Loss of Aly/ALYREF Suppresses Toxicity in Both Tau and TDP-43 Models of Neurodegeneration

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1 Loss of aly/ALYREF suppresses toxicity in both tau and TDP-43 models of neurodegeneration

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Abstract:

Background

Neurodegenerative diseases with tau pathology, or tauopathies, include Alzheimer's Disease and related dementia disorders. Previous work has shown that loss of the poly(A) RNA-binding protein gene *sut-2/MSUT2* strongly suppressed tauopathy in *C. elegans*, human cell culture, and mouse models of tauopathy. However, the mechanism of suppression is still unclear. Recent work has shown that MSUT2 protein interacts with the THO complex and ALYREF, which are components of the mRNA nuclear export complex. Additionally, previous work showed *ALYREF* homolog *Ref1* modulates TDP-43 and G₄C₂ toxicity in *D. melanogaster* models.

Methods

We used transgenic *C. elegans* models of tau or TDP-43 toxicity to investigate the effects of loss of ALYREF function on tau and TDP-43 toxicity. In *C. elegans*, there are three genes that are homologous to human *ALYREF*: *aly-1*, *aly-2*, and *aly-3*.

Results

We found that loss of *C. elegans aly* gene function, especially loss of both *aly-2* and *aly-3*, suppressed tau-induced toxic phenotypes. Loss of *aly-2* and *aly-3* was also able to suppress TDP-43-induced behavioral deficits. However, loss of *aly-2* and *aly-3* had divergent effects on mRNA and protein levels as total tau protein levels were reduced while mRNA levels were increased, but no significant effects were seen on total TDP-43 protein or mRNA levels.

Conclusions

48 Our results suggest that although *a/y* genes modulate both tau and TDP-43-induced toxicity phenotypes,
49 the molecular mechanisms of suppression are different and separated from impacts on mRNA and
50 protein levels. Altogether this study highlights the importance of elucidating RNA-related mechanisms in
51 both tau and TDP-43-induced toxicity.

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53 Keywords: tau, TAR DNA-binding protein 43, TDP-43, ALYREF, MSUT2, neurodegeneration

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Introduction

The microtubule-binding protein tau accumulates into abnormal aggregates in a number of age-associated neurodegenerative diseases called tauopathies[1]. Not only does the burden (extent) of tau tangles strongly correlates with cognitive decline in Alzheimer's disease [2–4], the most common dementia, multiple mutations in the *MAPT* gene, which encodes tau protein, have been directly linked with frontotemporal dementia with parkinsonism-17 [5]. However, there are no disease modifying pharmacological treatments for tauopathies and the molecular mechanisms underlying tauopathies are still unclear [6,7].

To discover novel genes and pathways that regulate tauopathy, our group previously generated a series of transgenic *C. elegans* disease models that express human 4R1N tau in all neurons [8]. Tau-induced phenotypes seen with this model include uncoordinated locomotion, accumulation of insoluble tau, neuron loss, and shortened life span [8]. Forward genetic screening in this model identified loss of function mutations in the RNA binding protein gene, *sut-2*, caused strong suppression of tau-induced toxicity in tau transgenic *C. elegans* [9]. This mechanism was shown to be conserved from *C. elegans* to mammalian models [10]. Additionally, removal of the RNA binding activity of MSUT2 is sufficient for tauopathy suppression [10]. The best-characterized functional consequence of MSUT2 knockdown is lengthening of global poly(A) mRNA tail lengths [11]; however, investigations into other poly(A) tail length regulators, such as poly(A) deadenylases, found that these proteins could act as enhancers or suppressors of tauopathy despite having similar protein functions [12]. Thus, while poly(A) tail length regulation is not the mechanism in which MSUT2 suppresses tauopathy, it is likely that the mechanism of action still relates to RNA binding and processing.

Another means of gaining insight into the molecular mechanisms by which *sut-2/MSUT2* loss of function suppresses tauopathy is to study the role of SUT-2/MSUT2 protein interactors on tauopathy. Recent work has demonstrated that MSUT2 interacts with the THO complex directly and ALYREF in a RNA-dependent manner [13]. While genetic knockout of *thoc* genes causes sterility and/or lethality in *C. elegans*, genetic ablation of the *aly* genes is tolerated, permitting their study in our transgenic *C. elegans* models.

ALYREF is a protein that couples the process of splicing mRNAs to nuclear export of mRNAs to the cytoplasm [14]. It primarily localizes to nuclear speckles but can shuttle between the nucleus and cytoplasm. While most literature has focused on ALYREF's role in nuclear export, other functions for ALYREF have been identified, including as a transcription factor/regulator [15], a splicing regulator [16], and a m⁵C reader [17]. As with MSUT2, additional ALYREF functions likely remain to be elucidated.

Interestingly, ALYREF has previously been linked to G₄C₂ hexanucleotide repeat and TAR DNA-binding protein 43 (TDP-43) toxicity in *Drosophila melanogaster* models for amyotrophic lateral sclerosis (ALS)-related toxicity [18–20]. The *D. melanogaster* homolog of ALYREF, *Ref1*, was first identified as a modifier of G₄C₂ repeat toxicity in an RNAi screen using a G₄C₂-58 repeat-induced eye degeneration *D. melanogaster* model [18]. A later study found that reducing *Ref1* levels in *D. melanogaster* not only suppressed G₄C₂-49 repeat-induced eye degeneration but TDP-43-induced eye degeneration as well [19]. Moreover, ALYREF and its homolog *Ref1* have been shown to be altered by changing G₄C₂ repeat or TDP-43 levels. Knockdown of TDP-43 in a human cell culture model caused ALYREF localization to shift more from the nucleus to the cytoplasm [20]. In *D. melanogaster* models, overexpression of G₄C₂ repeats or TDP-43 increased *Ref1* mRNA levels, and similarly ALYREF protein levels were increased in motor neurons from ALS patients with TDP-43 pathology [19]. Whether ALYREF may modify tau-induced

toxicity has not been thoroughly investigated; however, reduction of *Ref1* expression by RNAi did not alter tau protein or mRNA levels in a *D. melanogaster* tau model, which suggests that *Ref1* interacts differently with tau and TDP-43 [19].

