-type (Fig. 1C) and M337V forms of TDP-43 (Fig. 1D) showed diffuse localization, whereas Q331K showed some punctate immunoreactivity, as well (Fig. 1E, yellow arrows). Distinguishing the extent of cytoplasmic localization of a protein is difficult in the traditional apical view, since the nucleus falsely appears to occupy a considerable portion of the cytoplasm. Therefore, we performed a 3D projection of the confocal Z stacks to yield a more posterior view of the eye imaginal disc to facilitate a clear and accurate distinction of the nuclear and cytoplasmic layers. In the posterior view, the nuclear layer in control is visualized as a narrow band of Elav immunoreactivity (Fig. 1G), TDP-43 immunoreactivity largely colocalized with neuronal nuclei for wild-type (Fig. 1H) and M337V TDP (Fig. 1I) with very little localization in the cytoplasmic compartment. On the other hand, TDP-43 signal largely spread to the cytoplasmic compartment for Q331K (Fig. 1J). A large number of TDP43 positive punctae were also observed in the cytoplasmic region of the Q331K eye disc (Fig. 1E) indicating the formation of toxic aggregates. We further examined the neuronal phenotypes of these TDP-43 isoforms using the pan-neuronal driver elav-GAL4 (11). All three forms were embryonic lethal at 25°C. Q331K was also lethal when expression was restricted at 18°C, whereas wild-type and M337V were viable; these progeny demonstrated abnormal climbing ability using an automated activity assay (Fig. 1K). Using more restricted neuronal expression under control of the tyrosine hydroxylase (TH) promoter, we were able to ascertain selective adult onset cytoplasmic localization of TDP-43Q331K (Fig. 1L–N). In most cells of the PPL1 dopaminergic cluster, TDP-43 overlaps with nuclear staining (e.g. Fig. 1L–N, arrow). In a subset of cells that appear to be less healthy as indicated by loss of nuclear Elav staining; however, TDP-43 signal is excluded from the nucleus and appears in the vicinity of the cytoplasmic TH signal (dashed circle). These data using several different promoters suggest that the Q331K mutant is most toxic among the three variants tested and tends to aggregate and undergo displacement from the nucleus.

We further tested these three isoforms of human TDP-43 when expressed in a variety of different tissues using additional well-established GAL4 drivers. All isoforms were pre-adult lethal when expressed in a ubiquitous fashion under control of daughtercless (12) (Table 1). Like Q331K (Fig. 1), wild-type and M337V were viable when expressed in restricted subsets of neurons using TH-GAL4 (13), whereas all forms were lethal when expressed in motoneurons using the vesicular glutamate transporter promoter (14) (Table 1). Wild-type and M337V were viable when expressed in the mushroom body (15), which plays a role in learning and memory, whereas Q331K was lethal. All forms were lethal when expressed in embryonic mesoderm or muscle using either 24B- (16) or DMEG2-GAL4 (17). Finally, although all forms were lethal using the strong sevenless driver (18), all forms produced viable adults using GMR-GAL4 (Table 1). These data indicate that even wild-type human TDP-43 can be highly toxic when expressed in developing Drosophila tissues.

