

**PIKFYVE MODULATION MITIGATES TDP-43-DEPENDENT  
DISEASE PHENOTYPES IN A *DROSOPHILA* MODEL OF  
AMYOTROPHIC LATERAL SCLEROSIS**

by

Chuol Wal Kueth

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF CELLULAR AND MOLECULAR MEDICINE

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

2020

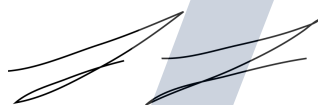
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As members of the Master's Committee, we certify that we have read the thesis prepared by Chuol Kueth, titled "*PIKFYVE modulation mitigates TDP-43-dependent disease phenotypes in a Drosophila model of Amyotrophic Lateral Sclerosis*" and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.



\_\_\_\_\_  
*Daniela C Zarnescu, PhD*

Date: 07/01/2020



\_\_\_\_\_  
*Lonnie Lybarger, PhD*

Date: 7/01/2020



\_\_\_\_\_  
*Frans E. Tax, PhD*

Date: 7/01/2020

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.



\_\_\_\_\_  
*Daniela C Zarnescu, PhD*  
Master's Thesis Committee Chair  
*Molecular and Cellular Biology*

Date: 07/01/2020

## **Acknowledgements**

**Funding Sources:** The University of Arizona Graduate College tuition support to (Chuo Kueth), RO1 NS01299 (to DCZ) and Fox Chase Drug Discovery Center.

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**Abstract**

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease affecting both upper and lower motor neuron and marked by progressive muscle weakness. However, the pathogenic mechanisms underlying motor neuron death remain unclear. Currently there is no cure for ALS. Therapies fully capable of mitigating complex disease processes are not well developed and greatly needed. So far, three drugs Riluzole, Radicava and recently Terasemtiv, have been approved for ALS, but none of them are very effective. Recently, a small molecule modulator of vesicle trafficking (Apilimod) has been reported to rescue patients motor neuron survival and improve the degree of degeneration in mouse model of ALS based on *C9ORF72* mutations. Here, I used a *Drosophila* model of ALS to test the therapeutic potential of Apilimod and its target, PIKFYVE, in TDP-43 proteinopathy. My results show that PIKFYVE knock down (PIKFYVE RNAi) in motor neurons rescue locomotor dysfunction caused by TDP-43. Consistent with the PIKFYVE knockdown results, Apilimod also rescues TDP-43-dependent locomotor dysfunction. PIKFYVE knockdown was also able to slightly improve lifespan in TDP-43 mutants. These findings confirm that PIKFYVE may provide a useful, albeit limited therapeutic target for TDP-43 proteinopathy.

## Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease affecting both upper and lower motor neurons and marked by progressive degeneration of motor neurons and muscle weakness leading to paralysis and death within 3-5 years after diagnosis due to respiratory failure (Taylor et al., 2016; Coyne et al., 2017). About 10% of patient populations have a family history of ALS (Taylor et al., 2016) and the other 90% have sporadic form of ALS (Estes et al., 2011). Several disease causative genes have been linked to ALS including, Superoxide Dismutase (SOD1), TAR DNA-Binding (*TARDBP*) which encodes the RNA binding protein TDP-43, and *C9ORF72* (chromosome 9 open reading frame 72) among others (Taylor et al., 2016).

TDP-43 is an RNA and DNA binding protein comprising two RNA recognition motifs RRM1 and RRM2 (Ayala et al., 2005), nuclear localization and export signals (NLS, NES) and a glycine-rich C terminal domain were most of disease-associated mutations are found (Coyne et al., 2017) is required for efficient regulation of splicing (Ayala et al., 2005). The RRM1 domain recognize and binds to the UG repeats while RRM2 is not required for UG rich repeat recognition (Ayala et al., 2005). TDP-43 involved in many cellular processes including transport, pre-mRNA splicing, and regulation of transcription (Ayala et al., 2005). Under stress conditions TDP-43 mislocalizes from the nucleus to the cytoplasm where it associates with stress granules, and consequently, affects many aspects of RNA metabolism including RNA splicing, synaptic mRNA transport and translation inhibition of many target mRNAs (Coyne et al., 2017). As disease progresses, motor neurons accumulate cytoplasmic aggregates containing TDP-43 as the main

component of ubiquitinated aggregates found in more than 97% of ALS cases (Ling et al., 2013). However, the pathogenic mechanisms underlying motor neuron death remain unclear.

Furthermore, many underlying molecular mechanisms have been implicated in ALS including defects in protein quality control, excitotoxicity caused by failure of astrocytes to uptake excess glutamate, and RNA metabolism defects affecting many of the RNA processing steps (Taylor et al., 2016). Similar defects are found in ALS caused by mutations in *C9orf72*, however the latter has additional pathological features including intracellular RNA foci, and ubiquitin-binding protein p62 containing cytoplasmic inclusions, which appear to be distinct from ubiquitinated TDP-43 containing inclusions (Taylor et al., 2016).

Currently there is no cure for ALS. Therapies and therapeutic targets fully capable of enhancing complex ALS processes are not well developed and greatly needed. Identification of novel therapeutic target depends greatly on complete understanding of disease mechanisms involved in motor neuron degeneration (Damme et al., 2017). Although there is no cure, three disease modifying drugs Radicava, Riluzole and recently Terasemtiv have been approved for ALS, but none of them are very effective. Therefore, an intense search for more effective disease modifying therapies including small molecules is underway. Much progress has been made understanding the mechanisms underlying ALS (Taylor et al., 2016). Model systems including yeast, zebrafish, mouse, *Drosophila* and induced pluripotent stem cells (iPSC) are used to model ALS for more insights into

the complex disease processes (Damme et al., 2017), and to perform drug screens (Shi et al 2018). Recently, a small molecule modulator of vesicle trafficking (Apilimod) has been reported to rescue patient motor neuron survival and improved degeneration caused by *C9ORF72* in mouse models (Shi et al., 2018).

Apilimod is a small molecule inhibitor that binds to Phosphoinositide Kinase, FYVE-Type Zinc Finger Containing (PIKFYVE) and blocks its phosphotransferase activity leading to selective inhibition of pro-inflammatory cytokines IL-12/IL-23p40 (Shi et al., 2018; Cai et al., 2013). PIKFYVE is a lipid kinase that regulates membrane trafficking (Shi et al., 2018). It phosphorylates phosphatidylinositol (PtdIns) and PtdIns 3-phosphate (PI3P) at the 5-hydroxyl position (Ikonomov et al., 2002) and converts phosphatidylinositol 3-phosphate (PI3P) into phosphatidylinositol (3,5)-bisphosphate (PI (3,5) P<sub>2</sub>), which ultimately disfavors the fusion of lysosome with endosomes and autophagosomes (Shi et al, 2018). Importantly, PIKFYVE inhibition increases PI3P levels which increases the fusion of autophagosomes with lysosomes (Shi et al, 2018) and mitigates *C9orf72*-dependent toxic processes by removing glutamate receptors and dipeptide repeats (Shi et al, 2018). These observations suggest that PIKFYVE inhibition has the potential to mitigate general ALS disease processes including TDP-43-dependent toxicities. Here, I describe my studies using a *Drosophila* model of ALS based on human TDP-43 overexpression that recapitulates key disease features including locomotor dysfunction, neuromuscular junction (NMJ) defects, formation of cytoplasmic aggregates, and reduced lifespan (Estes et al., 2011; Estes et al., 2013) to test the therapeutic potential role of PIKFYVE in TDP-43 proteinopathy. My results show that PIKFYVE knockdown (PIKFYVE RNAi) in motor

neurons rescues locomotor defects caused by TDP-43. Consistent with the PIKFYVE knockdown, Apilimod, a PIKFYVE inhibitor also rescued TDP-43-dependent locomotor dysfunction. However, when tested in the TDP-43 chronic overexpression model, PIKFYVE knock down caused massive pupal stage lethality and failed to improve the TDP-43 mediated reduction in lifespan. To overcome developmental effects, I used an inducible expression system and found that PIKFYVE knock-down slightly increases lifespan in adults expressing mutant TDP-43 at the adult stage only. Taken together, these findings indicate that PIKFYVE may provide a useful, albeit limited therapeutic target for TDP-43 proteinopathy.

