

## ORIGINAL ARTICLE

# Nuclear localized C9orf72-associated arginine-containing dipeptides exhibit age-dependent toxicity in *C. elegans*

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## Abstract

A hexanucleotide repeat expansion mutation in the C9orf72 gene represents a prevalent genetic cause of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia. Non-canonical translation of this repeat gives rise to several distinct dipeptide protein species that could play pathological roles in disease. Here, we show in the model system *Caenorhabditis elegans* that expression of the arginine-containing dipeptides, but not alanine-containing dipeptides, produces toxic phenotypes in multiple cellular contexts, including motor neurons. Expression of either (PR)<sub>50</sub> or (GR)<sub>50</sub> during development caused a highly penetrant developmental arrest, while post-developmental expression caused age-onset paralysis. Both (PR)<sub>50</sub>- and (GR)<sub>50</sub>-green fluorescent protein tagged dipeptides were present in the nucleus and nuclear localization was necessary and sufficient for their toxicity. Using an inducible expression system, we discovered that age-onset phenotypes caused by (PR)<sub>50</sub> required both continual (PR)<sub>50</sub> expression and an aged cellular environment. The toxicity of (PR)<sub>50</sub> was modified by genetic mutations that uncouple physiological aging from chronological aging. However, these same mutations failed to modify the toxicity of (GR)<sub>50</sub>, suggesting that (PR)<sub>50</sub> and (GR)<sub>50</sub> exert their toxicity through partially distinct mechanism(s). Changing the rate of physiological aging also mitigates toxicity in other *C. elegans* models of ALS, suggesting that the (PR)<sub>50</sub> dipeptide might engage similar toxicity mechanisms as other ALS disease-causing proteins.

Received: August 11, 2017. Revised: September 14, 2017. Accepted: September 21, 2017

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## Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are devastating and currently incurable neurodegenerative disorders. Most cases of both ALS and FTD with known genetic etiology are caused by the expansion of an intronic GGGGCC ( $G_4C_2$ ) hexanucleotide repeat in the chromosome 9 open reading frame 72 (C9orf72) gene (1,2). Several mechanisms for why this repeat leads to disease have been proposed, including loss of C9orf72 function, nuclear  $G_4C_2$  RNA foci formation and abnormal translation of dipeptide repeat proteins (DPRs) (3). Recent work shows that C9orf72-associated DPRs accumulate in the spinal fluid of C9 ALS/FTD patients and can cause cell death in *in vitro* and *in vivo* settings (4–8). While this suggests an important role for DPRs in C9 ALS/FTD pathogenesis, analysis of post-mortem brain samples shows that DPRs are present at very low levels in brain regions that undergo the most severe neurodegeneration (i.e. spinal cord motor neurons), thus calling into question the relevance of DPR proteins in disease pathogenesis (9,10). While this discrepancy could be due to a variety of factors (poor antibody detection of toxic species, loss of DPR-expressing neurons in end-stage samples), understanding the relationship between the individual DPRs and neurodegeneration in a variety of settings will be necessary to parse out the role of the DPRs in the development of C9orf72-associated diseases.

Translation of the intronic  $G_4C_2$  repeat occurs through a process termed repeat-associated non-AUG-dependent (RAN) translation (11). RAN translation of the  $G_4C_2$  repeats can give rise to five distinct DPRs—proline–alanine (PA), glycine–alanine (GA), proline–arginine (PR), glycine–proline (GP) and glycine–arginine (GR). While PA, GP and GA are not thought to cause significant toxicity, PR and to a lesser extent GR have been shown to be toxic in yeast, *Drosophila*, and mammalian cell models (6–8). Recent studies implicate several pathways in the toxicity of the PR RAN dipeptide (8,12,13). Given that both ALS and FTD exhibit age-related onset and that age-related phenotypes are not easily modeled in cellular systems, organismal models are needed to examine the role of cell non-autonomous aging regulatory pathways on the onset and severity of DPR toxicity. In this study, we establish *Caenorhabditis elegans* as a new model for investigating DPR toxicity mechanisms *in vivo*. We expressed codon-optimized transgenes for four DPRs (GA, PA, GR, PR) and characterized their toxicity and expression patterns. We found that (i) arginine-containing DPRs exhibited potent toxicity in neuronal and non-neuronal contexts; (ii) nuclear localization was necessary and sufficient for this toxicity; (iii) PR and GR toxicity was age-dependent and PR, but not GR, toxicity was influenced by mutations that uncouple physiological aging from chronological aging. Given that the toxicities of other ALS disease proteins are similarly modified by these mutations, our findings suggest that PR and other forms of ALS may share common disease mechanisms.