The GMR-GAL4 driver was used to coexpress DBT and the same three isoforms of TDP-43 in the retina (Fig. 2). As coexpression of DBT rendered Q331K a synthetic lethal at 25°C, these experiments were repeated at 18°C. Photomicrographs of adult retina were taken using an extended depth of focus algorithm as previously described (2). By 1–2 days post-eclosion, all genotypes produced eyes that, apart from pigmentation, were indistinguishable from the GMR-GAL4 driver alone (Fig. 2A), with the exception of Q331K; coexpression of DBT...
Figure 1. The Q331K mutation of human TDP-43 undergoes cytoplasmic localization and confers toxicity in transgenic Drosophila. (A) Schematic of conventional apical view of the third instar larval eye imaginal disc. Expression of transgenes using GMR-GAL4 commences posterior to the morphogenetic furrow (hatched area). The red square corresponds to the approximate fields viewed in (B)–(D). Arrowhead, morphogenetic furrow. (B–D) Apical views posterior to the furrow corresponding to GMR-GAL4 driver alone (A) or co-expressed with either WT (B), M337V (C) or Q331K UAS-TDP-43 transgenes (E). Green, neuronal nuclear protein Elav (which occupies most of the cytoplasm of developing photoreceptors in this view); red, TDP-43. The TDP-43 signal is diffuse for WT (C) and M337V (D) but somewhat punctate for Q331K UAS-TDP-43 (yellow arrows, E). (F) Schematic of the anterior view of the eye disc corresponding to the view in (A) rotated 90° into the plane of the page in order to distinguish nuclear from cytoplasmic layers. Elav staining (G) identifies the nuclei of developing photoreceptors, which overlaps the TDP-43 signal for WT (H) and M337V (I) TDP-43. For Q331K, however, the TDP-43 signal is displaced from the nuclei, and the eye disc appears thickened. (K) Negative geotaxis assessed automatically using the Drosophila activity monitor system (DAMS). Male flies with pan-neuronal expression of TDP-43 were reared at 18°C throughout development and then after eclosion transferred to an incubator with 12-h light–dark cycles at 25°C for behavioral testing. WT (∗∗ P < 0.05) and M337V TDP-43 flies (∗∗∗ P < 0.001) showed impaired climbing performance when compared with driver alone (Student’s t-test). Pan-neural Q331K was lethal even at 18°C. (L–M) Views of dopaminergic neurons in the adult brain (protocerebral posteriolateral cluster 1 (PPL1) at 10 days expressing UAS-TDP-43Q331K under control of TH-GAL4. Green, TDP-43; blue, tyrosine hydroxylase (TH); red, Elav. Most neurons (e.g. white arrow) show nuclear TDP-43 staining that overlaps the Elav signal; however, in a sick neuron (hatched circle) that has begun to lose its nuclear Elav staining, TDP-43 is displaced from the nucleus, with traces left in the cytoplasm in the vicinity of the TH signal. Scale bars, 5 μm (B–E and G–J); 7 μm (L–N). Genotypes: (B) w; GMR-GAL4/Y; +; (C and H): w; GMR-GAL4+/+, (C and G): w; GMR-GAL4+/+, (C and H): w; GMR-GAL4+/+, UAS-TDP-43M337V+/+. (E and J): w; GMR-GAL4+/+, UAS-TDP-43M337V+/+, (E and J): w; GMR-GAL4+/+, UAS-TDP-43Q331K+/+, K: ‘CTRL’, elav-GAL4/Y; ‘TDP-WT’, elav-GAL4/Y; ‘+’, UAS-TDP-43WT++; ‘M337V’, elav-GAL4/Y; ‘+’, UAS-TDP-43M337V+/+, ‘Q331K’, elav-GAL4/Y; ‘+’, UAS-TDP-43Q331K+/+, (E and J): w; GMR-GAL4+/+, UAS-TDP-43Q331K+/+.  

Table 1. Summary of TDP expression data with various drivers at 25°C

<table>
<thead>
<tr>
<th>Driver</th>
<th>Control</th>
<th>TDP-43WT</th>
<th>TDP-43M337V</th>
<th>TDP-43Q331K</th>
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<tbody>
<tr>
<td>Elav (neuronal)</td>
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<td>L(E)</td>
<td>L(E)</td>
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<tr>
<td>Da (ubiquitous)</td>
<td>+</td>
<td>L(L)</td>
<td>L(E/L)</td>
<td>L(L)</td>
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<tr>
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<td>L(L)</td>
<td>L(P)</td>
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<tr>
<td>TH (dopaminergic)</td>
<td>+</td>
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<tr>
<td>GMR (retina)</td>
<td>+</td>
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<tr>
<td>Sev (retina)</td>
<td>+</td>
<td>L(E)</td>
<td>Pharate</td>
<td>L(L/P)</td>
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<tr>
<td>OK107 (mushroom body)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>DMEF2 (muscle)</td>
<td>+</td>
<td>L(L)</td>
<td>L(E)</td>
<td>L(E)</td>
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<tr>
<td>24B (mesoderm)</td>
<td>+</td>
<td>L(L)</td>
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L(E), lethal (embryonic); L(L), lethal (larval); L(P), lethal (pupal); +, viable adults.