## **Materials and Methods:**

### ***Drosophila Genetics***

All stocks and crosses were maintained on standard yeast/cornmeal/ molasses food (25.0 °C using) a 12 hours light and dark cycle unless otherwise noted. The following stocks were used: (i) GAL4 motor neuron driver D42-GAL (Gustafson and Boulianne, 1996) was used to drive the expression of UAS transgenes using the GAL4-UAS bipartite expression system (Brand, A.H, Perrimon, 1993), (ii)  $w^{1118}$  as used as a genetic background control for TDP-43 transgenes, (iii)  $w^{1118}$ ; UAS-TDP-43<sup>WT</sup>- YFP and  $w^{1118}$ ; UAS-TDP-43<sup>G298S</sup>-YFP were used to drive TDP-43 expression in motor neurons. (iv)  $y[1]v[1]$ ;  $P\{Y[+(7.7) = GSYP\}attp2$  was used as genetic background control for PIKFYVE RNAi. All these stocks were generated by or available in the Zarnescu laboratory. In addition,  $y[1] sc[*] v[1]$ ;  $P\{y[+t7.7] v[+t1.8]=TRiP.GL00246\}attp2$ , used to knock-down PIKFYVE by RNAi was obtained from the Bloomington stock center (stock # 35793).

### **TDP-43 expression in motor neurons in Drosophila:**

TDP-43 was expressed in motor neurons using the D42 GAL4 (Gustafson and Boulianne, 1996). GAL4 motor neuron driver D42-GAL4 virgins were crossed with (i)  $w^{1118}$  as a control cross (ii)  $w^{1118}$  ; UAS-TDP-43<sup>WT</sup>- YFP (iii)  $w^{1118}$  ; UAS-TDP-43<sup>G298S</sup>- YFP as experimental crosses (referred to as D42> TDP-43<sup>WT</sup> or D42> TDP-43<sup>G298S</sup>).

### **PIKFYVE knock down by RNAi (PIKFYVE-RNAi) in motor in neurons in Drosophila:**

Similarly, PIKFYVE was knocked down in motor neurons by crossing GAL4 motor neuron driver D42-GAL4 with  $y[1] sc[*] v[1]; P\{y[+t7.7] v[+t1.8]=TRiP.GL00246\}attp2$ .  $y[1]v[1]; P\{Y[+(7.7) = GSYP\}attp2$  was crossed with D42 GAL4 as a genetic background control. For TDP-43 – PIKFYVE genetic interaction experiments, male progeny with TDP-43<sup>WT</sup> and disease associated mutants TDP-43<sup>G298S</sup> genotypes were generated by crossing GAL4 motor neuron driver D42-GAL4 with (i)  $w^{1118}$  ; UAS-TDP-43<sup>WT</sup>- YFP (ii)  $w^{1118}$  ; UAS-TDP-43<sup>G298S</sup>- YFP (referred to as D42> TDP-43<sup>WT</sup> or D42> TDP-43<sup>G298S</sup>). In order to generate more males, crosses were incubated at 22.0°C. D42>TDP-43<sup>WT</sup> males (genotype: UAS–TDP-43<sup>WT</sup>-YFP; D42-GAL4) were crossed with (i)  $y[1]v[1]; P\{Y[+(7.7) = GSYP\}attp2$  as a control (ii)  $y[1] sc[*] v[1]; P\{y[+t7.7] v[+t1.8]=TRiP.GL00246\}attp2$  as experimental crosses. D42> TDP-43<sup>G298S</sup> males (genotype: UAS-TDP–43<sup>G298S</sup>-YFP; D42-GAL4) were crossed with (i)  $y[1]v[1]; P\{Y[+(7.7) = GSYP\}attp2$  as a control and with (ii)  $y[1] sc[*] v[1]; P\{y[+t7.7] v[+t1.8]=TRiP.GL00246\}attp2$  as experimental crosses to study the effects of PIKFYVE knock down. Crosses were fed standard Drosophila food and incubated at 25.0°C to generate desired progeny with TDP-43 disease associated phenotypes. Third instar larvae were screened for the presence of Yellow Fluorescent

Protein (YFP) and assayed for locomotor function using larval turning assay, and lifespan studies.

### **Small molecules treatment: Apilimod**

Apilimod was received from Santa Cruz and dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 30 mM. The same DMSO used to dissolve Apilimod was aliquoted and used as a control. To deliver the compound, 120 mL of standard *Drosophila* food was reheated and allowed to cool. 40  $\mu$ l of 30 mM (millimolar) Apilimod was added to the melted food to achieve a final concentration of 10  $\mu$ M in the food; and 40  $\mu$ l DMSO was added to 120 mL volume of food which was used as a control. Similarly, 80  $\mu$ l of Apilimod was added to achieve a final 20  $\mu$ M concentration in the food. The supplemented *Drosophila* food was transferred into vials and allowed to cool, covered and stored in the cold room for up to 10 days. Experimental and control crosses fed either various concentrations of Apilimod or DMSO were brooded every two days. All crosses were incubated at 25.0°C with a 12 hours light and dark cycle. 33 third instar larvae were collected and assayed for locomotor function using larval turning assay (see Locomotor assays below). Newly emerged male and female (80 per genotype) were collected and transferred into new vials for lifespan studies and brooded every 5 days (see Lifespan studies below).

### **Locomotor assays:**

Larval turning assays were performed using third instar larvae as previously described (Estes et al., 2011; Estes et al., 2013). Third instar larvae are placed on the center of a Petri dish filled with a solidified grape juice/agar medium and allowed to accommodate moving around. Larvae are gently turned ventral side up with a clean paintbrush and

observed until they turned ventral side down made the first movement forward. The time it takes for each larva to turn ventral side down and make a forward movement is recorded. 33 larvae per genotype, per condition were evaluated for locomotor function and larval turning data was analyzed with Graphpad Prism7 for statistical significance.

### **Lifespan studies:**

Apilimod feeding: To study the effects of Apilimod on TDP-43<sup>WT</sup> and disease associated mutants TDP-43<sup>G298S</sup> survival, newly emerged male and female (40 per genotype) were collected into separate vials containing standard *Drosophila* food supplemented with (10 µM) of Apilimod or DMSO and transferred into a new vial with food containing (10 µM) Apilimod or DMSO every 5 days.

PIKFYVE Knock down survival: To study the effects of PIKFYVE knock down on TDP-43<sup>WT</sup> and disease associated mutants TDP-43<sup>G298S</sup> survival, newly emerged male and female were collected into separate vials containing standard *Drosophila* food and transferred into a new vial with food every 5 days. However, progeny with ALS associated genotypes and PIKFYVE knock down don't survive past pupal stage, the don't survive pass the pupa stage.

Survival analysis using inducible expression (with RU486): To overcome the pupal lethality observed when PIKFYVE RNAi and TDP-43 were co-expressed, I used the drug inducible elav-GS-GAL4 system (Kim et al, 2014) this system allows for control of TDP-43 expression, in adults only, under the control of RU486. 80 µg/ml of Mifepristone (RU486) was dissolved in 1 ml of ethanol (EtoH) to a final concentration of 80000 µg/ml. To deliver the compound, 63 mL of standard *Drosophila* food was reheated and allowed

to cool. 31.5  $\mu$ l of 80000  $\mu$ g/ml (microgram per milliliter) RU486 was added to the melted food to achieve a final concentration of 40  $\mu$ g/ml in the food; and 31.5  $\mu$ l of ethanol was added to 63 mL volume of food which was used as vehicle. Newly emerged males and females (80 per genotype) were collected and transferred into vials of fly food containing RU486 (40  $\mu$ g/ml) or, ethanol as vehicle (Kim et al, 2014). Crosses were brooded every 4 days into new vials containing RU486 (40  $\mu$ g/ml) or, ethanol as vehicle for the course of lifespan studies.

