Rapamycin alleviates pathogenesis of a new Drosophila model of ALS-TDP

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Abstract

TDP-43 is a multi-functional RNA/DNA-binding protein, well-conserved among many species including mammals and Drosophila. However, it is also a major component of the pathological inclusions associated with degenerating motor neurons of amyotrophic lateral sclerosis (ALS). Further, TDP-43 is a signature protein in one subtype of frontotemporal degeneration, FTLD-U. Currently, there are no effective drugs for these neurodegenerative diseases. We describe the generation and characterization of a new fly model of ALS-TDP with transgenic expression of the Drosophila ortholog of TDP-43, dTDP, in adult flies under the control of a temperature-sensitive motor neuron-specific GAL4, thus bypassing the deleterious effect of dTDP during development. Diminished lifespan as well as impaired locomotor activities of the flies following induction of dTDP overexpression have been observed. Dissection of the T1/T2 region of the thoracic ganglia has revealed loss of these neurons. To counter the defects in this fly model of ALS-TDP, we have examined the therapeutic effects of the autophagy activator rapamycin. Although harmful to the control flies, administration of 400 µM rapamycin before the induction of dTDP overexpression can significantly reduce the number of neurons bearing dTDP (+) aggregates, as well as partially rescue the diminished lifespan and locomotive defects of the ALS-TDP flies. Furthermore, we identify S6K, a downstream mediator of the TOR pathway, as one genetic modifier of dTDP. In sum, this Drosophila model of ALS-TDP under temporal and spatial control presents a useful new genetic tool for the screening and validation of therapeutic drugs for ALS. Furthermore, the data support our previous finding that autophagy activators including rapamycin are potential therapeutic drugs for the progression of neurodegenerative diseases with TDP-43 proteinopathies.

Introduction

TAR DNA-binding protein, TDP-43, is a 43 kD DNA/RNA-binding protein and is highly conserved from worms to mammals. TDP-43 is primarily expressed in the nucleus and has multiple cellular functions, especially in transcriptional repression (Ou et al., 1995; Wang et al., 2004), translational repression (Wang et al., 2008a), mRNA splicing (Bose et al., 2008; Buratti et al., 2001; Polymenidou et al., 2011; Tollervey et al., 2011), mRNA stability (Ayala et al., 2008; Fiesel et al., 2010; Strong et al., 2007), and microRNA biogenesis (Buratti et al., 2010; Ling et al., 2010). The protein contains two RNA-recognition motifs (RRM), RRM1 and RRM2, a C-terminal glycine-rich domain, a bipartite nuclear localization signal (NLS), and a nuclear export signal (NES) (Buratti & Baralle, 2009). Of these, the RRM domains of TDP-43 show a preference for binding UG-rich RNA and TG-rich DNA (Buratti & Baralle, 2001; Kuo et al., 2009). In addition to the RRM, TDP-43 can interact with members of the heterogeneous ribonucleoprotein (hnRNP) family through the C-terminal glycine-rich domain (Buratti et al., 2005), which also contains a glutamine/asparagine (Q/N)-rich prion-like element that mediates its aggregation with polyQ aggregates (Fuentesalba et al., 2010; Wang et al., 2008b).

TDP-43 has been identified as the major component of the cytoplasmic ubiquitinated inclusions (UBIs) characteristic of most forms of amyotrophic lateral sclerosis (ALS-TDP) and one subtype of frontotemporal lobar degeneration (FTLD-U or FTLD-TDP) (Chen-Plotkin et al., 2010; Ling et al., 2013; Neumann et al., 2006). UBIs containing full-length TDP-43, polyubiquinated TDP-43, phosphorylated TDP-43, the 35-kD/25-kD carboxyl TDP-43 fragments, and other proteins (Buratti & Baralle, 2009; Grad et al., 2015) have been identified in several other neurodegenerative diseases as well, including Alzheimer’s disease, other tauopathies, and Lewy body disorders (Aoki et al., 2015; Mackenzie et al., 2010). Both gain-of-toxicity and loss-of-TDP-43 function are involved in the generation and/or progression of TDP-43 proteinopathies (Gendron et al., 2010; Ling et al., 2013; Wu et al., 2012).