## Results

### Arginine-rich dipeptides are toxic in *C. elegans* motor neurons and muscle

Unconventional translation of the C9orf72-associated  $G_4C_2$  repeats can produce several types of dipeptide repeat proteins (DPRs) (14). The nematode *C. elegans* contains a C9orf72 homolog, *alfa-1*, which is involved in aging and stress responses, as well as stress-induced neurodegeneration (15). However, *alfa-1*

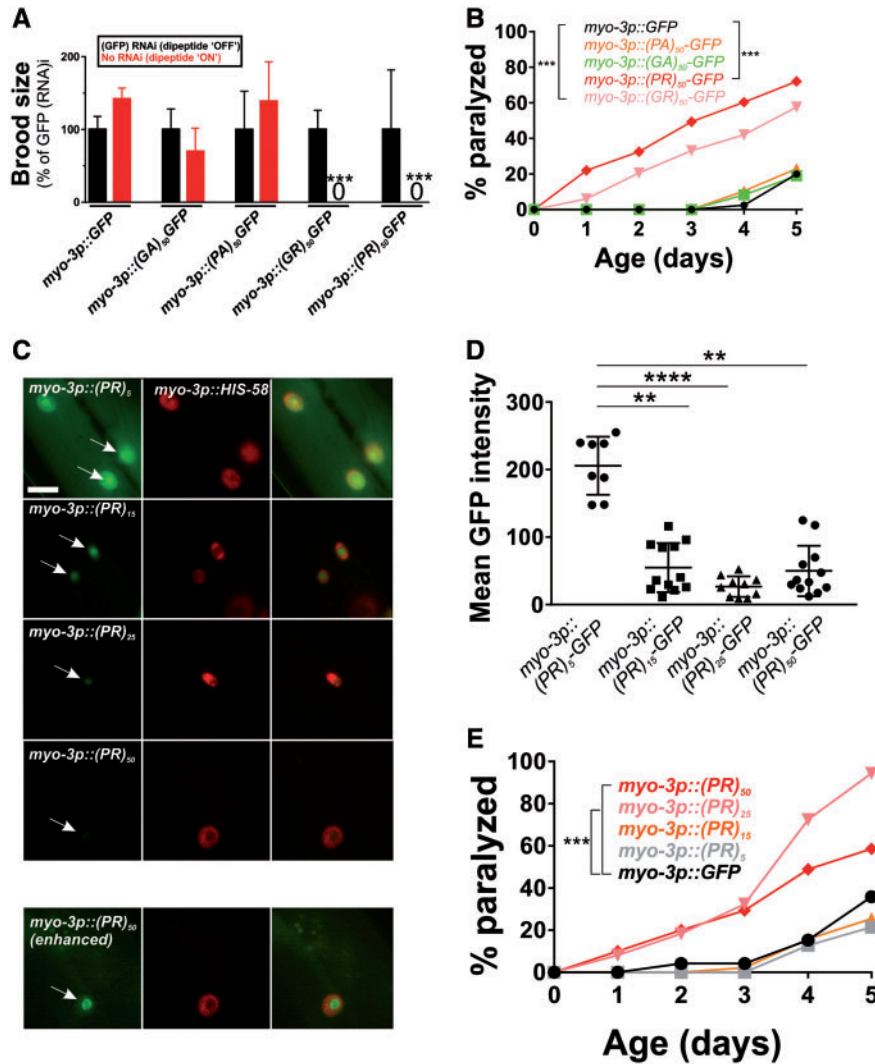
does not contain  $G_4C_2$  repeats and therefore does not model either RNA toxicity or RAN translation-induced DPR toxicity. To develop a *C. elegans* model for DPR expression, we generated constructs that express 50 repeats of 4 DPRs (GA, PA, GR and PR). Each of these constructs utilizes codon-optimized sequences that encode for one dipeptide but eliminates the  $G_4C_2$  repetitive sequence. Thus, our worm system is a ‘pure’ dipeptide model and does not contain repetitive GGGGCC sequences. To facilitate biochemical and cell biological detection of the DPRs, we inserted an in frame N-terminal FLAG epitope and a C-terminal green fluorescent protein (GFP) (Fig. 1A). Since ALS is a motor neuron disease, we used the motor neuron-specific *unc-47* promoter to control expression and generated transgenic animals expressing each individual DPR.

Consistent with previous studies in yeast, *Drosophila*, and mammalian cells (5–8), we found that the arginine-rich DPRs were highly toxic in *C. elegans*. Animals expressing arginine-rich DPRs were viable, consistent with previous studies showing that *unc-47*+ motor neurons are not required for viability in *C. elegans* (16). However, motor neuron expressed (PR)<sub>50</sub> and (GR)<sub>50</sub> each led to significant reductions in motility (Fig. 1B). Motor neurons expressing (PR)<sub>50</sub> exhibited morphological signs of degeneration commonly observed in other *C. elegans* models of neurodegenerative diseases (17–19), including commissure degeneration and membrane blebbing that were not observed in control animals or in animals expressing either (PA)<sub>50</sub> or (GA)<sub>50</sub> (Fig. 1C and D). These data show that arginine-containing dipeptides cause motility defects and neurodegenerative morphological changes when expressed in *C. elegans* motor neurons.