### **Western Blotting:**

To confirm TDP-43 protein expression using the drug inducible *elav-GS-GAL4* system (Kim et al, 2014), newly eclosed males and females (50 per genotype) were collected and fed RU486 at (40  $\mu$ g/ml) for 21 days. After 21 days of RU486 feeding, adults heads were collected, flash frozen and stored at – 80 °C until all genotypes were collected. To generate cellular fractions, 45 adult fly heads for each genotype were homogenized with 250  $\mu$ l LS buffer for per sample (1250  $\mu$ l LS buffer, 5.5  $\mu$ l PMSF, 10  $\mu$ l 1X protease inhibitor), using the Bullet Blender Blue and Bullet Blender Bead Lysis Kit Green. Samples were run in the Bullet Blender Blue for 3 minutes at intensity 8 to homogenize then spun down for 2 minutes with Mikro 20 centrifuge at 10,000 RPM. The supernatant was removed, out of which 30  $\mu$ l were taken and combined with 30  $\mu$ l of 2X Laemmli (protein input sample). The rest of the supernatant was centrifuged at 25,000g (24000rpm in TLA-100.3 rotor) for 30 minutes at 4° to generate the protein LS urea fractions. The supernatant was removed and combined with 150  $\mu$ l of 2X Laemmli (protein LS fraction). The remaining pellet was solubilized in urea buffer (250  $\mu$ l urea buffer, 5  $\mu$ l PMSF and 10  $\mu$ l protease inhibitor), and combined with 30  $\mu$ l 2X Laemmli (referred to as insoluble urea

fractions). All samples corresponding to input, LS and urea fractions were boiled for 5 minutes in 95-100° standard heat block and stored at – 20 °C.

Western blots on fractions was performed to detect TDP-43 via its YFP tag. Proteintech monoclonal mouse anti TDP-43 (human antibody) antibody at 1:1000 dilution, was used as primary antibody. The LI-COR goat anti-rabbit antibody lot number (C90220-05) was used a secondary (.5 µl of the secondary was diluted in 5 mL of 5% blocking milk).

For normalization, Cell Signaling polyclonal rabbit anti beta actin antibody was used at 1:2000 dilution in 5% blocking milk (0.25 g milk in 5 ml TBST) as primary antibody. ThermoFisher Alexa Fluor 700 antibody goat anti mouse IgG lot number (1981686) was used as secondary (5 µl of the secondary was diluted in 5 mL of 5% blocking milk). Westerns blots were analyzed using Odyssey Infrared Imager, image J for quantification and prism 7 for statistical analysis.

### **Statistics:**

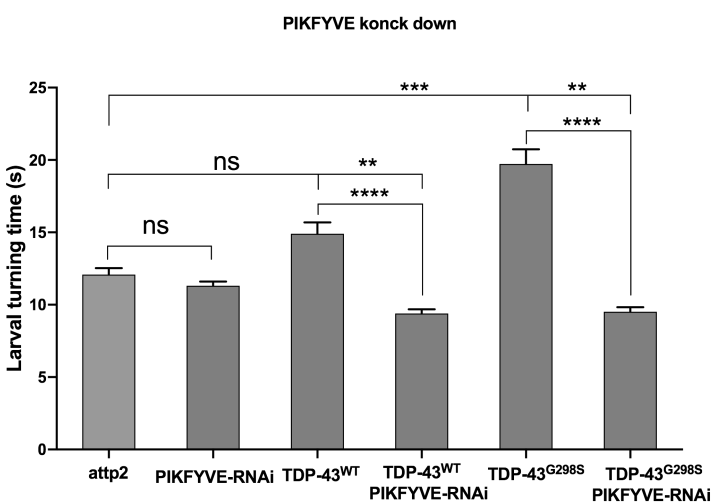
All larval turning assays data were entered into (Graphpad Prism 7) and analyzed using Kruskal-Wallis with multiple comparisons. Survival data were analyzed in Prism using Log-rank (Mantel-Cox) test. Asterisks indicate statistical significance (\*) P<0.05, (\*\*) P<0.01, (\*\*\*) P<0.001, (\*\*\*\*) P<0.0001, (ns) – not significant.

### **Results**

#### **PIKFYVE knock down in motor neurons rescues TDP-43 dependent locomotor dysfunction**

PIKFYVE was reported to be a therapeutic target in C9orf72 ALS (Shi et al., 2018). To determine whether PIKFYVE also has the ability to modulate TDP-43 proteinopathy, I

used a *Drosophila* model of ALS whereby chronic overexpression TDP-43 in motor neurons with the D42 GAL4 driver (Gustafson and Boulianne, 1996) recapitulates key pathological features of ALS including locomotor defects and reduced lifespan (Estes et al., 2011; Estes et al., 2013). More specifically, PIKFYVE was knocked down in motor neurons by crossing the motor neuron driver D42-GAL4 with  $y[1] \text{ sc}^* v[1]; P\{y[+7.7] v[+11.8]=\text{TRiP.GL00246}\} \text{attp2}$  in the context of third instar larvae expressing TDP-43 (see Materials and Methods for crossing scheme and genotypes). Locomotor function was measured using larval turning assays were performed as previously described (Estes et al., 2011; Estes et al., 2013). Interestingly, PIKFYVE knock down in motor neurons modulates TDP-43 dependent toxicity and significantly improves both TDP-43<sup>WT</sup> and mutant TDP-43<sup>G298S</sup> mediated locomotor defect in comparison to controls (attp2



background or PIKFYVE alone)

(Fig. 1). These results show that modulating PIKFYVE levels, specifically knocking down PIKFYVE, modifies TDP-43 dependent disease processes, and could be a potential therapeutic target for TDP-43 mediated ALS.

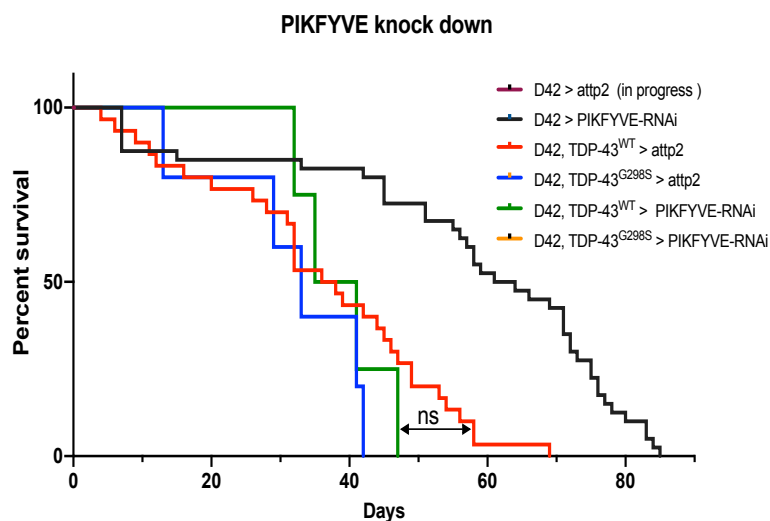
**Figure 1. PIKFYVE knock down in motor neurons rescues TDP-43-dependent locomotor defect.**

TDP-43 wild type and ALS associated mutant TDP-43<sup>G298S</sup> were expressed in motor neurons using GAL4-UAS. TDP-43 – PIKFYVE-RNAi genetic interaction larval turning. Control genotypes: *attp2 is yv; D42-GAL4/attp2*, and *PIKFYVE-RNAi is yv; D42-GAL4/UAS-PIKFYVE-RNAi-attp2*. TDP-43 wild type and mutant expression in motor neurons genotypes: TDP-43<sup>WT</sup> is *yv/w; UAS-TDP-43<sup>WT</sup>-YFP; D42-GAL4/attp2*,

and TDP-43<sup>G298S</sup> is *yv/w; UAS-TDP-43<sup>G298S</sup>-YFP; D42-GAL4/attp2*. TDP-43 – PIKFYVE-RNAi genetic interaction genotypes: TDP-43<sup>WT</sup> > PIKFYVE-RNAi is *yv/w; UAS-TDP-43<sup>WT</sup>-YFP; D42-GAL4/UAS-PIKFYVE-RNAi-attp2*. TDP-43<sup>G298S</sup> > PIKFYVE-RNAi is *yv/w; UAS-TDP-43<sup>G298S</sup>-YFP; D42-GAL4/UAS-PIKFYVE-RNAi-attp2*. N = 33 larvae per genotype. Larval turning data were analyzed using Kruskal-Wallis with multiple comparisons. Asterisks indicate statistical significance (\*\*) P<0.01, (\*\*\*) P<0.001, (\*\*\*\*) P<0.0001, (ns) – not significant.