FTLD is a progressive neurodegeneration presenting with loss in the frontal and anterior temporal lobes accompanying personality and behavioral changes as well as gradual impairment of language skills. On the other hand, degeneration of lower motor neurons and their axons as well as loss of upper

Keywords

Amyotrophic lateral sclerosis, D42-GAL4, Drosophila, lifespan, locomotor activity, TDP-43
motor neurons and their corticospinal axonal tracts results in ALS (Van Langenhove et al., 2012). Until now, there are no effective treatments for ALS, though the drug riluzole slows the progression and prolongs survival by three months (Rowland & Shneider, 2001). More than 40 mutations of TDP-43 in sporadic and familial ALS (sALS and fALS) and FTLD have been identified within the C-terminus (Lattante et al., 2013). Furthermore, the pathological inclusion of 97% of ALS cases and 45% of FTLD cases are TDP-43-positive (Ling et al., 2013). However, most ALS-TDP and FTLD-TDP cases are without detectable TDP-43 mutations (Gjišelinck et al., 2009; Guerreiro et al., 2008). These data suggest that targeting TDP-43 may offer a strategy to combat these neurodegenerative diseases.

Various animal models have been developed to explore the pathobiology of TDP-43 in vivo (Chiang et al., 2010; Diaper et al., 2013; Feiguin et al., 2009; Fiesel et al., 2010; Hanson et al., 2010; Hazelett et al., 2012; Huang et al., 2012; Igaz et al., 2011; Iguchi et al., 2013; Li et al., 2010; Lin et al., 2011; Lu et al., 2009; Tatom et al., 2009; Tsai et al., 2010; Uchida et al., 2012; Vaccaro et al., 2012; Wegorzewska et al., 2009; Wils et al., 2010; Wu et al., 2010; Wu et al., 2012). Previously, we have used the UAS/GAL4 system to generate fly models with transgenic overexpression or knockdown of the Drosophila ortholog of TDP-43, dTDP. This model exhibits pathologies similar to FTLD or ALS, depending on the tissues targeted for dTDP overexpression or knockdown (Lin et al., 2011). However, mouse TDP-43 and Drosophila dTDP are known to be involved in early development (Feiguin et al., 2009; Lin et al., 2011; Wu et al., 2010). Therefore, the UAS/GAL4 system has led to severe defects of the genetically engineered flies, limiting their application for drug screening/validations for ALS-TDP and FTLD-TDP, both of which are adult-onset diseases. Thus, it is desirable to design and generate more transgenic fly models of ALS-TDP or FTLD-TDP with adult stage-specific overexpression of the dTDP or hTDP-43 transgene.

TDP-43-positive aggregates are the hallmarks of ALS-TDP and FTLD-TDP, implying that TDP-43 loses its proteostasis, which is maintained by the ubiquitin proteasome system (UPS) and autophagy (Brady et al., 2011; Huang et al., 2014; Kim et al., 2009). Utilizing autophagy inducers may be beneficial for clearance of aggregates, which would therefore alleviate diseases. TDP-43 turnover is also required for maintenance of autophagy by stabilization of the ATG7 mRNA (Bose et al., 2011). Dysfunction of autophagy in neurons can cause the accumulation of aggregate-prone proteins and neurodegeneration (Harris & Rubinsztein, 2012). Recent studies have revealed that rapamycin, one of the autophagy inducers, can rescue mislocalization of TDP-43 (Caccamo et al., 2009), while also providing neuro-protective effects in other neurodegenerative disease models, including Huntington disease (HD) (Ravikumar et al., 2004), Alzheimer’s disease (AD) (Caccamo et al., 2010; Spilman et al., 2010), and Parkinson’s disease (PD) (Crews et al., 2010). Our group also reported that rapamycin can rescue the pathology observed in the FTLD-TDP mouse model (Wang et al., 2012).

In the following, we describe the generation of a fly model with temperature-controlled, motor neuron-specific overexpression of dTDP at early adulthood. The reduced lifespan and locomotive defect of this fly model could be partially ameliorated by rapamycin-mediated autophagy activation. The data support the idea that inhibition of the TOR pathway resulting in activation of autophagy or inhibition of more dTDP production indeed could be an effective strategy to counter ALS-TDP.