To better understand how *sut-2/MSUT2* suppresses tauopathy and to clarify whether *aly/ALYREF* could suppress multiple neurodegeneration-related protein-caused toxicities, we examined the effects of loss of *aly* gene expression on tau or TDP-43-induced toxicity in our tau and TDP-43 transgenic *C. elegans* models. We found that loss of *aly* genes could suppress tau-induced behavioral deficits as well as TDP-43-induced behavioral deficits. However, the effects on pathological protein accumulation were different; loss of *aly* function significantly reduced tau protein accumulation with little effect on TDP-43 protein accumulation, suggesting *aly* genes modulate tau and TDP-43-induced toxicity through different mechanisms of action.

Materials and Methods

***C. elegans* strains, genome editing and transgenics.** N2 (Bristol) was used as wild type *C. elegans* [21]. *C. elegans* mutants were generated by CRISPR-Cas9 (described below) and crossed into the tau transgenic (tau Tg) *C. elegans* strain CK10 (*bkl10[aex-3p::tau-V337M+ myo-2p::GFP]*) expressing human 4R1N tau with a V337M mutation [8] or TDP-43 transgenic (TDP-43 Tg) *C. elegans* strain CK426 (*bkl426[snb-1p::TDP-43-A315T + myo-2p::dsRED]*) expressing human TDP-43 with an A315T mutation [22]. The presence of alleles of interest was verified with PCR genotyping. All non-transgenic and tau-transgenic strains were maintained at 20°C on standard nematode growth media (NGM) plates containing OP50 *Escherichia coli* as previously described [21], while TDP-43 transgenic strains were maintained at 15°C and moved to 20°C for behavior and protein studies. For protein studies, worms were grown on NGM

plates containing 5x more peptone (5XPEP) at 20°C for at least 2 generations (one week) prior to collection.

C. elegans alleles generated in this study are listed in Table 1. Two to three independent null alleles were generated for *aly-1*, *aly-2*, or *aly-3* either in N2 or *aly* mutant backgrounds as indicated in the table using CRISPR-Cas9 genome editing technology by introducing purified active recombinant Cas9 protein and synthetic CRISPR guide RNAs (gRNAs) as previously described [23].

S.p. crRNA name	Protospacer	Relative Position in gene
aly-1 null 5'	CTGAGATGTAACATTGAAAG	Promoter -80
aly-1 null 3'	TTATGAAAACATGAGAACAAACAAGTAAAA	~stop codon
aly-2 null 5'	AAAAGAAAATAACGACATAT	Promoter -170
aly-2 null 3'	GGAACCTTTTATTAGGAATC	3'UTR +282
aly-3 null 5'	TCCTCGTCGAGCTCCTCCGA	~start codon
aly-3 null 3'	GACCGAAGCTGAACTCGACG	~stop codon

Cas9 gRNAs were prepared by mixing equimolar amounts (0.5 ug/uL) of Alt-R crRNA and Alt-R tracrRNA (Integrated DNA Technologies) and then the gRNAs were complexed to equimolar quantities of Cas9 protein (Alt-R S.p. Cas9 Nuclease V3, Integrated DNA Technologies as described). *Dpy-10* or *rol-6* were utilized as a gene co-conversion or co-injection marker as previously described [24] and removed by outcrossing. For each genome editing effort, two gRNAs were introduced targeting the promoter/start codon and 3'UTR/stop codon sequences of the *aly-1*, *aly-2*, or *aly-3* genomic loci respectively to generate null alleles.

Behavior in *C. elegans*. Swimming assays were performed as follows. NGM plates containing tau transgenic *C. elegans* 4 days post-hatch (roughly day 1 of adulthood) or non-transgenic *C. elegans* 3 or 4 days post-hatch (day 1 or 2 of adulthood) were flooded with 1 mL of M9 buffer. Swimming worms were then pipetted with a P1000 pipette onto a 35 mm unseeded NGM plate. Videos of swimming worms were recorded for 1 minute at 14 frames per second beginning 1 minute following the addition of M9 buffer. Recorded videos were analyzed using WormLab 2019 (MBF Bioscience). The frequency of body bends, defined as “turns” by the software, was quantified as follows. A turn was defined as a change in the body angle defined by the quarter points and midpoint of the worm that was at least 20 degrees positive or negative from a straight line. Worms that were tracked for less than 30 seconds were omitted from analysis. At least 100 worms from at least 3 independent samples were counted per strain.

Radial locomotion assays were performed as follows. 14-19 L4 stage worms were placed at the center of a 10 cm 5XPEP plate, as marked by a dot. They were allowed to disperse for 1 hour and the final location of each worm was marked. The assay plate was imaged with a scanner and the distance between the center dot and each worm’s final position was measured using ImageJ. At least 6 plates total were assayed on 3 separate occasions for a total of 90+ worms/strain.

Tau extraction and Immunoblotting. Staged young adult tau transgenic *C. elegans* were grown from eggs at 20°C for 3 days on 5XPEP plates, washed off plates in M9 buffer, and collected by centrifugation. Worms were snap frozen in liquid nitrogen and stored at -80°C. Tau fractions were obtained as described previously [12]. 2 µL of RAB Hi-Salt buffer (0.1 M MES, 1 mM EGTA, 0.5 mM MgSO₄, 0.75 M NaCl, 0.02 M NaF, pH 7.0) containing PMSF and protease inhibitors was added per mg of worm pellet and homogenized by sonication. A portion of the sample was saved for immunoblotting (for total tau, tubulin, SUT-2) while the rest was centrifuged at 40,000 x g, 4°C for 40 min. The supernatant was saved

as the RAB/soluble fraction. The pellet was extracted by adding 1 μ L of RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 0.5% deoxycholate, 0.1% SDS, pH 8.0) containing PMSF and protease inhibitors per mg of original worm pellet weight and centrifuged at 40,000 x g, 4°C for 20 min. The supernatant was saved as the RIPA/detergent soluble fraction. The pellet was extracted again by adding 2 μ L 70% formic acid (FA) per mg of original worm pellet weight and centrifuged at 13,000 x g, 4°C for 15 min. The supernatant was the FA/detergent insoluble fraction. This fraction was dried in a speed vacuum concentrator until only a dry film remained in the tube.