*Caenorhabditis elegans* motor neurons are extremely small (2–3  $\mu$ m in diameter), making cell biological analysis of DPR localization in these cells difficult. Additionally, *C. elegans* neurons are highly resistant to RNA-interference-mediated gene knock-down, which is commonly used to explore mechanisms of protein toxicity in *C. elegans* disease models (20,21). To overcome these limitations, we expressed the DPRs in the larger muscle cells, which regulate motility and are a common site of expression for other *C. elegans* disease models (22–24). As we observed in motor neurons, muscle expression of arginine-rich dipeptides was highly toxic. Expression of either (PR)<sub>50</sub> or (GR)<sub>50</sub> in muscle led to completely penetrant embryonic and larval lethality that could be suppressed by feeding animals *gfp(RNAi)*-expressing bacteria (Fig. 2A; Supplementary Material, Table S1). *gfp(RNAi)*-sensitive embryonic or larval lethality was not observed in animals expressing either (PA)<sub>50</sub> or (GA)<sub>50</sub>. (PR)<sub>50</sub> or (GR)<sub>50</sub> animals that were removed from *gfp(RNAi)* after embryonic development survived and went on to develop into adults. However, these DPR expressing adults exhibited age-onset paralysis (Fig. 2B). (PR)<sub>50</sub> animals showed a significantly reduced lifespan that was not observed in (GR)<sub>50</sub> expressing animals (Supplementary Material, Fig. S1). The enhanced paralysis observed in (PR)<sub>50</sub> and (GR)<sub>50</sub> animals was not due to higher mRNA expression, since qPCR showed that all DPRs were expressed at similar levels (Supplementary Material, Fig. S2 and Table S2). It was also not due to higher protein levels since *in vivo* imaging and flow cytometry quantification showed that (PR)<sub>50</sub>-GFP and (GR)<sub>50</sub>-GFP fluorescence levels were significantly below those of all other DPRs (Supplementary Material, Fig. S3). The toxicity phenotype required protein production and was not due to any potential RNA effects, since (PR)<sub>50</sub> transgenic animals lacking a start ATG exhibited no detectable toxicity (Supplementary Material, Fig. S4). (PR)<sub>50</sub> toxicity was dependent on the number of repeats, as animals expressing 5, 15 or 25







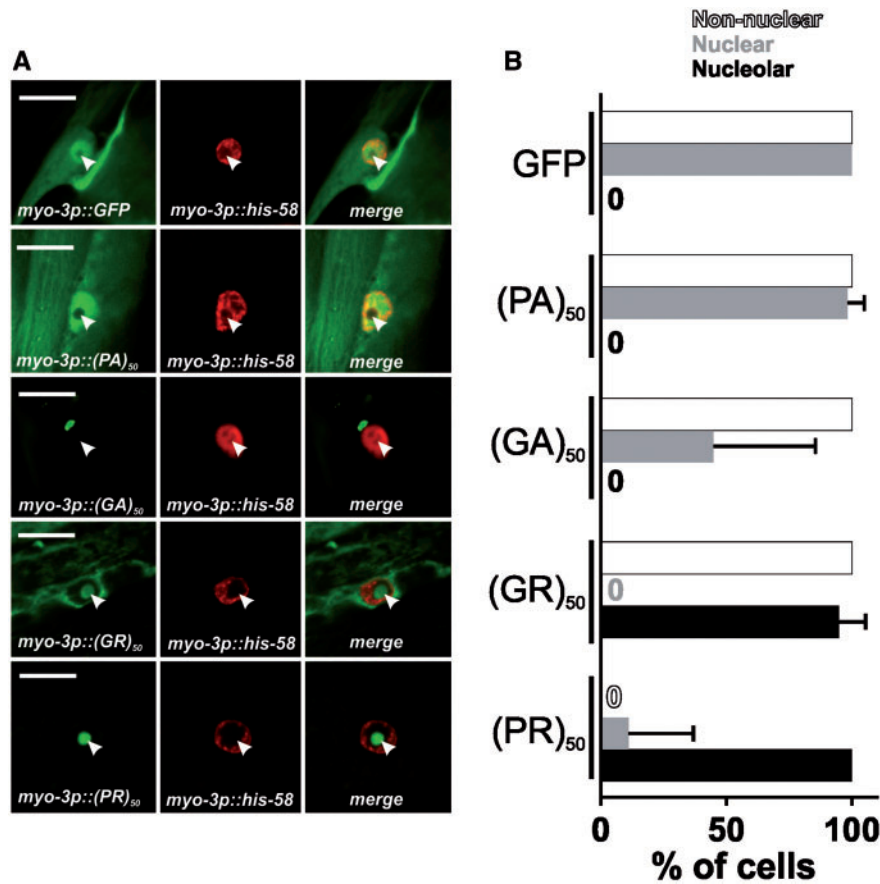
**Figure 2.** Muscle expressed arginine-containing dipeptides are toxic in *C. elegans*. (A) Brood size for worms expressing each integrated dipeptide protein in the presence or absence of *gfp(RNAi)* at 25 °C. Data for each strain are normalized to the mean brood size of animals grown on *gfp(RNAi)*. \*\*\* $P < 0.001$  (one-way ANOVA with post hoc Tukey's test). Raw brood size data for 25 °C are included in [Supplementary Material](#), Table S1. (B) Paralysis assay for adult animals raised in the absence of *gfp(RNAi)*.  $N \geq 43$ –49 animals per genotype. \*\*\* $P < 0.001$  (Log-rank test with Bonferroni adjusted  $P$ -value). (C) Fluorescent microscopy of Day 1 adult hermaphrodites expressing (PR)<sub>5</sub>-GFP, (PR)<sub>15</sub>-GFP, (PR)<sub>25</sub>-GFP or (PR)<sub>50</sub>-GFP (green) in the muscle. Muscle DNA (marked by a *myo-3p::his-58-mCherry* reporter) is in red. Arrows point to sites of nuclear GFP puncta. Scale bar = 10  $\mu$ m. All images were acquired using identical exposure settings. The row labeled '*myo-3p::GFP (PR)<sub>50</sub> (enhanced)*' was adjusted for brightness and contrast in the GFP channel differently from the other images so that the (PR)<sub>50</sub>-GFP signal is observable. (D) Quantification of the GFP levels from the indicated PR repeat animals. Each point shows the measured value for a single nucleus, horizontal line indicates population median with interquartile range. \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$  versus GFP control (one-way non-parametric ANOVA with Dunn's post hoc test). (E) Paralysis assay of animals expressing the indicated number of (PR) repeats under the control of the *myo-3* promoter. 'Day 0' animals were isolated as L4 stage animals.  $N = 48$ –50 animals per genotype. \*\*\*\* $P < 0.001$  versus GFP control (Log-rank test with Bonferroni adjusted  $P$ -value).