Materials and methods

Fly stocks and husbandry

UAS-dTDP#5-1 and UAS-dTDP#18-1 have been described previously (Lin et al., 2011). tubP-GAL80° (BL7108), motor neuron-specific GAL4 (D42-GAL4), UAS-S6K-TE (BL6912), and UAS-S6K-STDTE (BL6914) were obtained from Bloomington Drosophila Stock Center. D42-GAL4 was generated by tubP-GAL80° crossed with D42-GAL4. For overexpression of dTDP, UAS-dTDP#5-1 and UAS-dTDP#18-1 were crossed with D42w-GAL4; and for generation of the control D42w/+ flies, D42w-GAL4 was crossed with w wild-type line. All fly stocks were maintained in standard medium at 18°C and 60% humidity under a 12-h light–dark cycle. After rapamycin treatment for 10 days, flies were transferred to 29°C or 30°C for further analysis.

Rapamycin treatment

Rapamycin (LC Laboratories) was dissolved in ethanol. After eclosion for 4 days, male flies were fed food containing appropriate rapamycin concentrations (200 or 400 μM). For control food (0 μM), ethanol alone was added. Flies were then transferred to fresh food with rapamycin every 1–2 days.

Locomotor activity assay

The locomotor activity of the adult flies was measured in a dark room using a countercurrent apparatus as described (Benzer, 1967). Groups of 50 flies (25 males and 25 females) after 30°C induction for 12–14 days were given 15 s to move toward a white light source. They were then tapped down and moved to the next tube of the apparatus. After five runs, the distributions of the flies was measured, with the most active flies running into the most distal tube receiving a score of five and the least active flies staying in the original start tube receiving a score of zero. All the scores were then normalized against the average score of the control flies without rapamycin treatment.

Lifespan experiments

Male flies were kept in vials at a density of twenty flies per vial at 18°C. For rapamycin treatment, 4-day old adult flies were treated with rapamycin for ten days, and then transferred to 30°C. To examine the effect of constitutively active S6K on dTDP overexpression, 6-day old flies were transferred to 29°C. Flies were transferred to new vials every 1–2 days, and deaths were scored on a daily basis.

Western blotting

For analysis of dTDP overexpression at higher temperature, the heads and thoraces from 15 males were collected on day 5 after 30°C induction. For analysis of p62 and dTDP expression levels, after 29°C induction for 14 days, the heads and thoraces from 15 males treated with rapamycin were collected. All samples were lysed in cold RIPA buffer (0.1% SDS, 1%
Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.9) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, and a mixture of protease inhibitors, Roche), and then sonicated. Extracts were cleared by centrifugation and were run on 8% or 10% polyacrylamide gels and transferred to PVDF membranes. Primary antibodies included rabbit anti-dTDP 1:2000 (Lin et al., 2011), rabbit anti-p62 1:2500 (Pircs et al., 2012), and mouse anti-tubulin (1:10000, Millipore). After hybridizations, blots were incubated at room temperature with the appropriate secondary antibodies and WesternBright™ ECL (Advansta).

Immunostaining

For the analysis of dTDP expression, the thoracic ganglia of male adults at 29°C for 13–14 days were dissected in PBS, fixed with 4% paraformaldehyde in PBS with 0.25% Triton-X-100, and then irradiated using a microwave oven (Pelco BioWave, Pelco International). After blocking with 10% normal goat serum at room temperature for 1 h, the tissues were incubated overnight at room temperature with rabbit anti-dTDP (1:200). After washing, the tissues were incubated overnight at room temperature with biotinylated anti-rabbit antibody at 1:250 (Molecular Probes) and rat anti-ELAV antibody at 1:200 dilution (Developmental Studies Hybridoma Bank). They were then washed and incubated overnight with streptavidin-Alexa-546 antibody (1:1000; Molecular Probes) and Alexa-488 conjugated goat anti-rabbit antibody diluted 1:200 (Molecular Probes), respectively.

For neuron counting, the primary antibodies used were rabbit anti-HB9 antibody at 1:1000 dilution (Lacin et al., 2014) and rat anti-ELAV antibody at 1:200 dilution, followed by Alexa-555 conjugated goat anti-rabbit antibody diluted at 1:200 (Molecular Probes) and Alexa-488 conjugated goat anti-rabbit antibody diluted at 1:200, respectively.