Protein samples were diluted with 5x sample buffer (0.046 M Tris, 0.005 M EDTA, 0.2 M dithiothreitol, 50% sucrose, 5% sodium dodecyl sulfate, 0.05% bromophenol blue), boiled for 5 min., and centrifuged at 13,000 x g for 2 min. prior to being loaded onto 26-well 4-15% pre-cast Criterion sodium dodecyl sulfate polyacrylamide gel electrophoresis gradient gels and transferred to PVDF membranes as recommended by the manufacturer (Bio-Rad). Total protein samples were diluted 2-fold with 5x sample buffer. FA/insoluble fractions were resuspended with 2x sample buffer at 2 μ L per mg of original worm pellet weight. All other protein samples were diluted 5-fold with 5x sample buffer. 5-20 μ L of diluted sample was loaded for each analysis. Primary antibodies used were rabbit monoclonal anti-tau antibody (Rockland) at 1:5000, rabbit polyclonal anti-SUT-2 antibody RB841 at 1:2000, and mouse anti-tubulin antibody E7 (Developmental Studies Hybridoma Bank) at 1:5000. Secondary antibodies used were anti-rabbit HRP (Jackson Immuno Research) and anti-mouse HRP (Jackson Immuno Research) at 1:5000. ECL substrate (Bio-Rad) was added to the membrane and chemiluminescence signals were detected with a LICOR Odyssey Fc.

TDP-43 Immunoblotting. Staged young adult TDP-43 transgenic *C. elegans* were grown from eggs at 20°C for 3 days on 5XPEP plates, washed off plates in M9 buffer, and collected by centrifugation. Worms

were snap frozen in liquid nitrogen and stored at -80°C. 4 µL of 1x sample buffer (0.0092 M Tris, 0.001 M EDTA, 0.04 M dithiothreitol, 10% sucrose, 1% sodium dodecyl sulfate, 0.01% bromophenol blue) was added per mg of worm pellet and homogenized by sonication. Lysates were then boiled for 5 minutes and centrifuged at 13,000 x g for 2 minutes prior to being loaded onto 26-well 4-15% pre-cast Criterion sodium dodecyl sulfate polyacrylamide gel electrophoresis gradient gels and transferred to PVDF membranes as recommended by the manufacturer (Bio-Rad). 5 µL of diluted sample was loaded for each analysis. Primary antibodies used were mouse monoclonal anti-TDP-43 antibody ab57105 (Abcam) at 1:2500, mouse monoclonal anti-pS409/10 TDP-43 antibody TIP-PTD-M01 (Cosmobio) at 1:2000, rabbit polyclonal anti-SUT-2 antibody RB841 at 1:2000, and mouse anti-tubulin antibody E7 (Developmental Studies Hybridoma Bank) at 1:5000. Secondary antibodies used were anti-rabbit HRP (Jackson Immuno Research) and anti-mouse HRP (Jackson Immuno Research) at 1:5000. ECL substrate (Bio-Rad) was added to the membrane and chemiluminescence signals were detected with a LICOR Odyssey Fc.

GABAergic neuron loss assay. tau Tg;*aly-2(bk3079);aly-3(bk3069)* worms were crossed into CZ1200 (*juls76 [unc-25p::GFP + lin-15(+)]*) to generate tau Tg;*unc-25p::GFP* and tau Tg;*unc-25p::GFP; aly-2(bk3079);aly-3(bk3069)* worms. Worms were synchronized by timed egg lays and mounted at 4 or 7 days post-hatch on 2% agarose pads containing a droplet of 0.1% sodium azide as a paralytic. The number of visible GABAergic neurons in the ventral nerve cord were counted with a DeltaVision microscope (Applied Precision) using 60X magnification. At least 10 animals were counted per strain per replicate, and 3 replicates were done per time point.

Quantification of mRNA levels of target genes in *C. elegans*. Staged young adult tau transgenic *C. elegans* were grown from eggs at 20°C for 3 days on 5XPEP plates, washed off plates in M9 buffer, and collected by centrifugation. Worms were snap frozen in liquid nitrogen and stored at -80°C. RNA was

isolated from *C. elegans* pellets of ~50-100 mg using Tri-Reagent (Molecular Research Center) following the manufacturer's instructions and resuspended in RNase-free water. RNA was treated with DNase using DNA-free™ DNase Treatment and Removal Kit (Invitrogen) following the manufacturer's instructions. RNA was transcribed into cDNA using iScript Reverse Transcription Supermix (Bio-Rad) following the manufacturer's instructions. Quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) in a CFX Connect Real-Time PCR System (Bio-Rad) with primers against *rpl-32* (control), *tau*, *aly-1*, *aly-2*, and/or *aly-3*. Data was analyzed using CFX Manager 3.0 (Bio-Rad). Experimental samples were run in triplicate for each target.

Target gene	Forward primer	Reverse primer
<i>rpl-32</i>	GGTCGTCAAGAAGAAGCTCACCAA	TCTGCGGACACGGTTATCAATTCC
<i>tau</i>	GTGTGGCTCATTAGGCAACATCC	CGTTCTCGCGGAAGGTCAG
TDP-43	TGAGCCTTTGAGAAGCAGAA	ATCCATCACATCCCCGTACT

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8. Behavior, immunoblot, neuron loss, and relative mRNA measurement data with just two groups was analyzed by Student's t-test. Behavior and immunoblot data with three or more groups was analyzed by one-way ANOVA followed by post-hoc multiple comparison testing.