age-dependent paralysis (Fig. 5B). We further tested whether nuclear localization was sufficient for toxicity by fusing the (PR)<sub>50</sub> DPR to the coding sequence for the *his-58* gene, which encodes a DNA-binding histone only found in the nucleus. GFP-*his-58* expressing animals were viable and motile and exhibited strong nuclear GFP expression (Fig. 5C and D). However, GFP-*his-58*-(PR)<sub>50</sub> expressing animals were not viable unless they were cultured under *gfp(RNAi)* conditions [number of viable transgenic progeny (>L4)/total progeny in the absence of GFP after 72 h—GFP-*his-58*-348/406; GFP-*his-58*-(PR)<sub>50</sub>-9/56;  $P < 0.0001$ , Fisher's exact test]. Upon removal from *gfp(RNAi)*, *his-58*-(PR)<sub>50</sub> animals exhibited age-dependent paralysis (Fig. 5D) and this paralysis occurred with more rapid onset than in (PR)<sub>50</sub> animals

(Fig. 5E), suggesting that nuclear localization further enhances (PR)<sub>50</sub> toxicity. The enhanced *his-58*-(PR)<sub>50</sub> toxicity was not due to higher expression levels of the transgene since GFP fluorescence levels of *his-58*-(PR)<sub>50</sub> was not higher than (PR)<sub>50</sub> ([Supplementary Material](#), Fig. S5). Overall, these data show that nuclear localization is necessary and sufficient for (PR)<sub>50</sub> toxicity in *C. elegans*.

#### Post-mitotic age-dependent toxicity of (PR)<sub>50</sub> requires continuous dipeptide expression

Nuclear (PR)<sub>50</sub> exhibits age-dependent toxicity in *C. elegans*. This could be because (PR)<sub>50</sub> requires time to accumulate to

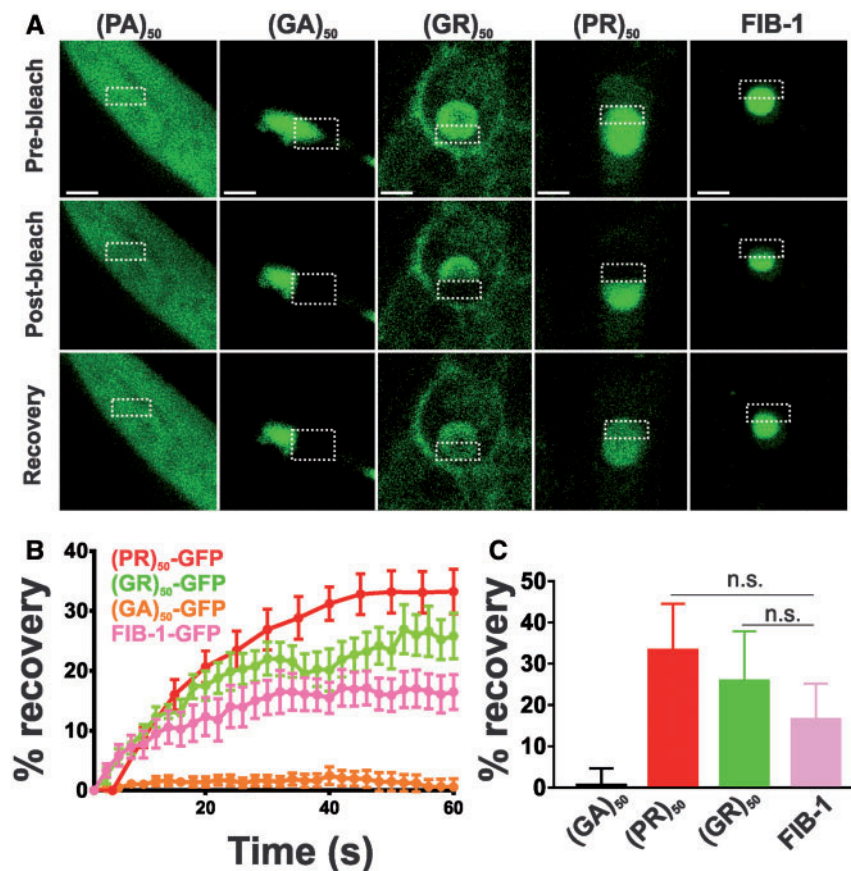


**Figure 3.** (PR)<sub>50</sub> and (GR)<sub>50</sub> are localized to the nucleolus in *C. elegans* muscle cells (A) Imaging of live, anesthetized Day 1 adult *C. elegans* hermaphrodites expressing the indicated dipeptide. Green shows dipeptide-GFP localization expressed in muscle, red shows histone-tagRFP within the muscle nucleus. Arrowhead points to the histone-free nucleolar region. Scale bar=10 μm. (B) Quantification of DPR-GFP signal that is observed in nucleoplasm, nucleolus, and non-nuclear cellular compartments. N = 55–78 nuclei from five to six animals. Data shown are means ± S.D.