The confocal images were acquired using Zeiss LSM710. ELAV and HB9-positive cells in the regions bordering T1 and T2 were counted using Imaris (Bitplane) and ImageJ (National Institutes of Health) software, respectively. Average cell counts were compared by the t-test, and significant differences were set at \( p < 0.05 \).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software. For survival analysis, the log-rank (Mantel–Cox) test was performed. For locomotor activity analysis and neuron counts, data are presented as mean ± SEM and analyzed by Student’s t-test. Significant statistical differences are denoted by * \( (p < 0.05) \), ** \( (p < 0.005) \), and *** \( (p < 0.0005) \).

RESULTS

Overexpression of dTDP in the motor neurons under temporal UAS/GAL4 control caused the formation of dTDP (+) aggregates and neuronal loss in the thoracic ganglia

Previously, we used the D42-GAL4 to drive dTDP overexpression in motor neurons from an early developmental stage (Lin et al., 2011). Since ALS is typically an adult-onset motor neuron disease, to avoid developmental defects caused by dTDP overexpression, we generated a temperature-sensitive D42-GAL4 line, D42-GAL4. Under permissive conditions (18°C), GAL80 functions normally and blocks GAL4-mediated transactivation of dTDP in the motor neurons. Therefore, even in fly lines bearing UAS transgenes of dTDP, D42-GAL4->dTDP#18-1 and D42-GAL4->dTDP#5-1, dTDP could not be highly overexpressed at the lower temperature (Figure 1A-a, lanes 2 and 3). After transfer to 30°C, GAL80 became dysfunctional and therefore allowed GAL4 to induce more than 10-fold dTDP overexpression in the motor neurons (Figure 1A-a, lanes 5 and 6). Further, we dissected the flies at day 13–14 after transfer to 30°C, to examine the possible formation of dTDP (+) aggregates. Indeed, there were some neurons bearing dTDP (+) aggregates and/or translocation of dTDP from nuclei to cytosol in the D42-GAL4->dTDP#18-1, but not in the D42-GAL4-> control flies (Figure 1A-b).

Since a previous study showed that there was no detectable loss of motor neurons in the T1/T2 region of the thoracic ganglia in ALS-SOD1 flies by counting GFP-positive nuclei (Watson et al., 2008), we next determined whether there was a similar phenotype in our ALS-TDP flies by counting cells positive for ELAV, a pan-neuron marker (Figure 1B-b and C-a) in the same anatomical region (Figure 1B-a). In male D42-GAL4->dTDP#18-1 flies, the neuron number in the thoracic ganglia was significantly reduced by ~8% after incubation at 29°C for 13–14 days compared with male D42-GAL4-> control flies (Figure 1C-a). To further dissect which type of neurons were lost in the T1/T2 region of the thoracic ganglia in ALS-TDP flies, we also analyzed HB9-positive cells (Figure 1B-c), since HB9 is expressed in some subsets of motor neurons and interneurons (Odden et al., 2002). However, there was no difference in HB9-positive cells between D42-GAL4-> and D42-GAL4->dTDP#18-1 flies (Figure 1C-b). This indicated that D42-GAL4 could tightly regulate dTDP expression under temporal control, and our ALS-TDP flies exhibited TDP-43 proteopathlike phenotypes, such as the formation of dTDP (+) aggregates, mislocalization of dTDP, and the neuronal loss.

Shortened lifespan and decreased locomotor activity were induced by the temporal UAS/GAL4 control

Next, we examined the lifespan of flies bearing ectopic dTDP. The median survival of +/+ and D42-GAL4-> was 40 (data not shown) and 34 days, respectively, while D42-GAL4-> dTDP#18-1 exhibited a median survival of only 23 days (Figure 2A, \( p < 0.0001 \)). Phototaxis locomotor assay further confirmed the diminished fitness accompanying dTDP overexpression. Compared with D42-GAL4->, D42-GAL4-> dTDP#18-1 maintained at 30°C for 12–14 days showed serious locomotor defects (Figure 2B, the two bars on the left indicated as 0 μM, **, \( p < 0.0005 \)). Since D42-GAL4-> dTDP#18-1 displayed similar pathogenesis of ALS under temporal control without affecting development, we used this fly line to study the preventive effect of rapamycin.