and Q331K was partially lethal at 18°C, with survivors showing a severely depigmented rough eye phenotype with patches of necrotic or hypermelanized tissue (Fig. 2H). It is likely that by examining depigmentation or other phenotypes of aged adult retina, especially in flies maintained at 23°C or more, we would also detect enhancement of the TDP-43 WT and M337V phenotypes by DBT. As the baseline TDP-43 external eye phenotype used here is essentially normal, we did not test for its suppression using RNAi.

Given the severe phenotype of dual DBT + Q331K transgenic eyes, we sought to examine the pattern of phospho-TDP-43 accumulation in the eye disc. We stained developing photoreceptor nuclei with Elav, total TDP-43, and phospho Ser409/410 TDP-43 using a mouse monoclonal. Background staining for phosphoTDP-43 in the absence of the DBT transgene was low (Fig. 3A–P), and there was no pTDP-43 signal in the presence of overexpressed DBT alone (Fig. 3Q–T). Dual DBT + TDP-43 transgenic eye discs showed a somewhat diffuse pattern of pTDP-43 immunoreactivity in the case of wild-type and M337V TDP-43, whereas dual DBT + Q331K discs showed a more punctate pattern of staining. Higher magnification views of aggregates in larval eye disc coexpressing DBT and TDP-43 are displayed in Figure 4. The pTDP-43 signal again appears somewhat diffuse in the case of wild-type and M337V.
TDP-43 (Fig. 4A–H), whereas more discrete foci of pTDP-43 signal appear for Q331K + DBT; these largely fail to overlap with nuclear Elav.

Given the enhanced retinal toxicity and prominent cytoplasmic aggregates of phosphorylated TDP-43 we observed in dual DBT + Q331K transgenics, we undertook a series of biochemical analyses to determine the conformational state of TDP-43 in these flies. Consistent with a previous report (19), expression levels of the wild-type, M337V and Q331K transgenes used here were indistinguishable (Fig. 5A and B). In agreement with immunohistochemical staining, there was no basal pSer409/410 staining of human TDP-43 in transgenic Drosophila (Fig. 5C, lanes 1–5); however, when coexpressed with DBT, all forms showed robust phosphorylation (lanes 6–8), which was most robust in the case of Q331K (Fig. 5G). We utilized a series of validated tau kinase reagents (20) to examine specificity of DBT in phosphorylating the Ser409/410 epitope. Neither CDK5 + p35 (21), Shaggy/GSK-3β (22) nor PAR1/MARK2 (23) elicited Ser409/410 phosphorylation when coexpressed with TDP-43 (Fig. 5D–F); nor was there clear phenotypic evidence of synergy in producing a rough eye phenotype (Supplementary Material, Fig. 1). Finally, TDP-43 phosphorylation by DBT required its kinase activity, as it was abolished by a kinase dead transgene (24) and markedly reduced by an ATP-binding mutant transgene (25) (Fig. 5H).