sufficiently high levels necessary for toxicity. Alternatively, it could be because the cellular environment in aged animals is more permissive for toxicity. To test these models, we generated animals expressing DPR-GFP under the control of a heat shock inducible promoter (*hsp-16.2*). Following a brief (30 min) heat shock, we observed robust GFP expression of all DPRs in young adult animals. In most cases, the subcellular expression patterns for each DPR matched what we had observed previously, although the proteins were expressed more broadly across tissues due to the global activity of the heat shock promoter (Fig. 6A). Induction of (PR)<sub>50</sub> during embryonic development caused significant toxicity that was not observed in the absence of induction (Fig. 6B). Animals that had been induced to express (PR)<sub>50</sub> on Day 1 of adulthood continued to exhibit normal motility and survival across their lifespan (Fig. 6C). However, continual daily induction of (PR)<sub>50</sub> protein eventually led to increased paralysis and death that was not observed in the control animals or in other DPR expressing animals, including those expressing (GR)<sub>50</sub>. A single induction of (PR)<sub>50</sub> expression later in life (Day 4 of adulthood) also failed to elicit any detectable phenotypes. The toxicity of (PR)<sub>50</sub> was not due to higher induction levels than other DPRs (Supplementary Material, Fig. S6). Therefore, the age-onset phenotypes of nuclear (PR)<sub>50</sub> in post-mitotic adult *C. elegans* require both continuous expression throughout life and an aged cellular environment.

### Mutations that alter the rate of physiological aging delay (PR)<sub>50</sub> age-dependent toxicity

The toxicity of (PR)<sub>50</sub> in an aged cellular environment could be due to the stochastic accumulation of toxic protein levels or conformations over long periods of time. Alternatively, the process of aging could lead to a decline in cellular processes that oppose (PR)<sub>50</sub> toxicity. To differentiate between these two hypotheses, we tested the role of the aging process in the toxicity of (PR)<sub>50</sub>. If (PR)<sub>50</sub> toxicity is due to accumulation of toxic protein levels/conformation over time, lifespan-extending conditions should have no effect on the rate of toxicity. However, if the aging process actively regulates (PR)<sub>50</sub> toxicity, alterations in the rate of aging should enhance (lifespan shortening) or suppress (lifespan extending) the toxicity of (PR)<sub>50</sub>. The insulin signaling pathway is the most well characterized pathway that regulates lifespan and youthfulness from worms to humans (27). Mutations in this pathway are thought to extend lifespan by promoting a youthful cellular physiological state and therefore separate chronological and physiological states of aging. In *C. elegans*, the *daf-2* gene encodes the homolog of the insulin/IGF receptor. Loss of function mutations in *daf-2* exert their effects via signaling-dependent activation of the FOXO transcription factor *daf-16*, as well as the heat shock transcription factor *hsf-1* (28). Mutations in *daf-16* and *hsf-1* suppress phenotypes associated with *daf-2* mutations and accelerate the rate of aging (27).



**Figure 4.** Arginine-containing DPRs are not aggregates. (A) Representative images from FRAP analysis of subcellular localized DPR proteins expressed in muscle. Dashed outline indicates site of photobleaching and post-bleaching quantification. Recovery images are 150 s post-bleach. Scale bar = 2  $\mu$ m. (B) Quantification of FRAP imaging. Data shown are mean  $\pm$  S.E.M. from 9 to 10 datasets per genotype. (C) Average equilibrium fluorescence recovery after 60 s. Data shown are mean  $\pm$  S.D. from 9 to 10 datasets per genotype. n.s. versus FIB-1-GFP (one-way ANOVA with Dunn's multiple comparison test).