Results

Loss of *aly* genes suppress tau-induced behavioral deficits

Although previous work had concluded that *ALYREF* did not modulate tauopathy because it did not alter total tau protein levels [19], we have previously seen that suppression of tau toxicity can occur without significant alterations to tau protein levels [9,12,25,26]. Therefore we wanted to reexamine whether *ALYREF* could modulate tauopathy using our tau transgenic *C. elegans* model, in which human 4R1N tau carrying the V337M mutation is expressed pan-neuronally [8]. There are three *aly* genes in *C. elegans*: *aly-1*, *aly-2*, and *aly-3*. To investigate a possible role for these *aly* genes in tauopathy, we generated *C. elegans* strains containing whole-gene deletions for one or two *aly* genes via CRISPR/Cas9 (Table 1, Fig. 1). Attempts to generate an *aly-1;aly-2;aly-3* triple knockout were unsuccessful, suggesting that loss of all three genes is lethal or sterile. We then crossed these CRISPR-generated *C. elegans* strains with tau transgenic *C. elegans* and performed swimming assays to determine if loss of one or two *aly* genes affected tau-induced behavioral deficits (Fig. 2).

Chromosome IV

Figure 1: *C. elegans aly* genes and alleles used in this study. Symbols are used to indicate the approximate location of each *aly* gene on *C. elegans* Chromosome IV. The genetic location is also listed for each gene. Due to the close proximity of *aly-1*, *aly-2*, and *aly-3* in the *C. elegans* genome, CRISPR/Cas9 was employed to introduce deletions of multiple *aly* genes, when desired, in a sequential manner (see Table 1). Gene exon/intron diagrams are adapted from Wormbase.org. The brackets underneath each exon diagram indicate the sequences that were targeted for deletion.

Table 1 – List of *C. elegans aly* strains used in this study

Strain name	<i>C. elegans</i> alleles (genes deleted)	Generated on
CK3029	<i>aly-1(bk3029)</i>	N2 (wild-type)
CK3030	<i>aly-1(bk3030)</i>	N2
CK3031	<i>aly-1(bk3031)</i>	N2
CK3032	<i>aly-2(bk3032)</i>	N2
CK3033	<i>aly-2(bk3033)</i>	N2
CK3050	<i>aly-1(bk3050);aly-2(bk3031)</i>	CK3031

CK3051	<i>aly-1(bk3051);aly-2(bk3032)</i>	CK3032
CK3069	<i>aly-3(bk3069)</i>	N2
CK3070	<i>aly-3(bk3070)</i>	N2
CK3079	<i>aly-2(bk3079);aly-3(bk3069)</i>	CK3069
CK3080	<i>aly-2(bk3080);aly-3(bk3069)</i>	CK3069
CK3098	<i>aly-1(bk3030);aly-3(bk3098)</i>	CK3030

Figure 2: Loss of *aly* genes on tau-induced behavior deficits in tau transgenic *C. elegans*. Worms 4 days post-hatch (approximately day 1 adult for tau transgenic, day 2 adult for non-transgenic) were recorded swimming in M9 Buffer for 1 minute, and the turning frequency was determined for each worm. A total

262 of 82-126 worms were analyzed per strain from 3 different replicates. Significance for all data except for
263 the non Tg data was determined by one-way ANOVA followed by multiple comparisons (Dunnett) with
264 control tau transgenic strain. Bars are means $\pm$ S.E.M. *** $p < 0.001$ **** $p < 0.0001$ of tau transgenic
265 mutants vs tau transgenic (tau Tg).

266

267 We found that loss of either *aly-1* or *aly-2* improved swimming ability of tau transgenic *C. elegans*
268 approximately 2-fold while loss of *aly-3* did not significantly improve swimming ability. Interestingly, loss
269 of two *aly* genes did not result in additive effects on suppression. Loss of both *aly-1* and *aly-2* caused
270 similar levels of suppression as loss of either *aly-1* or *aly-2* alone, while loss of both *aly-1* and *aly-3* or
271 *aly-2* and *aly-3* caused a synergistic improvement in swimming of tau transgenic *C. elegans*. This was not
272 a complete rescue in swimming ability, as non-transgenic *C. elegans* still swim significantly better than
273 these tau transgenic *C. elegans* mutants. Loss of *aly* genes caused either no significant or slightly
274 worsened swimming ability in non-transgenic *C. elegans*, indicating that the differences in suppression
275 of swimming ability seen in tau transgenic *C. elegans* is due to modulation of tau-induced deficits and
276 not hyperactivity caused by *aly* mutations independently (Additional File 1: Fig. S1).

Figure S1: Loss of *aly* genes on swimming behavior of non-transgenic *C. elegans*. 3-day-old worms (approximately day 1 adult) were recorded swimming in M9 Buffer for 1 minute, and the turning frequency was determined for each worm. 80-110 worms total were analyzed per strain from 3 different replicates. Significance was determined by one-way ANOVA followed by multiple comparisons (Dunnett) with control non-transgenic wildtype strain. Bars are means $\pm$ S.E.M. * $p < 0.05$ ** $p < 0.005$ **** $p < 0.0001$ vs non-transgenic (non-Tg).