### PIKFYVE knock down partially improves TDP-43-mediated reduction in lifespan

Next, I asked whether PIKFYVE knockdown (PIKFYVE-RNAi) can also improve TDP-43-dependent lifespan reduction using the method the lab reported previously (Estes et al., 2011; Estes et al., 2013). To address this issue, lifespan experiments were performed on newly emerged flies expressing TDP-43<sup>WT</sup> or mutant TDP-43<sup>G298S</sup> chronically throughout development. However, TDP-43 co-overexpression with PIKFYVE knockdown (PIKFYVE-RNAi) in motor neurons (TDP-43<sup>WT</sup> > PIKFYVE-RNAi or TDP-43<sup>G298S</sup> > PIKFYVE-RNAi) showed pupal stage lethality. Most or all TDP-43 – PIKFYVE-RNAi pupae were dead making it difficult to complete the lifespan experiment. (Fig. 2).

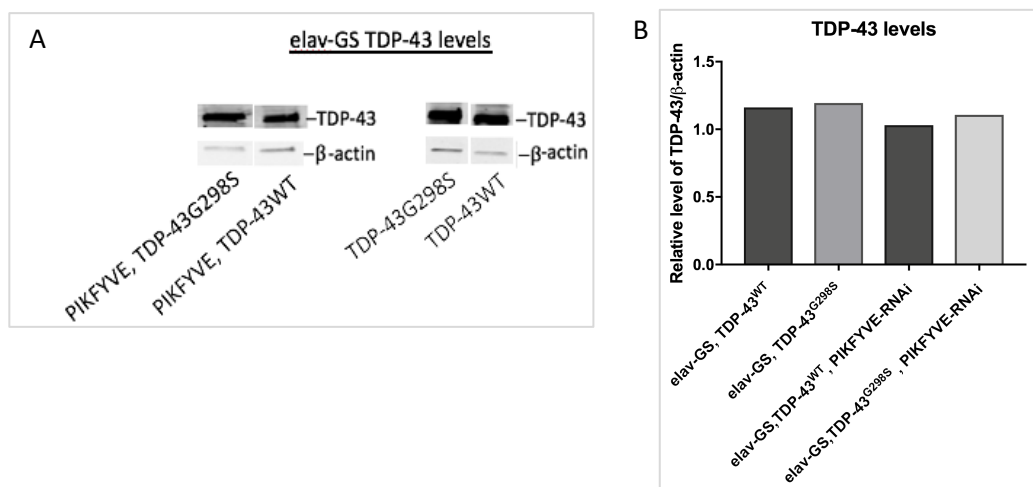


**Figure 2. PIKFYVE knock down is insufficient to improve TDP-43-mediated reduction in lifespan in a chronic over expression model.** TDP-43 – PIKFYVE-RNAi genetic interaction lifespan. genotypes: *D42 > attp2* is *yv; D42-GAL4/attp2* (40 flies in progress), *D42 > PIKFYVE-RNAi* is *yv; D42-GAL4/UAS-PIKFYVE-RNAi-attp2* (40

flies). TDP-43 wild type and mutant expression in motor neurons genotypes: TDP-43<sup>WT</sup> > attp2 is *yv/w; UAS-TDP-43<sup>WT</sup>-YFP; D42-GAL4/attp2* (30 flies), TDP-43<sup>G298S</sup> is *yv/w; UAS-TDP-43<sup>G298S</sup>-YFP; D42-*

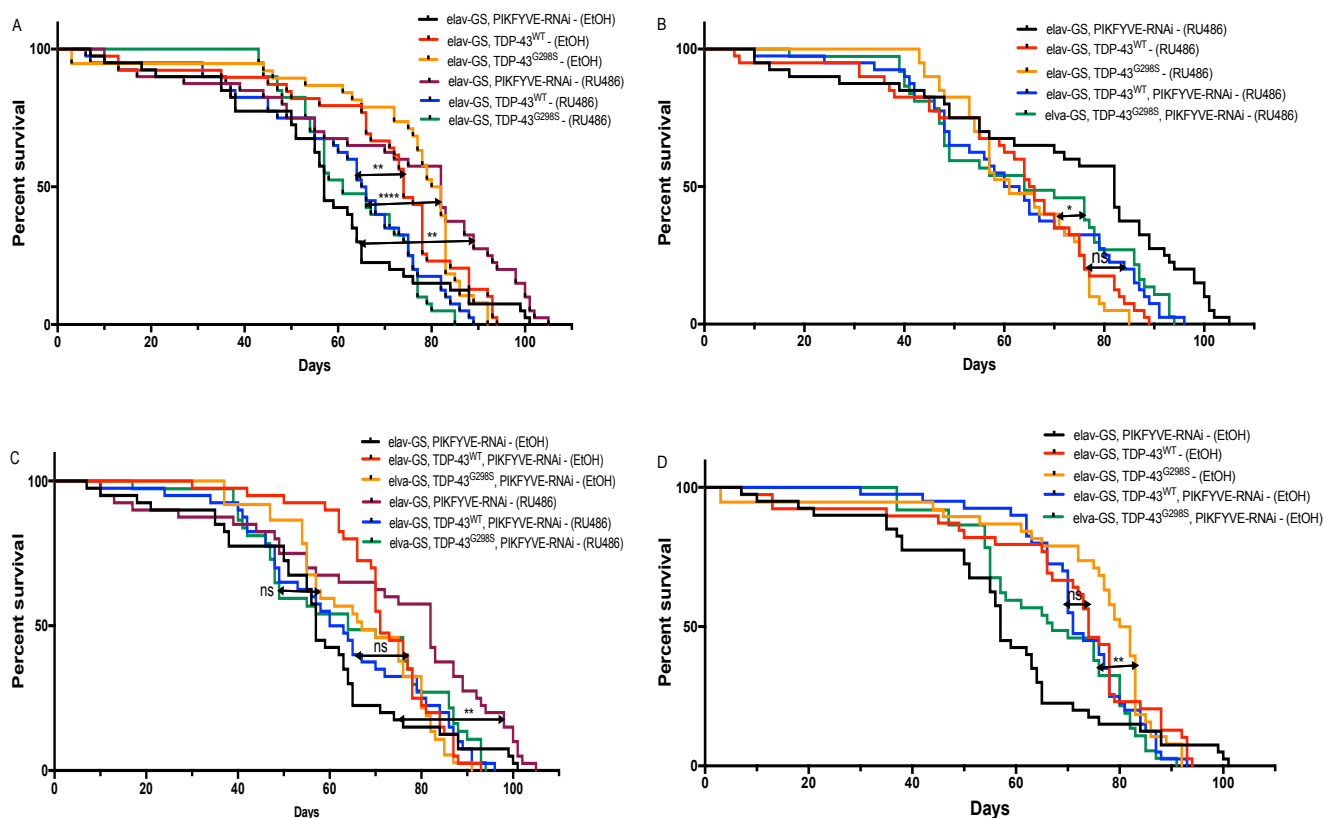
*GAL4/attP2* (5 flies). TDP-43 – PIKFYVE-RNAi genetic interaction genotypes: *TDP-43<sup>WT</sup> > PIKFYVE-RNAi* is *yv/w; UAS-TDP-43<sup>WT</sup>-YFP; D42-GAL4/UAS-PIKFYVE-RNAi-attp2* (4 flies), *TDP-43<sup>G298S</sup> > PIKFIVE-RNAi* is *yv/w; UAS-TDP-43<sup>G298S</sup>-YFP; D42-GAL4/UAS-PIKFVE-RNAi-attp2* (0 flies).

To overcome this difficulty, the drug inducible *elav-GS-GAL4* system (Kim et al, 2014) was used to turn on TDP-43 expression and knockdown PIKFYVE after the pupal stage. First, I confirmed TDP-43 expression is driven by *elav-GS* using Western blots of adult heads after 21 days on RU486 (40  $\mu$ g/ml) containing food compared with ETOH only food as a control (Fig. 3).



**Figure 3. TDP-43 expression driven by *elav-GS*.** (A) Western blot confirms TDP-43 expression induced with *elav-GS* by RU468 and (B) shows relative levels of TDP-43 in adults heads after 21 days of RU486 feeding (40  $\mu$ g/ml).

After confirming that I can induce TDP-43 expression using the inducible *elav GS* driver and RU486, I tested whether TDP-43 expression in adulthood only causes a reduction in lifespan as it does when chronically overexpressed. To this end I compared survival of TDP-43 WT or G298S expressing flies on RU486 versus ETOH (Fig. 4A).