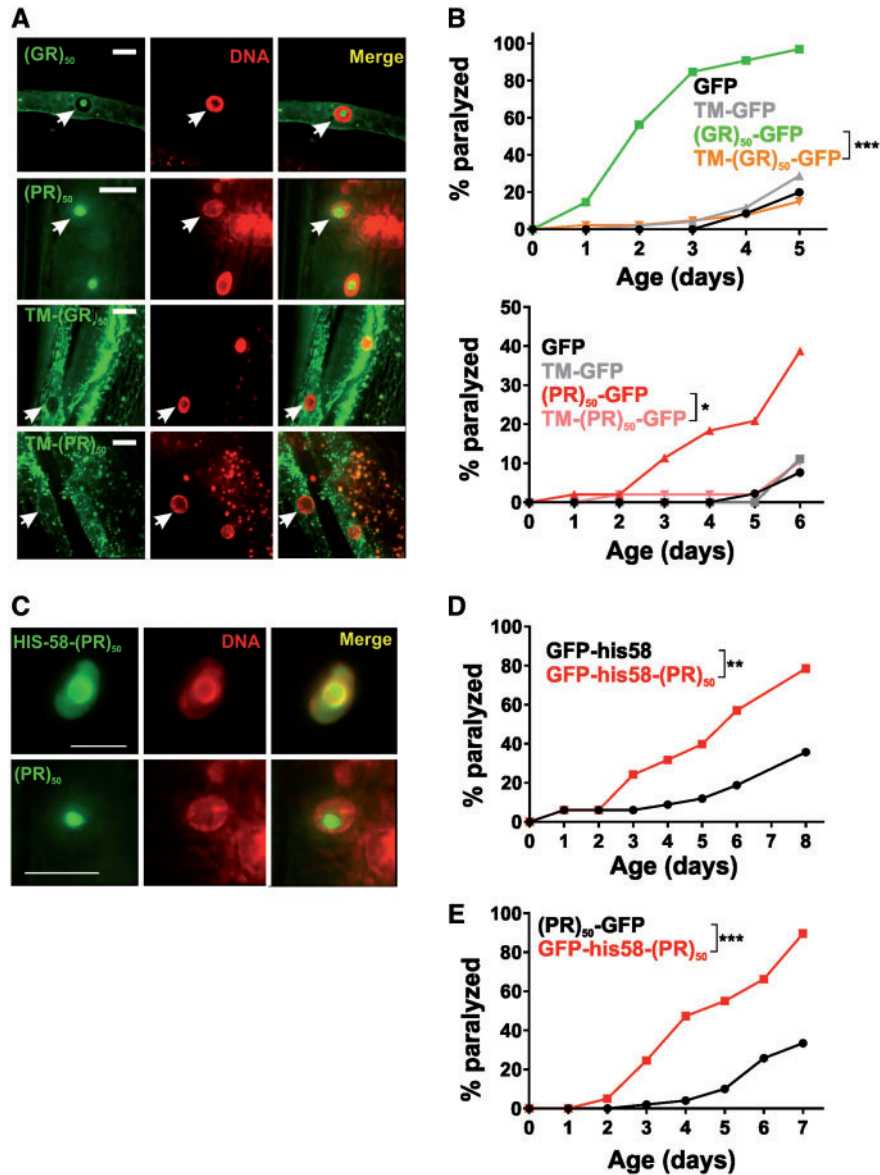
To determine whether the toxicity of (PR)<sub>50</sub> is stochastic or enabled by declining physiological processes, we crossed the (PR)<sub>50</sub> transgene into the *daf-2(e1370)*; *daf-16(mu86)* and segregated all possible (PR)<sub>50</sub> genotypes. If (PR)<sub>50</sub> toxicity is stochastic, then reduced insulin signaling should not alter the age-related phenotypes. However, if (PR)<sub>50</sub> toxicity is due to declining cellular physiology, then alterations in the rate of aging should slow the initiation and progression of toxicity. We found that in the *daf-2* mutant background, age-dependent declines in (PR)<sub>50</sub> motility were significantly reduced (Fig. 7A). These *daf-2*-dependent phenotypes were suppressed in a *daf-2*; *daf-16* double mutant and were accelerated in the *daf-16* mutant background (Fig. 7B). Likewise, a mutation in the heat shock transcription factor *hsf-1*, which accelerates the rate of aging (29), enhanced the toxicity of (PR)<sub>50</sub> (Fig. 7C). Interestingly, the protective effects of the *daf-2* mutant were not observed in (GR)<sub>50</sub> expressing animals (Fig. 7A). The protective effects of insulin signaling appear to act downstream from DPR accumulation and nuclear localization, as DPR abundance and subcellular distribution did not appear to be altered in any of these genetic backgrounds (Fig. 7D–F). We also found that in motor neurons expressing (PR)<sub>50</sub>, *daf-2* reduced the levels of neurodegeneration-associated membrane blebbing (Supplementary Material, Fig. S7). These data demonstrate that alterations in the rate of aging impact (PR)<sub>50</sub> toxicity in both *C. elegans* muscle cells and motor neurons and suggest that (PR)<sub>50</sub> toxicity is not stochastic but is dependent on the cellular

aging process. Additionally, they suggest that (PR)<sub>50</sub> and (GR)<sub>50</sub> are toxic through partially distinct mechanisms since enhanced DAF-16/FOXO activation protects (PR)<sub>50</sub> animals but has no protective effects on (GR)<sub>50</sub> animals.