Loss of *aly-2* and *aly-3* reduces total and insoluble forms of tau, but increases tau mRNA levels

We next investigated whether loss of *aly* function had effects on tau protein. Although loss of *aly-1* and *aly-3* had the overall strongest suppression of tau-induced behavioral deficits, we had two alleles of *aly-2* and *aly-3* that showed similar effects on tau-induced behavioral deficits, supporting the idea that the effect seen was not due to background effects. Therefore, we focused further characterization of *aly* loss on tau phenotypes on loss of *aly-2* and *aly-3*. While previous work in a TDP-43 *D. melanogaster* model showed that RNAi against *aly* homolog *Ref1* caused no change in total tau protein levels [19], the use of RNAi, which only reduced *Ref1* mRNA ~40%, instead of complete gene knockouts, may lessen effects on tau protein. Since loss of the related gene *sut-2* causes significant decreases in insoluble tau [9], we tested whether loss of *aly-2* and *aly-3* would have a similar effect on tau protein. We found that loss of *aly-2* and *aly-3* caused a significant decrease in both total and insoluble tau (Fig. 3A-C, Additional File 2: Fig. S2). The significant decrease in insoluble tau may be driven by the decrease in total tau protein.

RNAi against *aly* homolog *Ref1* in *D. melanogaster* models reduced toxic TDP-43 or G4C2-repeat protein levels with accompanying decreases in mRNA levels [19]. Therefore, we determined whether the decrease in total tau protein seen with loss of *aly-2* and *aly-3* might be due to reduced tau mRNA levels. Using quantitative RT-PCR, we saw the opposite effect; relative tau mRNA levels were significantly increased with loss of *aly-2* and *aly-3* gene expression in tau transgenic *C. elegans* (Fig. 3D), suggesting that reduction of tau protein with loss of *aly-2* and *aly-3* is not simply a reflection of tau mRNA levels.

Figure 3: Loss of *aly-2* and *aly-3* on tau levels in tau transgenic *C. elegans*. Sequential extractions of tau protein using buffers of increasing solubilizing strength were performed on tau transgenic (tau Tg) and tau Tg;*aly-2*(*bk3079*);*aly-3*(*bk3069*) worm lysates, starting with RAB high-salt buffer, followed by detergent-containing RIPA buffer, and finally 70% formic acid (FA). Tubulin was detected in the total lysate as a loading control. [A] Representative immunoblots for total, FA (or insoluble) tau, and tubulin. [B] Quantification of total tau/tubulin. 7 replicates of tau Tg and tau Tg;*aly-2*; *aly-3* were quantified and significance was determined by Student's t-test ($p = 0.032$). [C] Quantification of FA tau/total tau. 6 replicates of tau Tg and tau Tg;*aly-2*; *aly-3* were quantified and significance was determined by Student's t-test ($p = 0.20$). [D] Quantitative RT-PCR for tau mRNA in tau Tg and tau Tg;*aly-2*(*bk3079*);*aly-3*(*bk3069*) *C. elegans*. Tau mRNA levels were normalized to *rpl-32*. 5 replicates of tau Tg and tau Tg;*aly-2*; *aly-3*

318 were quantified and significance was determined by Student's t-test ($p = 0.0047$). For all graphs, bars are
 319 means $\pm$ S.E.M. * $p < 0.05$ compared to tau transgenic ** $p < 0.005$ compared to tau transgenic.

KEY: 1 = tau Tg 2 = tau Tg;aly-2(bk3079);aly-3(bk3069) 3 = tau Tg;aly-2(bk3080);aly-3(bk3069)

320
 321 **Figure S2: Total tau, RAB-soluble tau, RIPA-soluble tau, insoluble (formic acid) tau, and tubulin**
 322 **(loading control) immunoblots.** Immunoblots for all 7 replicates quantified in Figure 3B and 6 replicates

quantified in Figure 3C, including immunoblotting of a second tau transgenic;*aly-2*;*aly-3* triple mutant. Sequential extractions of tau protein using buffers of increasing solubilizing strength were performed on tau transgenic worm lysates, starting with RAB high-salt buffer, followed by detergent-containing RIPA buffer, and finally 70% formic acid (FA). No sequential extraction was performed on the last/rightmost set.

Loss of *aly-2* and *aly-3* reduces tau-induced neuron loss

To investigate whether loss of *aly-2* and *aly-3* could modulate tau-induced neuron loss, we crossed our tau transgenic *C. elegans* with or without loss of *aly-2* and *aly-3* with a *C. elegans* strain that expresses green fluorescent protein in all GABAergic motor neurons and quantified the number of GABAergic motor neurons present at 4 days post-hatch (roughly day 1 of adulthood) and 7 days post-hatch (Fig. 4). Although we saw no significant differences in the number of neurons lost at 4 days post-hatch, we found that loss of *aly-2* and *aly-3* significantly protected against neuron loss seen at 7 days post-hatch.

Figure 4: Loss of *aly-2* and *aly-3* on GABAergic motor neuron loss in tau transgenic *C. elegans*. Using a GFP reporter to mark GABAergic motor neurons, the number of neurons were counted in tau transgenic or tau Tg;*aly-2(bk3079);aly-3(bk3069)* *C. elegans* at 4 and 7 days post-hatch (roughly day 1 and day 4 of adulthood). Dots indicate counted neurons while arrows indicate missing neurons. [A] Representative image of *unc-25::GFP*, the GABAergic motor neuron reporter strain, at 7 days old. [B] Representative image of tau Tg;*unc-25::GFP* at 7 days old. [C] Representative image of tau Tg;*unc-25::GFP;aly-2(bk3079);aly-3(bk3069)* at 7 days old. [D] Quantification of neuron loss. 3 replicates for a total of 35-39 worms per strain per time point were counted. Significance was determined by Student's t-test. 4 days old $p = 0.049$. 7 days old $p < 0.0001$. Bars are means $\pm$ S.E.M.