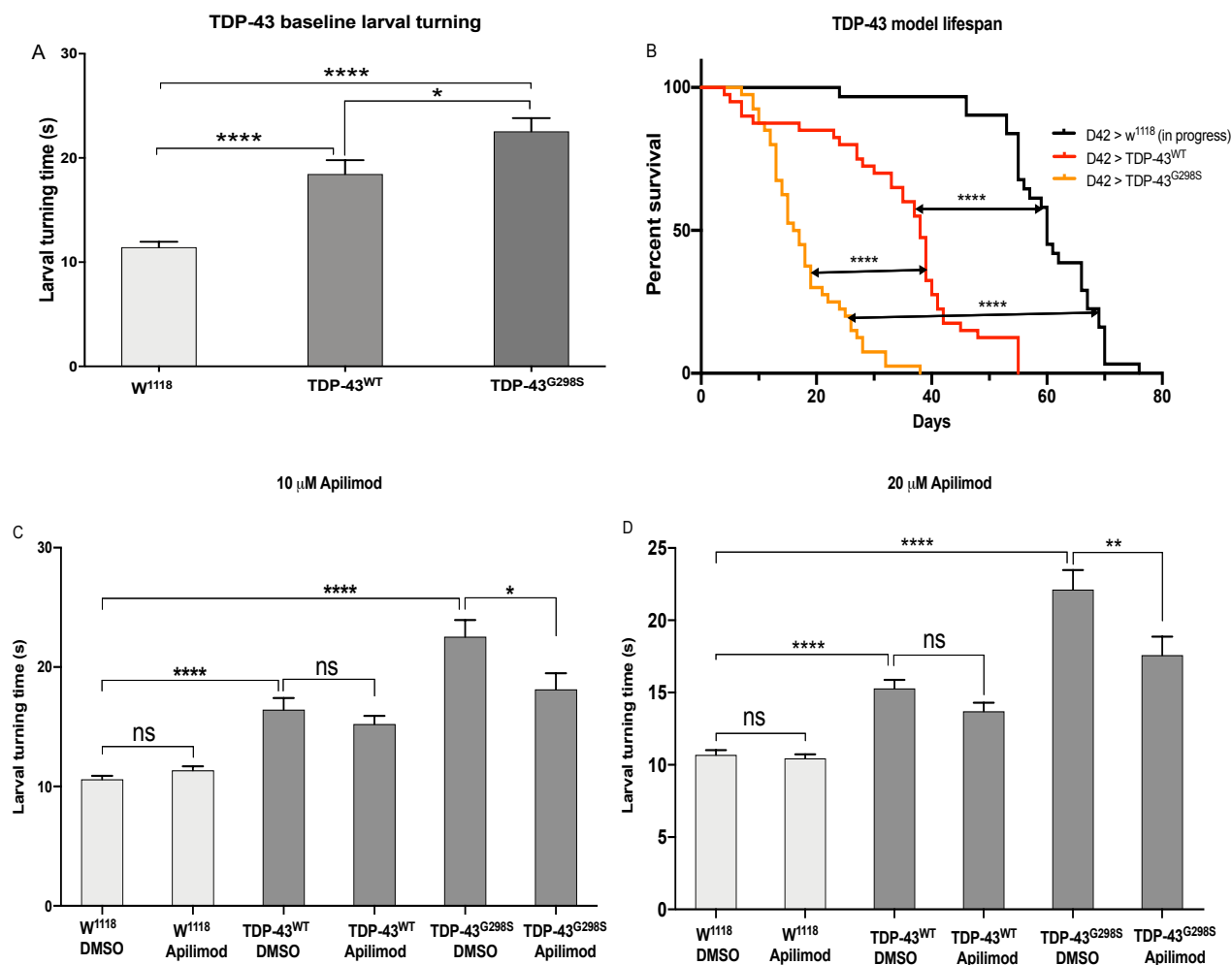
**Figure 4. PIKFYVE knock down slightly improves mutant but not WT TDP-43-mediated reduction in lifespan.** *elavGS-GAL4* was used to turn on TDP-43 expression after the pupal stage, in adults only. (A-D) lifespan of flies raised on either RU486 (40  $\mu$ g/ml) or, ethanol as vehicle. (A) TDP-43 induction in adults causes a slight but statistically significant reduction in lifespan. (B) PIKFYVE RNAi increases lifespan in TDP-43 G298S expressing adults but has no effect on TDP-43 WT. (C, D) Lifespan comparisons on RU486 versus Et-OH (C) or EtOH only (D). Genotypes: *elav-GS > PIKFYVE-RNAi is elav-GS-GAL4/UAS-PIKFYVE-RNAi-attp2* (n = 80 flies), *elav-GS > TDP-43<sup>WT</sup> is UAS-TDP-43<sup>WT</sup>-YFP*; *elav-GS-GAL4* (n = 79 flies); *elav-GS > TDP-43<sup>G298S</sup> is UAS-TDP-43<sup>G298S</sup>-YFP*; *elav-GS-GAL4* (n = 78 flies). TDP-43 – PIKFYVE-RNAi genetic interaction genotypes: *PIKFYVE-RNAi > TDP-43<sup>WT</sup> is UAS-TDP-43<sup>WT</sup>-YFP*; *elav-GS-GAL4/UAS-PIKFYVE-RNAi-attp2* (n = 80 flies); *PIKFYVE-RNAi > TDP-43<sup>G298S</sup> is UAS-TDP-43<sup>G298S</sup>-YFP*; *elav-GS-GAL4/UAS-PIKFYVE-RNAi-attp2* (n = 74 flies). All survival data were analyzed using Log-rank (Mantel-Cox) test. Asterisks indicate statistical significance (\*) P<0.05, (\*\*) P<0.01, (\*\*\*) P<0.001, (\*\*\*\*) P<0.0001, (ns) – not significant (Prism GraphPad v7.0).

These experiments showed that TDP-43 expression in adults causes a significant reduction in lifespan (94 days for elav-GS, TDP-43 WT on EtOH vs 89 days on RU486,  $P_{\text{value}} = 0.0074$ , 92 days for elav-GS, TDP-43 G298S on EtOH vs 85 days on RU486,  $P_{\text{value}} = < 0.001$  and 101 days for elav-GS, PIKFYVE-RNAi on EtOH vs 105 days on RU486,  $P_{\text{value}} = 0.0037$ ). Next, I tested the ability of PIKFYVE-RNAi to rescue TDP-43 dependent reduction in lifespan (**Fig. 4 B-D**). As shown in Figure 4B, reducing PIKFYVE levels by RNAi knock-down had no effect on TDP-43 WT ( 89 days for elav-GS TDP-43 WT vs 96 days for elav-GS, PIKFYVE-RNAi on RU486,  $P_{\text{value}} = 0.3597$ ), but caused a slight yet statistically significant increase in lifespan for TDP-43 G298S-expressing flies ( 85 days for TDP-43 G298S vs 94 days for TDPG298S PIKFYVE RNAi on RU486 ,  $P_{\text{value}} = 0.0401$ ). The positive effect of PIKFYVE RNAi on TDP-43 is further substantiated by findings that TDP-43 (WT or G298S) PIKFYVE RNAi expressing adults have comparable lifespans on RU486 versus EtOH (Fig. 4C). It is surprising that the different genotypes examined exhibit some differences on ETOH (Fig. 4D), however this does not affect our interpretation that PIKFYVE RNAi improves TDP-43 G298S survival significantly, albeit slightly (Fig. 4B).

### **Apilimod rescues TDP-43 induced locomotor dysfunction**

Next, I investigated whether Apilimod, a PIKFYVE inhibitor, can mitigate TDP-43 mediated locomotor dysfunction in *Drosophila* models of ALS in a manner comparable to PIKFYVE knockdown. To test this, I first used larval turning assays to measure locomotor function in third instar larva expressing TDP-43<sup>WT</sup> or mutant TDP-43<sup>G298S</sup> and w<sup>1118</sup> controls as previously described (Estes et al., 2011; Estes et al., 2013). Indeed, consistent

with published results, TDP-43<sup>WT</sup> and ALS-associated mutant TDP-43<sup>G298S</sup> flies raised on standard *Drosophila* food had higher turning time and reduced lifespan in comparison to control (**Fig. 5 A-B**). Using this assay I found that Apilimod treatment significantly improved ALS associated mutant TDP-43<sup>G298S</sup> mediated locomotor dysfunction at 10  $\mu$ M and 20  $\mu$ M concentrations (**Fig. 5 C**), however it was unable to rescue the TDP-43<sup>WT</sup> locomotor defect when tested at either 10  $\mu$ M or 20  $\mu$ M in comparison to control (**Fig. 5 C-D**). These findings show that Apilimod has the ability to improve locomotor dysfunction although unlike PIKFYVE RNAi it only mitigated TDP-43 G298S and not TDP-43<sup>WT</sup> phenotypes, possibly due to the limited range of concentrations tested here. Supporting this is our finding that 20  $\mu$ M shows a rescue trend for TDP-43 WT.