## Discussion

In this study, we developed the first *C. elegans* model for C9orf72 dipeptide toxicity. We discovered that (1) (PR)<sub>50</sub> and (GR)<sub>50</sub> dipeptides are toxic in neuronal and non-neuronal contexts, (2) both toxic dipeptides exhibit a shared nucleolar localization, (3) nuclear localization of both (PR)<sub>50</sub> and (GR)<sub>50</sub> dipeptides is required for toxicity, (4) post-mitotic toxicity of (PR)<sub>50</sub> is age-dependent, (5) age-dependent toxicity of (PR)<sub>50</sub> is modified by genetic pathways that accelerate or decelerate the rate of physiological aging in both muscle and motor neurons while (GR)<sub>50</sub> toxicity is not modified by these pathways. While some of these findings (i.e. Points 1 and 2) are consistent with recent studies from yeast, *Drosophila*, and mammalian cells, others leverage the unique strengths of *C. elegans* to examine questions that either have not or cannot be addressed in other systems (i.e. Points 3–5). While many of our findings are consistent with studies using other C9 animal models, there are also significant differences. For example, a recent report describes toxicity associated with (GA) dipeptides in zebrafish (30). However, we find no evidence of (GA) toxicity in *C. elegans* when expressed in either motor neurons or muscle or more globally after heat



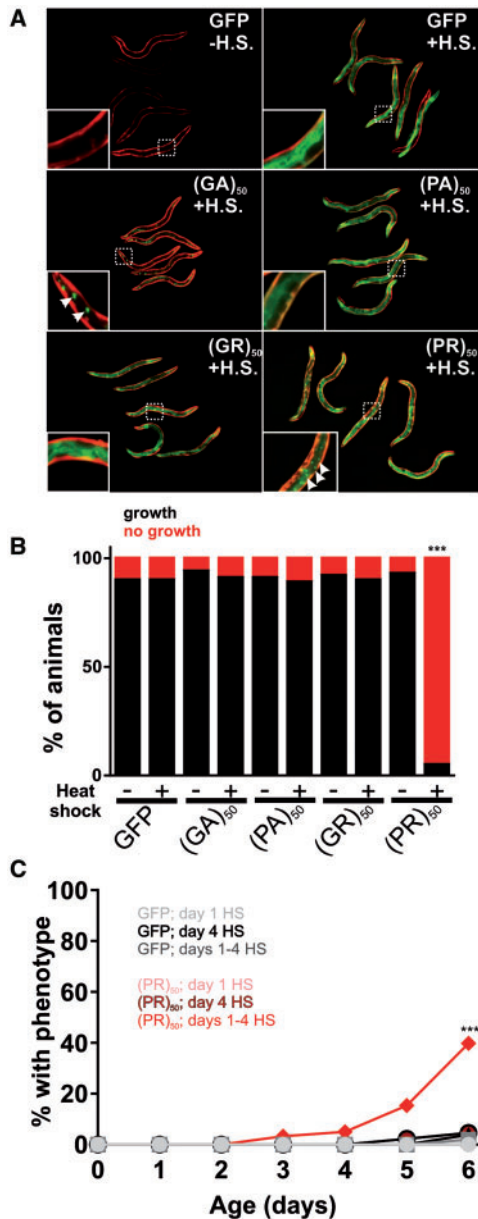


**Figure 5.** Nuclear localization of muscle expressed arginine-containing DPRs is necessary and sufficient for toxicity. (A) Representative images of the indicated soluble or membrane anchored DPR (green), the HIS-58-mCherry nuclear marker (red), and a merged image. Arrows point to nuclear regions defined by the HIS-58 mCherry signal. Coincident green and red signals in the TM-(PR)<sub>50</sub> image is intestinal autofluorescence. Scale bar = 10  $\mu$ m. (B) Paralysis assay comparing animals expressing soluble or transmembrane localized (GR)<sub>50</sub> (top) or (PR)<sub>50</sub> (bottom). N = 48–50 animals per genotype, \*\*\*P < 0.001, \*\*P < 0.01, \*P < 0.05 (Log-rank test with Bonferroni adjusted P-value). (C) Representative images of HIS-58 anchored (PR)<sub>50</sub> (green; top) or unanchored (PR)<sub>50</sub> (green; bottom), HIS-58-mCherry nuclear marker (red) and a merged image. Scale bar = 5  $\mu$ m. (D) Paralysis assay comparing animals expressing nuclear localized GFP or (PR)<sub>50</sub>. n  $\geq$  30 animals per genotype, \*\*P < 0.01 (Log-rank test with Bonferroni adjusted P-value). (E) Paralysis assay comparing soluble (PR)<sub>50</sub> with his58-(PR)<sub>50</sub>. n  $\geq$  50 animals [(PR)<sub>50</sub>] or 80 animals [his58-(PR)<sub>50</sub>], \*\*\*P < 0.001 (Log-rank test with Bonferroni adjusted P-value).

shock, even though (GA) aggregates are observed in all of these conditions. The lack of toxicity of GA in worms compared with fish could be due to differences in repeat number (50 in worms versus 80 in fish) or differences in expression levels. In another example, we found prominent nucleolar (GR)<sub>50</sub> localization, whereas a recent post-mortem study of C9orf72 carrier brains revealed perinuclear GR aggregates (31). Such discrepancies could reflect differences in dipeptide repeat length (50 repeats in our model versus unknown repeats in patient cells) or in live animal versus post-mortem sample fixation and preparation. Overall, our findings suggest that this new *C. elegans* model, in conjunction with high throughput genetic and pharmacological

screening methods, will be a useful and complementary system for exploring the mechanisms underlying C9orf72-associated DPR toxicity.