Loss of *aly-2* and *aly-3* suppresses TDP-43-induced behavioral deficits but does not significantly alter TDP-43 protein or mRNA levels

Because our investigation into the role of *aly* genes in tau-induced toxicity differed from previously published work [19], we wanted to determine whether or not *aly* genes modified TDP-43 induced toxicity as previously published. Since loss of *aly-2* and *aly-3* had one of the strongest effects on tau-induced toxicity, we investigated whether loss of *aly-2* and *aly-3* also had a significant effect on TDP-43-induced toxicity in a transgenic TDP-43 *C. elegans* model in which human TDP-43 carrying the A315T mutation is expressed pan-neuronally [22]. This TDP-43 transgenic worm model will not thrash in a swimming assay. Therefore, we used a different motility assay, radial locomotion, which quantifies movement on solid agar. With this assay, we found a significant increase in dispersion of TDP-43 transgenic *C. elegans* lacking *aly-2* and *aly-3* compared to controls (Fig. 5A) that was not due to hyperactivity (Fig. 5B), indicating that loss of *aly-2* and *aly-3* ameliorates the TDP-43-induced locomotor deficit.

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365 **Figure 5: Loss of *aly-2* and *aly-3* on TDP-43-induced behavior deficits in TDP-43 transgenic *C. elegans*.**

366 Radial locomotion assays were performed on L4-stage worms and the distance traveled from the center
 367 of the assay plate was measured after 1 hour. 90-96 total worms from 3 different replicates were
 368 analyzed per strain. [A] Radial locomotion assay on non-transgenic, TDP-43 transgenic (TDP-43 Tg), and
 369 TDP-43 Tg;*aly-2(bk3079);aly-3(bk3069)* *C. elegans*. [B] Radial locomotion assay on wild type non-
 370 transgenic and non-transgenic *aly-2(bk3079);aly-3(bk3069)* *C. elegans*. For [A], significance was
 371 determined only between TDP-43 transgenic *C. elegans* and TDP-43 tg;*aly-2(bk3079);aly-3(bk3069)* *C.*
 372 *elegans* by Student's t-test ($p < 0.0001$). For [B], significance was determined by Student's t-test ($p =$
 373 0.57). Bars are means $\pm$ S.E.M. **** $p < 0.0001$.

374

375 To determine whether loss of *aly-2* and *aly-3* might suppress any toxic neuronal protein accumulation
 376 phenotype, we examined the loss of *aly-2* and *aly-3* on a polyglutamine-induced neuronal toxicity *C.*
 377 *elegans* model, in which there is pan-neuronal expression of Q86::YFP, or 86 consecutive glutamine
 378 residues fused to yellow fluorescent protein [27]. We found no significant differences in swimming

ability between Q86::YFP with or without *aly-2* and *aly-3* (Fig. 6), suggesting that loss of *aly-2* and *aly-3* causes suppression of toxic neuronal protein accumulation in a pathway shared by tau and TDP-43 but not by polyQ.

Figure 6: Loss of *aly-2* and *aly-3* on polyQ-induced behavior deficits in Q86::YFP transgenic *C. elegans*.

Worms 4 days post-hatch (approximately day 1 adult) were recorded swimming in M9 Buffer for 1 minute, and the turning frequency was determined for each worm. Q86::YFP worms and Q86::YFP worms lacking *aly-2* and *aly-3* (Q86::YFP;*aly-2*(*bk3079*);*aly-3*(*bk3069*)) from 3 different replicates (total n of 111 and 107 respectively) were analyzed. Significance was determined by Student's t-test ($p = 0.42$). Bars are means $\pm$ S.E.M.

We next examined whether loss of *aly-2* and *aly-3* affected TDP-43 protein. Previous work in the TDP-43 *D. melanogaster* model showed that RNAi against *aly* gene homolog *Ref1* decreased total TDP-43 protein and mRNA levels [19]. In contrast, we found that loss of *aly-2* and *aly-3* did not significantly change TDP-43 total or phosphorylated protein levels (Fig. 7A-C, Additional File 3: Fig. S3), although changes in total TDP-43 protein were trending as decreased. This not only differs from the results seen

in *D. melanogaster* [19] but also differs from the effect loss of *aly-2* and *aly-3* had on tau protein in the tau transgenic *C. elegans* model.

Because we saw an increase in tau mRNA levels despite a decrease in tau protein levels with loss of *aly-2* and *aly-3* in tau transgenic *C. elegans*, we investigated the effect of loss of *aly-2* and *aly-3* on TDP-43 mRNA levels in TDP-43 transgenic *C. elegans*. We observed a trending but not significant increase in TDP-43 mRNA levels in TDP-43 transgenic *C. elegans* with loss of *aly-2* and *aly-3* (Fig. 7D). Altogether, this shows that unlike with tau, loss of *aly-2* and *aly-3* does not cause significant changes in protein and mRNA levels, although the effects are trending in the same direction.

A

B

C

D

Figure 7: Loss of *aly-2* and *aly-3* on TDP-43 levels in TDP-43 transgenic *C. elegans*. Tubulin was detected in the total lysate as a loading control. [A] Representative immunoblots for total, phosphorylated (S409/S410) TDP-43, and tubulin in TDP-43 transgenic (TDP-43 Tg) and TDP-43 Tg;*aly-2*(*bk3079*);*aly-3*(*bk3069*) *C. elegans*. [B] Quantification of total TDP-43/tubulin. 6 replicates of TDP-43 Tg and TDP-43 Tg;*aly-2*; *aly-3* were quantified and significance was determined by Student's t-test ($p = 0.15$). [C] Quantification of phosphorylated TDP-43/total TDP-43. 6 replicates of TDP-43 Tg and TDP-43 Tg;*aly-2*; *aly-3* were quantified and significance was determined by Student's t-test ($p = 0.35$). [D] Quantitative RT-PCR for TDP-43 mRNA in TDP-43 Tg and TDP-43 Tg;*aly-2*(*bk3079*);*aly-3*(*bk3069*) *C. elegans*. TDP-43 mRNA levels were normalized to *rpl-32*. 3 replicates for each strain were quantified and significance was determined by Student's t-test ($p = 0.46$). For all graphs, bars are means $\pm$ S.E.M.