High-molecular weight species consistent with oligomers have been isolated from FTLD brain and transfected HEK293 cells, as well as from recombinant TDP-43. Wu and colleagues have also reported that synthetic peptides derived from the carboxy terminal prion domain form protofibrils and fibrils (5). However, many animal models of TDP-43 proteinopathy lack even histopathological evidence of aggregates, and with the exception of one other fly model (26), oligomers composed of TDP-43 have never been reported from any animal model. Using extraction in low detergent buffer and low denaturing conditions, we were able to detect abundant high-molecular weight species consistent with oligomers (Fig. 6A, arrows). Using low exposures, these were most apparent with Q331K and were increased in dual Q331K + DBT transgenics (Fig. 6A). However, oligomers were apparent for all genotypes even in the absence of the DBT transgene (Fig. 6B, arrows). High exposures in the presence of DBT showed oligomers for all genotypes (Fig. 6C, arrows). Separate experiments using the antibody recognizing phosphoSer 409/410 TDP-43 suggest that these high-molecular weight species are phosphorylated (data not shown). A high-molecular weight band consistent with the previously described 25 kDa fragment was reliably produced in dual Q331K + DBT transgenics (asterisk).

We were able to detect abundant oligomeric species using stirred preparations of recombinant TDP-43, consistent with the work of Gitler and colleagues (27). Incubation of these preparations with recombinant rat CKI markedly enhanced cytotoxicity when applied exogenously to SY5Y human neuroblastoma cells when compared with untreated TDP-43 oligomers further emphasizing the role of CKI phosphorylation in mediating TDP-43-induced cell death (Fig. 6D and E).
DISCUSSION

Hyperphosphorylation occurs in pathological forms of TDP-43, with the serines 409/410 epitope being most robustly phosphorylated. In a C. elegans model of TDP-43 proteinopathy, mutation of these serines to phosphorylation-resistant alanines largely abolishes pathological phenotypes (28). Recombinant CKI phosphorylates TDP-43 in vitro and appears to promote formation of high-molecular weight species consistent with oligomers. Recently, CDC-7 was identified using kinome-wide screening in the worm as a pathological kinase contributing to TDP-43 phosphorylation and neurodegeneration in vivo (29), but this kinase did not appear to have effects on overall conformation of TDP-43.

Figure 3. DBT phosphorylates wild-type human TDP43 and its ALS-associated mutant variants, TDP-43M337V and TDP-43Q331K in vivo. (A–FF) Confocal images of third instar larval eye discs stained with antibodies against phosphorylated TDP43 (green), total TDP43 (red) and Elav (Blue). (D), (H), (L), (P), (T), (X), (BB) and (FF) are merged confocal images showing the staining pattern of the three antibodies, phospho-TDP43, total TDP43 and Elav. There is no background staining for pSer409/410 TDP-43 in the absence of the DBT transgene or when DBT is expressed without TDP-43 (A–T). Phospho-TDP signal is modest when DBT is coexpressed with WT or M337V TDP (U–BB), whereas DBT promotes formation of punctae containing phosphorylated TDP-43 in the presence of the Q331K mutant protein (CC–FF), particularly in the posterior portions of the disc. Scale bar, 200 μm. (A–D) w1118; GMR-GAL4/+; UAS-TDP-43M337V/+. (E–H), w1118; GMR-GAL4/++; UAS-TDP-43M337V/+. (I–P), w1118; GMR-GAL4/++; UAS-TDP-43Q331K/+. (Q–T), w1118; GMR-GAL4/++; UAS-Tdp43M337V/+. (U–X), w1118; GMR-GAL4/++; UAS-Tdp43Q331K/+. (Y–BB), w1118; GMR-GAL4/++; UAS-TDP-43WT/+. (CC–FF), w1118; GMR-GAL4/++; UAS-TDP-43Q331K/++; UAS-dbT. (U–BB), w1118; GMR-GAL4/++; UAS-TDP-43M337V/++; UAS-dbT. CC–FF, w1118; GMR-GAL4/++; UAS-TDP-43Q331K/++; UAS-dbT.