**Figure 5. Apilimod rescues TDP-43 induced locomotor dysfunction.** TDP-43 or ALS associated TDP-43<sup>G298S</sup> were expressed in motor neurons using GAL4-UAS. N = 33 third instar larvae per genotype per condition were assayed using larval turning assay. **(A)** Locomotor function of *Drosophila* models of ALS raised on standard *Drosophila* food. Control and treatment genotypes: w<sup>1118</sup> is control, TDP-43<sup>WT</sup> and disease associated mutant TDP-43<sup>G298S</sup> genotypes: TDP-43<sup>WT</sup> is w<sup>1118</sup>; UAS-TDP-43<sup>WT</sup>- YFP; D42-GAL4, TDP-43<sup>G298S</sup> is w<sup>1118</sup>; UAS-TDP-43<sup>G298S</sup>- YFP; D42-GAL4. **(B)** Lifespan of *Drosophila* models of ALS. Genotypes: D42 > W<sup>1118</sup> is control (n = 33 flies), D42 > TDP-43<sup>WT</sup> is w<sup>1118</sup>; UAS-TDP-43<sup>WT</sup>- YFP; D42-GAL4 (n = 40 flies), and D42 > TDP-43<sup>G298S</sup> is w<sup>1118</sup>; UAS-TDP-43<sup>G298S</sup>- YFP; D42-GAL4 (n = 40 flies). **(C-D)** Locomotor function of flies treated with either (10 μM or 20 μM) concentrations of DMSO and Apilimod. Larval turning assay data were analyzed using Kruskal-Wallis with multiple comparisons, and survival data were analyzed using Log-rank (Mantel-Cox) test. Asterisks indicate statistical significance (\*) P<0.05, (\*\*) P<0.01, (\*\*\*) P<0.001, (\*\*\*\*) P<0.0001, (ns) – not significant (Prism GraphPad v7).

## Discussion

Despite the considerable progress towards defining novel therapeutic strategies for ALS treatment and intervention there is no cure for this disease. Intense efforts are underway to identify and develop disease modifying small molecules and this approach is aided by studies in various disease models including *Drosophila* models of ALS based on TDP-43 proteinopathy (Estes et al., 2011; Estes et al., 2013). Therapeutic success depends on understanding the toxicities caused by proteins such as TDP-43, an RNA binding protein that associates with stress granules and insoluble cytoplasmic aggregates and is implicated in more than 97% of ALS cases (Ling et al., 2013). Thus, the relative contribution of TDP-43 toxicity to the development and progression of disease deserves important consideration in therapeutic development. Another ALS gene of interest is C9ORF72, which causes the largest proportion of familial ALS but is also associated with some sporadic cases; in addition to similarity with TDP-43 pathology, it contains distinct

pathological features including dipeptide repeats and intracellular RNA foci, and ubiquitin-binding protein-p62 containing cytoplasmic aggregates distinct from ubiquitinated TDP-43 containing inclusions (DeJesus-Hernandez et al., 2011; Shi et al., 2018; Taylor et al., 2016). Recently, Apilimod, a small molecule modulator of vesicle trafficking that act as a PIKFYVE inhibitor, has been reported to rescue motor neuron survival from ALS patients and to improve degeneration caused by *C9ORF72* in patient-derived iPSC motor neuron and mouse models (Shi et al., 2018). Interestingly, inhibition of PIKFYVE increases PI3P production similar to the activation of Rab5 (Lodhi et al., 2008), which associates with early endosomes. Since we recently showed that Rab5 overexpression mitigates TDP-43 dependent phenotypes in vivo (Liu et al., 2017) we reasoned that Apilimod may also protect TDP-43 proteinopathy.

Here I tested the therapeutic potential of PIKFYVE inhibition and Apilimod in TDP-43 proteinopathy. My results show that PIKFYVE knockdown in the context of two models of TDP-43 proteinopathy, TDP-43 wild type and disease-associated TDP-43G298S mutant over-expression in motor neurons is sufficient to mitigate disease phenotypes. PIKFYVE knockdown in motor neurons significantly rescue TDP-43 dependent locomotor defects. Given that TDP-43 clearance can be enhanced by Rab5 overexpression (Liu et al., 2017), these results suggest that PIKFYVE knockdown may increase the clearance of cytoplasmic aggregates by increasing PI3P levels which promotes early endosome maturation, early phases of lysosome biogenesis and the fusion of autophagosomes with lysosomes (Shi et al, 2018). Thus, it will be interesting to determine whether PIKFYVE

inhibition or Apilimod treatment reduce TDP-43 aggregation or levels in vivo, something we can do in our model.

Furthermore, a recent report showed that reduction in *C9ORF72* activity caused neurodegeneration through accumulation of glutamate receptors causing excitotoxicity mediated motor neuron death (Shi et al., 2018), due to the incapacity of astrocytes to uptake glutamate caused by poly (PR) repeat expression (Shi et al., 2018). It will be interesting to investigate whether modulating PIKFVE levels in astrocytes improve their capacity to clear poly (PR) repeats, increase glutamate uptake and modulate TDP-43-dependent functional defects. My results further suggest that genetic modulation of vesicle trafficking is sufficient to mitigate TDP-43-dependent locomotor dysfunction and is consistent with our previously published results (Liu et al., 2017).

Consistent with the PIKFYVE knockdown results, Apilimod, a PIKFYVE inhibitor, also rescues TDP-43-dependent locomotor dysfunction. Interestingly, both concentrations tested significantly rescue TDP-43 G298S motor neuron dysfunction but showed only a minor effect on TDP-43 wild type overexpression. Given that PIKFYVE knock-down rescued both TDP-43 models, these findings suggest that a different Apilimod concentration might be needed to mitigate TDP-43 overexpression. It is also possible that different mechanisms might be at play in TDP-43 WT versus mutant G298S, which is consistent with previous publications showing that TDP-43 WT and mutant G298S overexpression cause toxicity through partially distinct mechanisms (Coyne et al., 2017). We speculate that Apilimod accelerates aggregate clearance by stimulating autophagy

and might limit the association of TDP-43 with stress granules preventing new cytoplasmic aggregate formation and this might be more effective for mutant TDP-43. Additional experiments with different concentrations or perhaps a more active PIKFYVE inhibitor will need to be performed to provide an explanation for the trending rescue seen with TDP-43 wild type. Nevertheless, both approaches demonstrated that modulating PIKFYVE, specifically PIKFYVE inhibition, modifies TDP-43 dependent disease processes, and that Apilimod could have therapeutic benefits for TDP-43-mediated ALS.

Although PIKFYVE knockdown significantly improved TDP-43-dependent locomotor defects, PIKFYVE knockdown and TDP-43 co-expression in motor neurons caused pupal stage lethality and failed to improve TDP-43-dependent reduction in lifespan in our chronic overexpression model. Although this was unexpected, previous reports in *C. elegans* and *Drosophila* showed that loss of PIKFYVE cause pupal stage lethality (Shisheva et al., 2008) suggesting that vesicle trafficking pathways might be insensitive to the reduction of phosphatidylinositol (3,5)-bisphosphate (PI (3,5) P2) levels than pathways necessary to maintain normal pupal stage and that involved lifespan. For example, the removal of glutamate receptors might be insensitive to PIKFYVE knockdown during this developmental stage. Recently, reduction in *C9ORF72* activity was shown to cause neurodegeneration through accumulation of glutamate receptors causing excitotoxicity and reduced ability of astrocytes to uptake glutamate due to poly (PR) repeat expansion (Shi et al., 2018). It would be interesting to examine the effect of PIKFYVE knockdown on the synaptic architecture at the neuromuscular junction and astrocytes in the future. Nevertheless, the fact that PIKFYVE knock-down did improve

TDP-43 G298S survival in our inducible, adults only expression models (with the elav Gene Switch) suggests that PIKFYVE's ability to modulate TDP-43 toxicity is promising but limited beyond its significant effect on locomotor function.