KEY: 1 = TDP-43 Tg 2 = TDP-43 Tg;*aly-2*(*bk3079*);*aly-3*(*bk3069*)

Figure S3: TDP-43 immunoblots: phosphorylated (pS409/S410) TDP-43, total TDP-43, and tubulin (loading control). Immunoblots for all 6 replicates quantified in Figure 7B-C.

Loss of *aly-2* and *aly-3* does not suppress toxicity by affecting SUT-2 expression

Since the protein products of *ALYREF* and *MSUT2*, the mammalian homologs of the *aly* genes and *sut-2*, have been shown to interact in a complex [13], we hypothesized that loss of *aly* genes might lead to suppression of toxicity by altering SUT-2 expression levels. Additionally, the differences we observed in tau and TDP-43 protein regulation could be explained by a *sut-2*-dependent mechanism, since complete deletion of *sut-2* suppresses tau protein levels greatly but not TDP-43 (personal communication – Nicole Liachko). However, when we measured SUT-2 protein in tau or TDP-43 transgenic *C. elegans* with or without *aly-2* and *aly-3*, we found SUT-2 levels were highly variable between replicates and not consistently altered with loss of *aly-2* and *aly-3* (Fig. 8, Additional File 4: Fig. S4). This suggests that the mechanism in which loss of *aly* genes suppress tau or TDP-43 toxicity is not via changes in SUT-2 expression levels.

A

B

C

D

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Figure 8: Loss of *aly-2* and *aly-3* on SUT-2 levels in tau or TDP-43 transgenic *C. elegans*. Tubulin was

437

detected in the total lysate as a loading control. [A] Representative immunoblots for total tau, SUT-2,

438

and tubulin in tau transgenic *C. elegans* with or without *aly-2* and *aly-3* genes (tau Tg;*aly-2*(*bk3079*);*aly-*

439

3(*bk3069*)). [B] Quantification of total SUT-2/tubulin in tau transgenic *C. elegans*. 7 replicates of each

440

strain were quantified and significance was determined by Student's t-test ($p = 0.28$). [C] Representative

441

immunoblots for total TDP-43, SUT-2, and tubulin in TDP-43 *C. elegans* with or without *aly-2* and *aly-3*

442

genes (TDP-43 Tg;*aly-2*(*bk3079*);*aly-3*(*bk3069*)). [D] Quantification of total SUT-2/tubulin in TDP-43

443

transgenic *C. elegans*. 6 replicates of each strain were quantified and significance was determined by

444

Student's t-test ($p = 0.15$). For all graphs, bars are means $\pm$ S.E.M.

445

KEY: 1 = tau Tg 2 = tau Tg;*aly-2*(bk3079);*aly-3*(bk3069) 3 = tau Tg;*aly-2*(bk3080);*aly-3*(bk3069)

KEY: 1 = TDP-43 Tg 2 = TDP-43 Tg;*aly-2*(bk3079);*aly-3*(bk3069)

446

447 **Figure S4: SUT-2, tau or TDP-43, and tubulin (loading control) immunoblots.** These are for all 6-7

448 replicates quantified in Figure 8C-D as well as for an additional tau Tg;*aly-2*;*aly-3* triple mutant.

Discussion

Our investigation into the role of *aly*/*ALYREF* in both tau and TDP-43 proteinopathies has shown that *aly*/*ALYREF* modulates both tau and TDP-43-induced toxicity, although it has different effects on mRNA expression and protein accumulation. While significant improvement in locomotor behavior was observed with loss of *aly* genes in both tau and TDP-43 transgenic *C. elegans* models, loss of *aly-2* and *aly-3* caused over a 2-fold decrease in tau protein levels but no significant decreases in TDP-43 protein levels, while causing a ~1.9-fold increase in tau mRNA levels and no significant increase in TDP-43 mRNA levels. Although loss of *aly-2* and *aly-3* in TDP-43 transgenic *C. elegans* resulted in changes that trended in similar directions as that seen in tau transgenic *C. elegans*, the fact that we did not observe significant changes in TDP-43 protein and mRNA levels despite a significant effect on locomotor behavior suggests that amelioration of TDP-43-induced locomotor deficits by loss of *aly* genes does not require significant changes in TDP-43 protein levels. Instead, it may act through changes TDP-43 protein functionally (such as its solubility, aggregation, cellular localization) in a way that causes less toxicity and/or ameliorates the downstream toxicity induced by TDP-43.

Interestingly, the opposite result on tau and TDP-43 protein levels was observed in *D. melanogaster* models of tau and TDP-43 toxicity [19]. RNAi knockdown against *Ref1*, the *D. melanogaster* homolog of *aly*/*ALYREF*, caused no significant changes in tau mRNA or protein levels while decreasing TDP-43 mRNA and protein levels significantly. It is possible that the differences in our results versus their results are due to our use of genetic null alleles in this study versus their use of RNAi, which causes partial knockdown of the targeted gene. Another possibility is that loss of *aly* genes in *C. elegans* triggers a different signaling pathway leading to suppression than loss of *Ref1* in *D. melanogaster*. Nevertheless,

the ability of *aly*/ALYREF to suppress neurodegeneration, especially that caused by TDP-43, appears to be conserved across species even if the mechanism may not be exactly the same.

Indeed, the reported activities of *aly*/ALYREF in the literature appear mixed. While the ability of ALYREF to modulate nuclear export of mRNAs has been shown to be conserved across species, the literature seems contradictory on whether ALYREF or its homologs are required for proper nuclear export of mRNAs. In *C. elegans*, RNAi knockdown of *aly* gene expression resulted in no change in poly(A) accumulation or splicing of mRNAs [28,29], however deletion of *aly-2* increased poly(A) accumulation [30]. Likewise, depletion of REF1 suggests that *Ref1* is not required for nuclear export in *D. melanogaster* [31]. However, in yeast and humans, ALYREF appears to be required for nuclear export [32–34].