Rapamycin partially prevented the development of pathology of the fly model of ALS-TDP

Since rapamycin has been reported to rescue TDP-43 mislocalization in vitro (Caccamo et al., 2009), we first tested the efficacy
Figure 1. dTDP of D42ts dTDP#18-1 and D42ts dTDP#5-1 was overexpressed at higher temperature, and this ectopic expression caused the formation of dTDP (+) aggregates and loss of neurons in the thoracic ganglia. (A) Analysis of dTDP expression in the male flies with the temperature-sensitive motor neuron GAL4 overexpressing dTDP. (a) Western blotting analysis of the dTDP expression in the heads and thoraces at 18°C and 30°C. Lanes 1–3: Flies kept at 18°C, including D42ts dTDP#18-1 and D42ts dTDP#5-1, had low-level expression of dTDP. Lanes 4–6: After flies were transferred to 30°C for 5 days, the higher temperature induced more than 10-fold increase in dTDP overexpression in the heads and thoraces of male D42ts dTDP#18-1 and D42ts dTDP#5-1, but not in D42ts control flies. (b) Formation of dTDP (+) aggregates, but not in the D42ts control flies, in the D42ts dTDP#18-1 flies at 13–14 days after dTDP induction at 30°C. Left panels, D42ts; right panels, D42ts dTDP#18-1. Red, dTDP; green, ELAV. As seen, dTDP was translocated to the cytosol (arrowhead) and dTDP (+) aggregates formed (arrows) in some neurons bearing overexpressed dTDP. (B) Neuron numbers were determined by counting ELAV or HB9-positive cells in the T1/T2 border (indicated by rectangle) in confocal stacks of whole-mounted thoracic ganglia. (a) Representative images of the thoracic ganglion of genotype D42ts control flies (left panels) and D42ts dTDP#18-1 flies (right panels). Ab, abdominal ganglion. (b) ELAV-positive cells; and (c) HB9-positive cells. Both (b) and (c) are high magnifications of pictures from the rectangular region of (a). Green, ELAV; white, HB9. (C) Comparisons of the effect of different dosages of rapamycin (Rapa) on the (a) total neuron numbers, and (b) HB9-positive cells of D42ts+ control flies and D42ts dTDP#18-1 flies are shown. (a) Without rapamycin treatment, significantly fewer neurons were detected in the T1/T2 border in D42ts dTDP#18-1 compared with D42ts+ control flies (*, p < 0.05). The number of neurons in D42ts dTDP#18-1 did not show obvious change following rapamycin treatment, but rapamycin reduced neuron numbers in D42ts+ control flies (*, p < 0.05). (b) There was no significant change in the number of HB9-positive cells between D42ts+ control and D42ts dTDP#18-1 flies; and rapamycin treatment did not affect the numbers of HB9-positive cells in either genotype. The cell numbers were normalized to D42ts control flies without rapamycin treatment within each experiment; average ± S.E.M. from 4–6 flies is presented.
of rapamycin on our Drosophila model of ALS-TDP. Both male \(D42^w\) and \(D42^w > dTDP\#18-1\) flies were reared on normal food at 18°C and then fed food supplemented with 200 or 400 \(\mu M\) rapamycin (Rapa) at the 4-day-old adult stage. After 10 days of rapamycin treatment, flies were transferred to 30°C to induce dTDP overexpression and were transferred to fresh food with rapamycin every 1–2 days. Rapamycin treatment at 200 and 400 \(\mu M\) significantly extended the median lifespan of \(D42^w > dTDP\#18-1\) (200 \(\mu M\): \(p = 0.002\); 400 \(\mu M\): \(p = 0.0004\), log-rank test compared to \(D42^w > dTDP\#18-1\) without rapamycin treatment). \(N = 195–216\). (B) Movement defects of \(D42^w > dTDP\#18-1\) could be partially rescued by 400 \(\mu M\) rapamycin treatment. Phototaxis was examined after transfer to 30°C for 12–14 days. The movement indexes were normalized to that of the control line \(D42^w\) without rapamycin treatment. \(N = 13\), *, \(p < 0.05\); **, \(p < 0.005\); ***, \(p < 0.0005\).