The ability of the PIKFYVE inhibitor Apilimod to mitigate TDP-43 proteinopathy and rescue both wild type and mutant TDP-43-dependent reduction in lifespan is yet to be determined. It will be interesting to determine whether Apilimod will be sufficient to modify other TDP-43-dependent disease processes including toxic cytoplasmic TDP-43 aggregates and improve lifespan. Notably, chemical or genetic modulation of vesicle trafficking rescues the survival and degeneration phenotypes caused by *C9ORF72* repeat expression (Shi et al., 2018). A recent report showed that treatment with Apilimod improves motor defects by increasing the clearance of Poly (PR) expression (Shi et al., 2018). Therefore, future experiments investigating Apilimod's sufficiency to clear TDP-43 aggregates and rescue lifespan would be interesting. Moreover, Apilimod is known to inhibit the release of pro-inflammatory cytokines IL-12 and IL-23 (Cai et al., 2013). It would be interesting to investigate whether inhibition of pro-inflammatory cytokines impairs TDP-43 incorporation into stress granules, or association with aggregates (Dewey et al., 2011). Modulation of vesicle trafficking pathway by PIKFYVE inhibitors might mitigate cytokine-dependent disease processes.

TDP-43 has been previously shown to interact with protein partners including *Drosophila* fragile X mental retardation protein (dFMRP) leading to synaptic defects and toxic aggregate formation (Coyne et al 2015). Furthermore, TDP-43 proteinopathy reduces

*futsch* protein expression at the neuromuscular junctions, and *futsch* overexpression reduced TDP-43 aggregation, improved locomotor dysfunction and increased lifespan in *Drosophila* (Coyne et al 2014). Therefore, strategies that modify TDP-43-positive aggregates might provide neuroprotective effects. A recent report showed that small molecules can prevent TDP-43 mRNA recruitment into stress granules and prevent its accumulation in iPSC-derived motor neurons (Fang et al., 2019). This suggest modulation of stress granule properties as a potential therapeutic strategy. It will be interesting to investigate Apilimod's effects on stress granules and aggregation in the future.

Currently available treatments for ALS include Radicava, a free radical scavenger that provides protective effects against damage caused by reactive oxygen species (Ikeda et al., 2015) its therapeutic mechanism of action seems to decrease lipoxygenase-mediated arachidonic acid production by trapping hydroxyl radicals (Ikeda et al., 2015). Although its exact mechanism of action is not known, it is thought to slow disease progression by mitigating oxidative stress implicated in motor neuron degeneration (Cruz et al., 2018). Additionally, Riluzole, the first approved treatment for ALS, protects against excitotoxicity-induced motor neuron degeneration by limiting glutamate release (Cheah et al., 2010). Although it has been shown to prevent glutamate-mediated motor degeneration *in vitro* (Cheah et al., 2010), it extended life in patients by only up to 3 months (Kim et al., 2014). Recently, Terasemtiv, a fast skeletal muscle troponin activator that sensitize sarcomeres to calcium, increasing skeletal muscle contraction, has been approved as a treatment for ALS (Shefner et al., 2019). However, Terasemtiv activity does not change the decline of slow vital capacity (SVC), or significantly improve muscle strength (Shefner et al., 2019).

Overall, none of these current treatments are effective to fully improving muscle weakness, extend life, or fully rescue motor neuron degeneration and death.

In summary, my results provide further support for PIKFYVE as an excellent therapeutic target in ALS. I provided data that chemical and genetic modulation of vesicle trafficking mitigate TDP-43-dependent toxicities and rescue locomotor dysfunction. Although TDP-43 and PIKFYVE knock down co-expression in motor neurons is larval stage lethal and failed to improve TDP-43-dependent reduction in lifespan, adult-only expression showed a protective effect of PIKFYVE on lifespan in TDP-43 G298S expressing flies. These results suggest that locomotor dysfunction and lifespan might be regulated by different cellular pathways. Elucidation of PIKFYVE genetic interactions and the pathways involved in the modulation of TDP-43 proteinopathy might uncover disease mechanisms involved in ALS and further confirm PIKFYVE potential to provide therapeutic benefits for TDP-43 proteinopathy.

**Supplemental Figure 6.** Crossing scheme used to generate the desired progeny used in analysis of disease associated phenotypes.

### Control crosses

$$\frac{X}{X'} \cdot \frac{+}{+} ; \frac{D42-GAL4}{D42-GAL4} \quad \times \quad \frac{YV}{YW} \cdot \frac{+}{+} ; \frac{attp2}{attp2}$$

$$\boxed{\frac{YV}{W} \cdot \frac{+}{+} ; \frac{D42-GAL4}{attp2}} \quad \dashrightarrow \text{Referred to as } attp2$$

$$\frac{X}{X'} \cdot \frac{+}{+} ; \frac{D42-GAL4}{D42-GAL4} \quad \times \quad \frac{YV}{Y} \cdot \frac{+}{+} ; \frac{UAS-PIKFYVE-RNAi-attp2}{UAS-PIKFYVE-RNAi-attp2}$$

$$\boxed{\frac{YV}{W} \cdot \frac{+}{+} ; \frac{D42-GAL4}{UAS-PIKFYVE-RNAi-attp2}} \quad \dashrightarrow \text{Referred to as PIKFYVE-RNAi}$$

### Crosses used to generate male progeny

$$\frac{X}{X'} \cdot \frac{+}{+} ; \frac{D42-GAL4}{D42-GAL4} \quad \times \quad \frac{X}{Y} \cdot \frac{UAS-TDP-43-WT-YFP}{UAS-TDP-43-WT-YFP} \cdot \frac{+}{+}$$

$$\boxed{\frac{X}{Y} \cdot \frac{UAS-TDP-43-WT-YFP}{+} ; \frac{D42-GAL4}{+}}$$

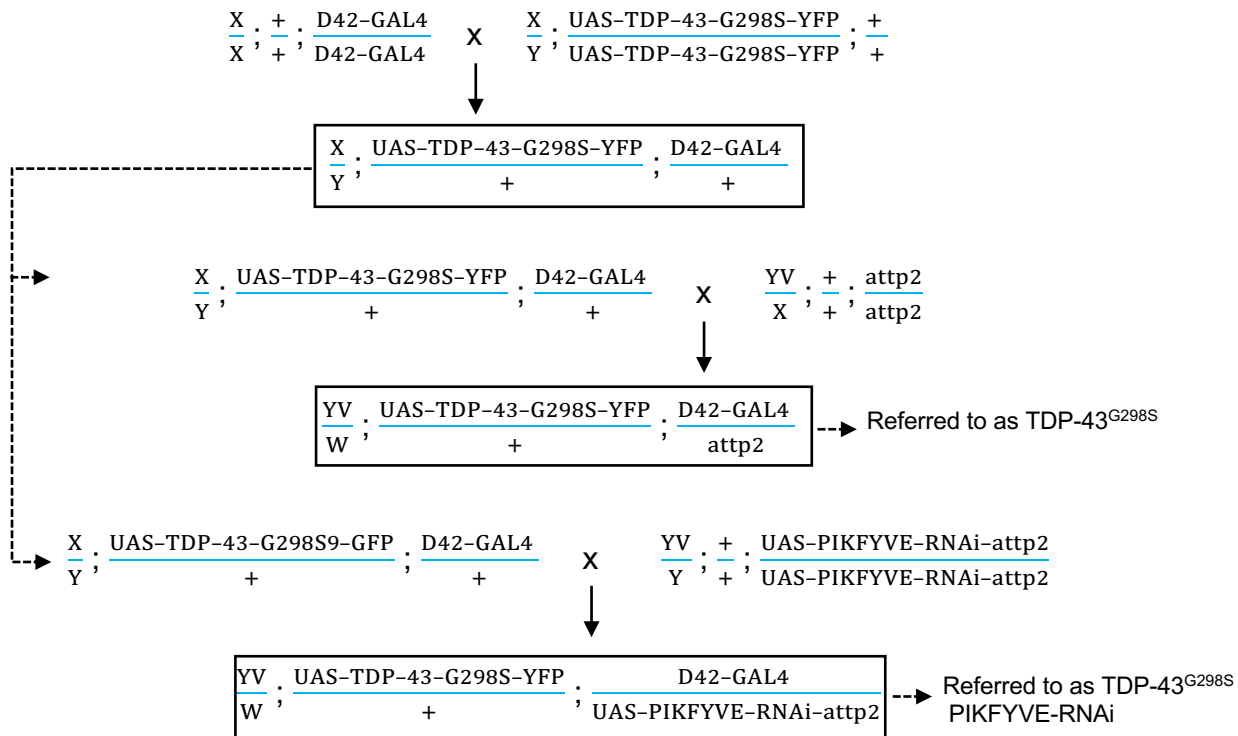
$$\frac{X}{Y} \cdot \frac{UAS-TDP-43-WT-YFP}{+} ; \frac{D42-GAL4}{+} \quad \times \quad \frac{YV}{X} \cdot \frac{+}{+} ; \frac{attp2}{attp2}$$

$$\boxed{\frac{YV}{W} \cdot \frac{UAS-TDP-43-WT-YFP}{+} ; \frac{D42-GAL4}{attp2}} \quad \dashrightarrow \text{Referred to as } TDP-43^{WT}$$

$$\frac{X}{Y} \cdot \frac{UAS-TDP-43-HWT21L-YFP}{+} ; \frac{D42-GAL4}{+} \quad \times \quad \frac{YV}{X} \cdot \frac{+}{+} ; \frac{UAS-PIKFYVE-RNAi}{UAS-PIKFYVE-RNAi}$$