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Early characterization of aggregates in TDP-43 proteinopathies showed scant fibril formation with negative thioflavin S staining (30). More recently, skeins derived from a subset of ALS patients have demonstrated fibrillar morphology, and a modified protocol has described more robust thioflavin S staining in FTLD-TDP and ALS (31). Despite the production of fairly robust neurodegenerative phenotypes, mouse models expressing TDP-43 have generally not produced conspicuous aggregates, in contrast with human FTLD and ALS. Wu and coworkers observed high-molecular weight species consistent with oligomers in human FTLD brain and transfected cell lines but not in transgenic Drosophila; this group reported that carboxy terminal peptides formed fibrils which were the toxic species (5). The ability of recombinant TDP-43 to form oligomeric species, including pore-forming oligomers (27), has been well documented by Gitler and colleagues. The only animal model in which robust oligomers have been observed previously was that reported by Lecourtios and coworkers (26), which used an inducible pan-neuronal model in flies expressing wild-type human TDP-43; these were detected using antibodies recognizing both total and phospho-TDP and were increased by a mutation of the nuclear export signal. However, whether modulation of kinase activity affected TDP-43 conformation was not reported. Thus, the relationship between TDP-43 phosphorylation, aggregation into oligomers and/or fibrils and pathogenicity is far from clear. Indeed, it has even been suggested on the basis of cell culture work that TDP-43 aggregates are neuroprotective (32).

Disruption of diurnal rhythms is a common feature of FTLD and other dementias, including dementia with Lewy bodies and Alzheimer’s disease (33). Phosphorylation of period proteins by CK1ε regulates circadian rhythms in both Drosophila and mammals (34). Mutations in dbt, the Drosophila CK1ε homolog, cause long periods (35); dbt is also known as discs overgrown (36) and plays a role in regulation of cell death via interactions with an inhibitor of apoptosis proteins (37). A spontaneous mutation in the golden hamster CK1ε protein causes a short period (38), and mutations in human CK1ε cause familial advanced sleep phase syndrome (39). It is thus intriguing to note that a kinase that regulates circadian rhythms in organisms

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**Figure 4.** DBT promotes the formation of aggregates containing phosphorylated TDP-43Q331K. (A–L) confocal images of third instar larval eye discs stained with antibodies against phospho-TDP43 (green), total TDP43 (red) and Elav (blue). (D, H and L) Images representing the colocalized pixels of phospho- and total TDP43. Many phosphorylated aggregates immunoreactive for both total and phospho-TDP in eye discs coexpressing TDP-43Q331K and DBT (I, K and L) are seen as yellow in merged images (I and K; see L for coregistered pixels) that largely fail to overlap with the nuclear stain (blue channel in I and J). Scale bar, 5 μm. (A–D) w1118; GMR-GAL4+/+; UAS-TDP-43T337V/UAS-dbth. (E–H) w1118; GMR-GAL4+/+; UAS-TDP-43M337V/UAS-dbth. (I–L) w1118; GMR-GAL4+/+; UAS-TDP-43Q331K/UAS-dbth.
as diverse as Diptera and humans also regulates TDP-43 phosphorylation and conformation in vivo. This effect appears to require kinase activity of DBT, and we favor a direct activity on TDP-43 rather than possible indirect effects such as through modulation of hedgehog signaling.

This work for the first time identifies a kinase that regulates phosphorylation, oligomerization and neurodegeneration mediated by TDP-43 in vivo and supports the hypothesis that oligomers are the most toxic species of TDP-43 aggregates. Whether the interaction between CKI\textsuperscript{1} and TDP-43 also affects circadian rhythm disturbances remains to be determined. Regardless, recognition of the robust synergy between CKI\textsuperscript{1} and TDP-43 in vivo provides a tool that should lead to major steps forward in models of TDP-43 proteinopathy.