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A drug screening model of ALS-TDP 63

Figure 2. Effect of rapamycin on lifespan and locomotor activity of flies with motor neuron-specific overexpression of dTDP. (A) The lifespan of male \(D42^w\) and \(D42^w > dTDP\#18-1\) were maintained at 18°C for 4 days before being switched to a diet supplemented with 0, 200, or 400 \(\mu M\) rapamycin (Rapa). After ten days of rapamycin treatment, flies were transferred to 30°C to induce dTDP overexpression and were transferred to fresh food with rapamycin every 1–2 days. Rapamycin treatment at 200 and 400 \(\mu M\) significantly extended the median lifespan of \(D42^w > dTDP\#18-1\) (200 \(\mu M\): \(p = 0.002\); 400 \(\mu M\): \(p = 0.0004\), log-rank test compared to \(D42^w > dTDP\#18-1\) without rapamycin treatment). \(N = 195–216\). (B) Movement defects of \(D42^w > dTDP\#18-1\) could be partially rescued by 400 \(\mu M\) rapamycin treatment. Phototaxis was examined after transfer to 30°C for 12–14 days. The movement indexes were normalized to that of the control line \(D42^w\) without rapamycin treatment. \(N = 13\), *, \(p < 0.05\); **, \(p < 0.005\); ***, \(p < 0.0005\).

Therapeutic effect of rapamycin was associated with activation of autophagy but did not improve neuron counts significantly in the thoracic ganglia of ALS-TDP flies

Protein levels of the autophagy substrate p62 have been established recently as an indicator for basal autophagic activity in mammals and Drosophila. Following induction of ectopic dTDP expression in motor neurons for 14 days, the expression of p62 in the lysates of heads and thoraces was significantly decreased compared to that in \(D42^w\) flies (Figure 3A, lanes 1 and 4 in the upper panel, and Figure 3B). When \(D42^w\) or \(D42^w > dTDP\#18-1\) flies were administered rapamycin, they both exhibited reduced p62 levels in a dose-dependent manner. However, the reduction of the p62 level of the \(D42^w > dTDP\#18-1\) appeared to be less, in proportion to the control \(D42^w\) flies (Figure 3A, upper panel, and
Figure 3. Effects of rapamycin treatment on p62, an autophagy marker, and dTDP (+) aggregates in the head and thoracic regions. (A) Western blotting analysis of expression levels of p62 and dTDP in the heads and thoraces of male adult flies were examined. Comparisons among D42ts++ control and D42ts dTDP18-1 flies treated with different dosages of rapamycin are shown in the histograms for (B) p62 and (C) dTDP normalized with tubulin, and then compared with the samples of D42ts++ control flies without rapamycin treatment. (B) Without rapamycin treatment, the protein level of p62 in D42ts dTDP18-1 was lower than that in D42ts++ control flies. Under rapamycin treatment, p62 expression in both genotypes decreased. (C) The expression level of dTDP in D42ts dTDP18-1 flies was much higher than that in D42ts++ control flies. At 400 μM, rapamycin dTDP expression was slightly reduced in both genotypes, but insignificantly. N = 4. (D) Quantitative analysis of the relative numbers of neurons bearing dTDP (+) aggregates in the T1/T2 border of the thoracic ganglia in the ALS-TDP flies with/without rapamycin. Note that the number of neurons with dTDP (+) aggregates in the ALS-TDP flies was significantly reduced after treatment with 400 μM rapamycin. N = 5. Average ± S.E.M. is presented. *, p < 0.05; **, p < 0.005; ***, p < 0.0005.

Notably, rapamycin treatment had no significant effect on the level of RIPA-soluble dTDP (Figure 3A, middle panel, and Figure 3C). Next, we analyzed whether rapamycin had any effect on the formation of dTDP (+) aggregates in the ALS-TDP flies. Indeed, after 400 μM rapamycin treatment, the number of neurons bearing dTDP (+) aggregates were decreased (Figure 3D), although the neuron number of the thoracic ganglia in D42ts > dTDP18-1 was not significantly rescued by rapamycin treatment (Figure 1C-a, bars 4 to 6). However, we observed a loss of 5% and 5.5% in neuron numbers (Figure 1C-a, bars 1 to 3) in the thoracic ganglia of D42ts++ control flies receiving 200 μM and 400 μM rapamycin treatment, respectively. These data suggest that the preventive efficacy of rapamycin is rooted in its modulation of autophagy activation following by reducing the dTDP (+) aggregates.

Increased S6K activity reduced the lifespan of the ALS-TDP flies

Since the phenotypes in the D42ts > dTDP flies might result from disturbance of the TOR pathway in part, we also manipulated the expression level of S6K and 4E-BP1 in the D42ts > dTDP flies. Co-overexpression of wild-type S6K or 4E-BP1
Figure 4. The shortening of lifespan is more severe as there is co-overexpression of constitutively active S6K and dTDP in motor neurons. Flies were maintained at 18°C until day 6 of the adult stage, and then were transferred to 29°C. Both D42osaic> S6K-TE and D42osaic> S6K-STDETE are constitutively active S6K fly lines, and these lines prolonged lifespan compared to D42osaic> + (Median survival- D42osaic> 43 days, D42osaic> S6K-TE: 47 days, and D42osaic> S6K-STDETE: 48 days; both are within p < 0.0001, log-rank test).