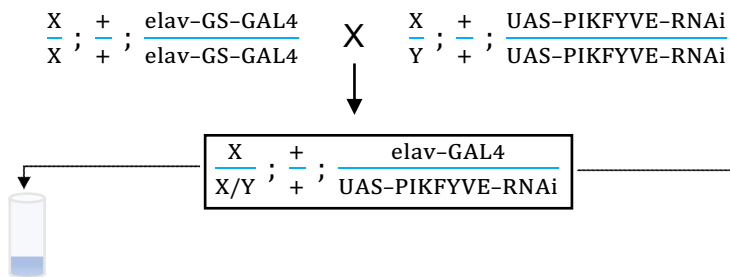
$$\boxed{\frac{YV}{W} \cdot \frac{UAS-TDP-43-HWT21L-YFP}{+} ; \frac{D42-GAL4}{UAS-PIKFYVE-RNAi-attp2}} \quad \dashrightarrow \text{Referred to as } TDP-43^{WT} \text{ PIKFYVE-RNAi}$$

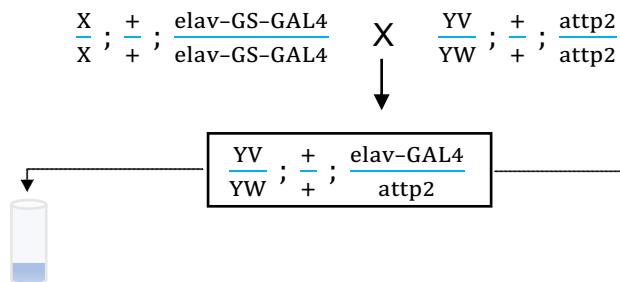
### Crosses used to generate male progeny



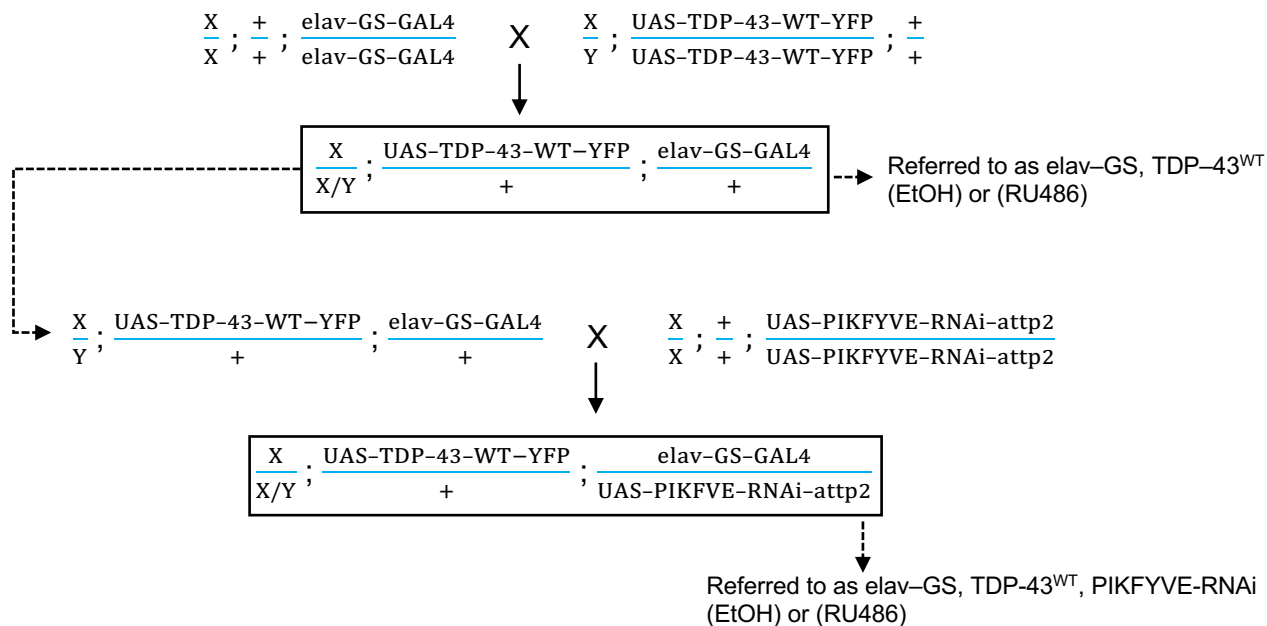
**Supplemental Figure 7.** Crossing scheme used for the lifespan assay using the drug inducible *Elav-GS-GAL4* system, to induce the expression of disease associated genotypes used to assess lifespan. Newly emerged males and female (40 per genotype) from the following crosses were collected and fed RU486 for experimental lifespan, and ethanol for experimental controls.

### Control crosses

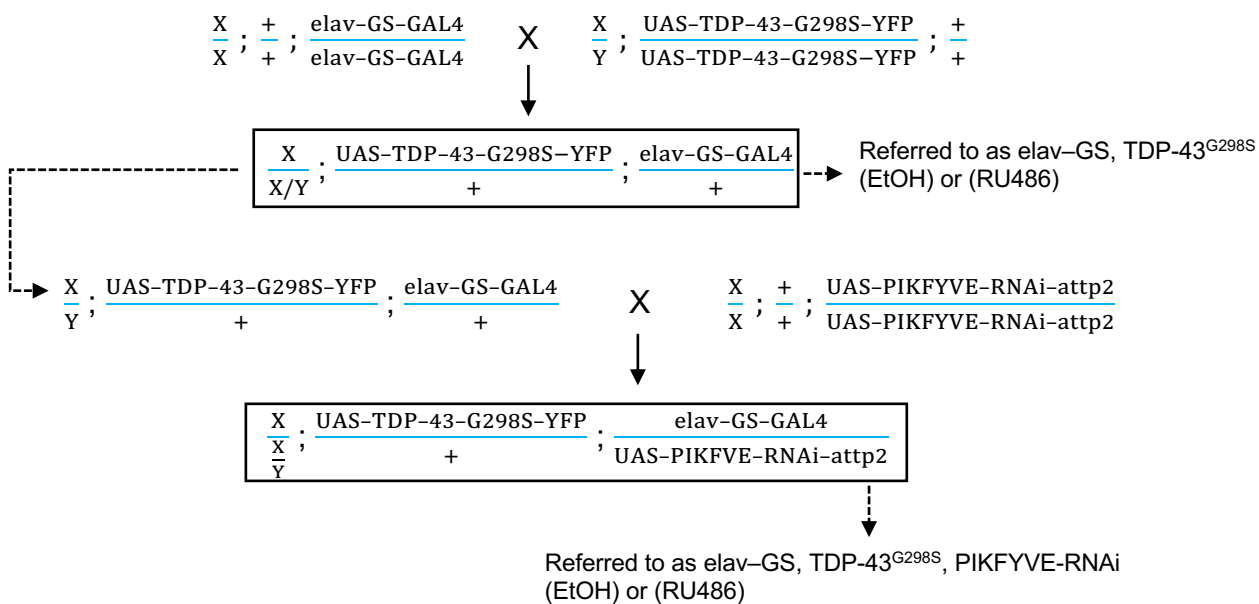




### Crosses used to generate progeny TDP-43<sup>WT</sup> genotypes



### Crosses used to generate progeny with mutant TDP-43<sup>G298S</sup> genotypes



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