**MATERIALS AND METHODS**

**Transgenic flies**

Flies expressing human wild-type TDP-43 (UAS-hTDP-43), ALS mutant TDP-43\textsuperscript{M337V} (UAS-hTDP43-M337V) and ALS mutant TDP-43\textsuperscript{Q331K} (UAS-hTDP43-Q331K) were obtained from Dr Fen-Biao Gao (19). Flies expressing *Drosophila dbt* (UAS-db\textit{t}) were obtained from the Bloomington Stock Center. Additional lines of epitope-tagged UAS-db\textit{t}\textsuperscript{WT} and UAS-db\textit{t}\textsuperscript{K38R} were the gift of Dr Jeff Price (24). UAS-db\textit{t}\textsuperscript{D132N} (37) was provided by Dr Thomas Neufeld. UAS-sgg was obtained from Dr Esther Siegfried (22), UAS-PAR-1 was obtained from Dr Bingwei Lu (23), and UAS-CDK5 and UAS-P35 were provided by Dr Ed Giniger (21). The following GAL4 drivers were

Immunohistochemistry

For eye imaginal disc staining, tissues were fixed in 4% paraformaldehyde containing 0.1% PTX (phosphate buffered saline + 0.1% Triton X 100) for 2 h on ice. After fixation, discs were washed four times with 0.1% PTX and then incubated in blocking buffer (PTX + 5% goat serum) for 1 h at room temperature. Tissues were then dissected to remove the entire brain. Brains were then fixed for 2 h on ice. After fixation, brains were washed four times for 15 min each in PTX and then incubated in blocking buffer for 1 h at room temperature. Tissues were incubated with primary antibody overnight at 4°C. Tissues were then washed four times with 0.1% PTX followed by incubation in Alexafluor-conjugated goat anti-mouse, -rabbit or -rat secondary antibodies (1:400, Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. Tissues were then washed four times for 15 min each in 0.1% PTX and mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Image analysis and quantitation

Samples were imaged using a Zeiss LSM 510 Meta confocal microscope. For comparisons of fluorescent intensities across different genotypes, samples were dissected and fixed identically and imaged under precisely identical conditions (identical exposures and light intensities) and in succession. For all data, images were acquired at the maximum level of brightness, while avoiding saturation. Images were analyzed using the LSM Image browser (for Z-stack processing) and NIH Image J software (for quantitation). The experimenter was blinded to genotypes during both imaging and analysis. Statistical analysis was performed using one-way ANOVA for comparison of samples within an experimental group. All histograms and measurements are given as means ± SEM. For detection of changes in phospho-TDP43, the Analyze, Measure RGB plugin of Image

Figure 6. TDP phosphorylation by DBT promotes oligomerization and toxicity. Analysis under low detergent conditions shows high-molecular-weight species (arrows) of TDP-43331K in addition to monomer, which are increased with DBT coexpression (A). Higher exposure (B and C) shows abundant high-molecular species for all genotypes. At this exposure, modest signal for cleavage products corresponding to the 25 kDa fragment is observed using extracts from dual DBT/ TDP-43 transgenics (all forms). (D) AFM analysis of recombinant human TDP-43WT stirred overnight confirms the presence of abundant oligomers. (E) Recombinant oligomeric TDP-43 is toxic to SY5Y human neuroblastoma cells as assessed using a dye reduction assay (P < 0.05, ANOVA with Bonferroni’s comparison; n = 4 biological replicates each. Pre-treatment of oligomeric TDP-43 with recombinant rat CKI approximately doubles its toxicity (P < 0.01 versus control), whereas CKI alone has no effect on SY5Y cells. 

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J was used. Using this plugin, intensities of phospho-TDP43 (p-TDP43) and Elav were measured in the stacked z-projected Image of the larval eye discs. The quantitative index of phospho-TDP43 levels was calculated by dividing the p-TDP43 intensity by the Elav intensity. Colocalization of p-TDP43 and TDP43 in aggregates formed was obtained using the Image J, RG2B Coloc Plugin in single planar eye disc sections.