Co-overexpression of dTDP and constitutively active S6K (D42osaic> dTDP#18-1,S6K-TE and D42osaic> dTDP#18-1,S6K-STDETE), shortened lifespan. N = 110–127. (Median survival- D42osaic> dTDP#18-1: 29 days, D42osaic> dTDP#18-1, S6K-TE: 22 days, and D42osaic> dTDP#18-1, S6K-STDETE: 25 days, p < 0.0001 and p = 0.0106, log-rank test, respectively).

Discussion

ALS is the most common type of adult-onset motor neuron disease and the third most common neurodegenerative disease, with approximately 10% of the cases being familial (fALS), while others are sporadic (sALS) (Renton et al., 2014). Since the peak age at onset is within 47–63 years (Logrosino et al., 2010) and most ALS-TDP cases are without detectable TDP-43 mutation, we generate the fly model of ALS-TDP (D42osaic> dTDP) with temporal control using the UAS/GAL4 system (the TARGET system) to overexpress wild-type dTDP in motor neurons from an early adult stage. This strategy has allowed us to circumvent the developmental defects due to misregulation of dTDP. Flies with overexpression of dTDP induced by the warmer temperatures of 29–30°C would develop ALS-TDP-like pathological phenotypes including the formation of dTDP (+) aggregates, the shortening of lifespan, locomotive defects, and reduced neuron number in the thoracic ganglia. Although such a ‘TARGET’ system has been utilized to identify the genetic modifiers of hTDP-43 (Gregory et al., 2012), it has not been tested before to serve as a platform for drug screening and validation. Notably, many groups have tested the effects of overexpressing wild-type as well as mutant TDP-43 in mouse, C. elegans, and Drosophila, and these animal models developed disease phenotypes that mimic FTLD-TDP or ALS-TDP (Estes et al., 2011; Huang et al., 2012; Igaz et al., 2011; Janssens et al., 2013; Li et al., 2010; Liachko et al., 2010; Tatom et al., 2009; Tsai et al., 2010; Uchida et al., 2012; Voigt et al., 2010; Wegrzewska et al., 2009; Wils et al., 2010; Xu et al., 2010). While the higher temperatures might have some undesired effects on flies, such as reduced mating, our fly model of ALS-TDP has turned out to be suitable for examining the therapeutic effects of potential drugs, such as rapamycin.

The reduced neuron number in the thoracic ganglia (Figure 1C-a) of the ALS-TDP flies is mainly due to neuronal loss, rather than insufficient neurogenesis, since the adult flies (D42osaic> dTDP) without heat shock develop normally under permissive temperatures. Although the lost neurons are not derived from the HB9-positive cells (Figure 1C-b), we cannot exclude the possibility that they are other subtypes of motor neurons. Interestingly, a transgenic mouse model of FTLD-TDP with overexpression of mouse TDP-43 in the hippocampus and cortex exhibits caspase-3-dependent neuronal apoptosis at the age of 6 months (Wang et al., 2012). We speculate that the neuronal loss in our fly model of ALS-TDP may also result from apoptosis.

We show in this study that rapamycin administration prior to dTDP overexpression partially ameliorates the lifespan as well as locomotor activity in the ALS-TDP flies (Figure 2). Rapamycin is a TORC1-dependent inducer of autophagy. In different tissues including the nervous system, autophagy maintains the normal balance between the formation and degradation of cellular proteins (Mizushima, 2007). Defects in the autophagy pathway have been reported in patients with neurodegenerative diseases such as PD, AD, and HD (Ghavami et al., 2014; Harris & Rubinsztein, 2012; Tooze & Schiavo, 2008). Therefore, autophagy-modulating drugs are increasingly being tested for therapeutic efficacy against neurodegenerative diseases (Barmuda et al., 2014; Sarkar et al., 2007; Wang et al., 2012; Wang et al., 2013). The therapeutic applications of rapamycin have been widely studied. However, rapamycin may not be effective against some diseases. For example,
treatment of SOD1<sup>G93A</sup>-ALS mice has resulted in shortened lifespan and exacerbated symptoms instead (Zhang et al., 2011). Nevertheless, the pathology of ALS-SOD1 is thought to be distinct from other types of ALS with TDP-43 and/or FUS inclusions (Mackenzie et al., 2007).