Not only may there be divergent activities of the various *aly*/ALYREF homologs between species, by using genetic knockouts of specific combinations of *aly* genes in *C. elegans*, we may have identified distinct roles for *aly-1* and *aly-2* compared with *aly-3*. Loss of *aly-1* or *aly-2* alone suppresses tau toxicity but loss of *aly-3* does not. Additionally, we found that loss of *aly-1* and *aly-2* was redundant in suppression of tau toxicity but loss of both *aly-1* and *aly-3* or both *aly-2* and *aly-3* was synergistic. The fact that *aly-1* and *aly-2* suppress tau-induced toxicity while *aly-3* does not is surprising, since there are no obvious differences in homology to ALYREF. All ALY proteins share ~30% identity to the C-terminal half of ALYREF and all show nuclear expression [35]. Not much is known about *aly-3*; the only reported finding so far is that it appears, unlike *aly-1* and *aly-2*, to not be involved in retention of *tra-2* mRNA in *tra-1(null)* *C. elegans* [36]. Further investigation into *aly-3* function specifically may be warranted to understand how it can synergistically improve suppression of tau and TDP-43 toxicity by *aly-1* or *aly-2*.

496 Because *ALYREF* and *MSUT2* have been shown to interact in human cells [13], we had wondered if the
 497 effect of *aly* genes on tau and TDP-43 might be mediated by changes in *sut-2*. The fact that *SUT-2*
 498 protein levels did not change with loss of *aly-2* and *aly-3* suggests this is not the mechanism in which *aly*
 499 genes modulate tau and TDP-43-induced toxicity. However, amelioration of toxicity, especially of tau-
 500 induced toxicity, might occur via some shared mechanisms. Interestingly, when we compare loss of *aly*
 501 genes to loss of *sut-2* in *C. elegans* models of tau toxicity, loss of *sut-2* has a stronger effect, causing
 502 nearly complete suppression of tau-induced toxicity [9], while loss of *aly-1* or *aly-2* and *aly-3* lead to
 503 ~70% suppression at most. In contrast, loss of *sut-2* does not have a significant effect on TDP-43
 504 (personal communication – Nicole Liachko) whereas loss of *aly-2* and *aly-3* does significantly suppress
 505 TDP-43-induced toxicity. This suggests that, in the case of tau, tau suppression is greater with loss of *sut-*
 506 2 either because loss of *aly* activates the same pathways less strongly and/or loss of *sut-2* activates
 507 additional pathways that loss of *aly* does not. In contrast, in the case of TDP-43, TDP-43 suppression is
 508 likely activated by loss of *aly* via pathways not activated by loss of *sut-2*. One possible pathway may be
 509 via the regulation of splicing, which both *ALYREF* and TDP-43 have shown involvement in [14,37,38],
 510 with disease-causing mutations or loss of function in TDP-43 causing aberrant splicing patterns [38,39].
 511
 512 The *aly/ALYREF* and *sut-2/MSUT2* genes are just two of numerous RNA binding proteins that have been
 513 linked to neurodegenerative diseases of aging. Other RNA binding proteins that have been shown to
 514 modulate tau-related neurodegeneration include Musashi, PABPN1, SRRM2, TIA1, TOE1, and U1-70K
 515 [10,12,40–44]. Additionally, expression of numerous RNA binding proteins, such as ones located in
 516 nuclear speckles and ones involved in RNA splicing, has been shown to change significantly in
 517 Alzheimer’s disease compared to controls [45]. This finding is consistent with the observation that RNA
 518 potently stimulates aggregation of tau [46] and that tau aggregates interact with RNA [47,48]. TDP-43
 519 and other RNA-binding proteins like FUS, HNRNPs, Ataxin-2, etc., have mutations that cause pathological

protein aggregation and neurodegenerative diseases, especially ALS [49–51]. Moreover, there is increasing evidence that TDP-43 and tau, when they overlap in disease-affected brain regions, lead to more severe neurodegenerative phenotypes from enhanced tau-induced toxicity [52,53]. Since synergistic toxicity was demonstrated in transgenic models expressing tau and TDP-43 cDNA [53], aberrant splicing by TDP-43 does not seem to be a necessary driver of synergistic toxicity. One possible mechanism instead could be that aberrant control of tau mRNA in the presence of toxic TDP-43 leads to an increase in toxic tau and RNA interactions. Further studies will be necessary to elucidate the mechanisms of synergistic toxicity.

Conclusions

The identification of *aly/ALYREF* as a suppressor of both tau- and TDP-43-induced toxicity suggests there may be common, RNA-related pathways that modulate toxicity caused by proteins. Moreover, with tau, TDP-43, and other RNA-binding or interacting proteins either causing or facilitating neurodegeneration, elucidating the mechanisms in which these RNA-related proteins modulate neurodegeneration may substantially increase our understanding of the genesis and/or progression of these diseases. Thus a gene like *aly/ALYREF*, with its ability to suppress both tau and TDP-43-induced toxicity, merits further investigation, as we may not just elucidate mechanisms of tau-induced toxicity but of TDP-43-induced toxicity and synergistic toxicity.

List of abbreviations

ALS = Amyotrophic Lateral Sclerosis
gRNAs = guide RNAs
NGM = nematode growth media

544 non Tg = non-transgenic
545 TDP-43 = TAR DNA-binding protein 43
546 Tg = Transgenic
547

548 **Declarations**

549

550 Availability of data and materials

551 The data generated during this study is included in the published article and linked supplementary files.

552

553 Competing interests

554 The authors declare that they have no competing interests.

555

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560

561 Authors' contributions

562 Conceptualization: BK, RK. Methodology: AB, AS, BK, NL, RK. Resources: AB, AS, BK, NL, RK. Performing

563 experiments: AB, RK. Data analysis: AB, RK. Visualization: AB, RK. Supervision: RK, BK; Funding

564 Acquisition: RK, BK; Project Administration: RK, BK. Writing – Original Draft: BK, RK. Writing – Revision

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574

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