Sample preparation for SDS–PAGE
At least 30 flies per genotype were decapitated and heads were homogenized using an Argos battery-operated pestle motor mixer for 1 min on ice in lysis buffer (0.137 M NaCl + 20 mM Tris, pH 8.0, + 10% glycerol + 1% NP-40 + 0.1% SDS + 0.1% sodium deoxycholate + 1 mM DTT + cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN, USA). Samples were then centrifuged at 4°C for 10 min at 18 000g. The supernatant was collected and pellets discarded. An appropriate amount of Laemmle sample loading buffer including β-mercaptoethanol (Bio-Rad, Hercules, CA, USA) was added to the supernatant and samples were boiled 10 min. Samples were then centrifuged briefly with pulse centrifugation and loaded on appropriate gels for electrophoresis.

For detection of high-molecular-weight species, samples were prepared as described above except that PBS including protease and phosphatase inhibitors + 2% NP-40 was used as homogenization buffer. Non-reducing sample loading buffer (Nupage sample buffer, Life Sciences, Grand Island, NY, USA) was added to the supernatant; samples were not boiled. SDS–PAGE and immunoblotting were performed as described previously (20). For immunoblots, primary antibodies included mouse clonal antibody (1:500, CAC-TIP PTD-MO1 Cosmobio, Tokyo, Japan). Mouse -actin monoclonal antibody (1:1000; Ambion, Austin, TX, USA) and β-actin antibody (1:1000, E-7 clone, DSHB, Iowa City, IA, USA) were used as loading controls. Secondary antibodies including HRP-conjugated sheep anti-mouse IgG (1:5000, NA931X, GE Healthcare) and donkey anti-rabbit antibodies (1:3000, NA934V, GE Healthcare) were used.

Light microscopy
Retinal images were acquired using a Nikon AZ100M light microscope (Melville, NY, USA). Extended depth of focus images were taken using a Nikon DSFi1 camera and Nikon NIS-Elements AR 3.0 software as described previously (45).

TDP43 purification and oligomerization
TDP43 was purified according to the protocol of Gitler and colleagues (4). Briefly, GST-TEV-TDP43 was overexpressed in E. coli BL21 (DE3), induced with 0.8 mM IPTG for 3–4 h and purified using the Pierce GST Spin Purification kit (Thermo Fisher, Rockford, IL, USA). GST was removed subsequently by cleavage with tobacco etch virus protease (Invitrogen) and both components were removed by dialysis. Untagged TDP43 proteins were found to be 95% pure on an SDS–PAGE gel. For oligomeric preparations, purified TDP-43 (30 μM) was mixed with hexafluoroisopropanol and slowly added to PBS with constant stirring. The hexafluoroisopropanol was gradually allowed to evaporate and the mixture stirred at 2000 rpm in a thermomixer at room temperature. A 10 μl aliquot of each sample was applied to a piece of freshly cleaved mica and atomic fluorescence microscopy images were acquired by standard procedures as described previously (46).

In vitro kinase reactions
Oligomerized TDP-43 (20 μM) was phosphorylated by CKI at a concentration of 200 nM. The phosphorylation reaction was carried out in 1 mM ATP + 40 mM HEPES + 130 mM KCl + 10 mM MgCl2 + 10 mM ATP + 5 mM DTT + 5 mM glycerol phosphate + 0.2 mM sodium orthovanadate at 30°C for 2 h in a 100 μl volume. The reaction was then quenched with 40 mM EDTA.

Cell viability assay
To study the effects of oligomerized TDP43 on cells, SHSY5Y cells were seeded on 24-well plates (1 × 104 cells/well). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2. Cells were incubated with CK1-treated and untreated oligomerized TDP-43 (10 μM). Cell viability was determined using a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. Averages from four replicate wells were used for each sample and control, and each experiment was repeated four times. The viability of control cells was set to 100% and the percentage of viable cells collected for each treatment was calculated relative to the control group.

Statistical analysis
Densitometric analysis was carried out using the NIH Image J software and graphs were plotted using the Microsoft Excel software. Statistical analysis was done using one-way ANOVA with Bonferroni’s correction. For paired analysis, Student’s t-test was used with two-tailed distribution of equal variance.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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