The partial rescue of the D42<sup>m</sup> > dTDP flies by rapamycin is at least in part due to the activation of autophagy (Figure 3A, upper panel, and Figure 3B). Therefore, the number of neurons with dTDP (+) aggregates is reduced by ~33% as compared to ALS-TDP flies without rapamycin treatment (Figure 3D). However, the expression level of dTDP was reduced by only 7% (Figure 3A, middle panel, and Figure 3C), possibly because the abundance of the ectopic dTDP is too high to be degraded efficiently by autophagy. Notably, the level of p62 in the ALS-TDP flies is lower than that in the control flies (compare lane 1 and 4 in the upper panel of Figure 3A and B). We reason that the time-point of analysis is the key. As mentioned in Materials and methods, we have analyzed the level of p62 at day 14 after transfer of the flies to 29°C. At that time, more than 90% of ALS-TDP flies were still alive (red line, Figure 4) and the autophagy could be activated then by the cellular defense system against the initial increase of the level of dTDP. It should be mentioned that the higher dose of rapamycin (400 μM) produced the opposite effect in control flies, with reduced lifespan, locomotor activity, and thoracic ganglia neuron counts (Figures 1C and 2), likely because the TOR pathway is involved in many essential cellular functions. Also, clinically relevant, we have attempted administering rapamycin after the onset of motor dysfunction. However, even at 400 μM rapamycin, we have observed no lifespan extension (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/01677063.2015.1077832) and no rescue of the locomotive ability (data not shown).

Different fly models have been generated before to test the therapeutic efficacies of drugs for ALS-TDP (Gregory et al., 2012; Joardar et al., 2015; Kim et al., 2014; Salado et al., 2014). For example, the PERK inhibitor, GSK2606414, reduced eIF2α phosphorylation and mitigated defective climbing ability in flies with pan-neuronal overexpression of human TDP-43 under the weak control by ELAV<sup>3A</sup> GAL4 (Kim et al., 2014). Based on a pupal lethal phenotype, Zarnescu’s group has reported that the PPARγ agonist pioglitazone can rescue TDP-43-dependent locomotor dysfunction in motor neurons in the ALS-TDP or ALS-FUS flies, but not the ALS-SOD1 flies (Joardar et al., 2015). It should be cautioned though that therapeutic effect observed in the above two fly models might be due to rescue of the defects of development, instead of the neurodegeneration directly. Finally, Salado et al. (2014) have tested the therapeutic effect of drugs in flies with the pan-neuronal inducible GeneSwitch-GAL4 system (Salado et al., 2014). This gene-switch system also supports a temporal and spatial control of the overexpression of hTDP-43 in the flies. However, the pathological phenotypes induced by pan-neuronal overexpression of hTDP-43 may not be fully representative of ALS, since the major characteristic of ALS is the degeneration of motor neurons. Our results suggest that in addition to improving motor function, rapamycin may extend lifespan in these other ALS-TDP models as it did in our fly model of ALS-TDP.

In sum, in combination with our previous study of a transgenic mouse model of FTLD-TDP (Wang et al., 2012), the current study supports the therapeutic potential of rapamycin and possibly other autophagy activators for the prevention and/or rescue of the ALS-TDP as well as FTLD-TDP. However, identifying the efficacious dosages and appropriate timing for administration of the drugs should be carefully evaluated in future clinical applications.

**Conclusions**

Here we describe a fly model of ALS-TDP for drug validation and screening, in which ectopic dTDP expression is manipulated under temporal control. Using this model, we have found that rapamycin can partially improve the shortened lifespan and impaired locomotor activity of ALS-TDP flies with overexpressed dTDP induced in the motor neurons from an early adult stage. The therapeutic efficacy of rapamycin may be partially mediated by autophagy activation, as reflected in part by the reduction of the number of neurons with the dTDP (+) aggregate. This study further supports the idea that the autophagy activators are potentially useful drugs for ALS-TDP.

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**Declaration of interest**

The authors report no declarations of interest.

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