Results from our work suggest that the localization of (PR) and (GR) dipeptides to the nucleus is essential for their toxicity. This is consistent with several recent studies that suggest both PR and GR DPRs exert their toxicity by interfering with nuclear functions and nuclear pores (8,12,13,32,33). For example, in yeast, overexpression of nuclear import pathways, ribosome biogenesis pathways and nuclear RNA-binding proteins strongly modify (PR)<sub>50</sub> toxicity (8). Likewise, knockdown of *Drosophila* genes implicated in nuclear transport also modify PR

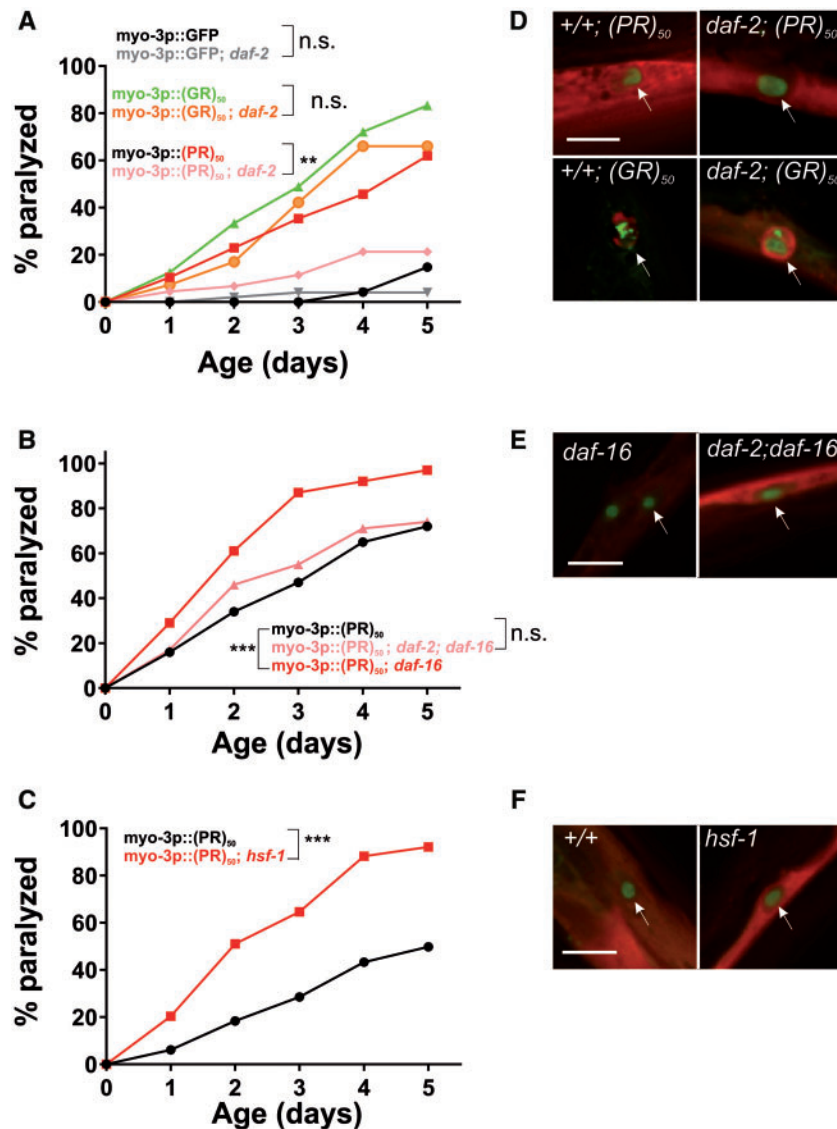


**Figure 6.** Age-onset toxicity requires continuous DPR expression. (A) Images of worms expressing the indicated DPR under the control of a heat shock inducible promoter. Red fluorescence shows the *myo-3p::mCherry* transgenic marker. Green fluorescence shows the *hsp-16.2p::DPR-GFP* signal. All DPRs are shown 2–4 h after heat shock. Insets show an enlarged view of the region identified by the dashed boxes. Arrows in the (GA)<sub>50</sub> inset point to putative protein aggregates, suggesting that (GA)<sub>50</sub> rapidly aggregates after synthesis. Arrows in the (PR)<sub>50</sub> inset point to sites of nuclear localization in the intestine. No such nuclear localization was observed in the (GR)<sub>50</sub> line after heat shock. (B) Developmental toxicity of transgenic embryos expressing the indicated *hsp-16.2p::DPR-GFP*. Embryos were heat shocked at 35 °C for 30 min and then returned to 20 °C. The number that reached ≥L4 after 48 h were scored as ‘growth’ while the remaining animals were scored as ‘no growth’.  $N \geq 81$  transgenic animals per condition. \*\*\* $P < 0.001$  (Fisher’s exact test versus GFP + H.S.). (C) Adult toxicity of animals expressing either *hsp-16.2p::GFP* or *hsp-16.2p::(PR)<sub>50</sub>-GFP*. Animals were subjected to either a heat shock (30 min, 35 °C) on Day 1, Day 4 or on Days 1, 2, 3 and 4. Every 24 h, animals were scored as either mobile, censored or affected (paralyzed + dead = % with phenotype). Heat shock induction of (GA)<sub>50</sub>, (PA)<sub>50</sub> and (GR)<sub>50</sub> produced phenotypes identical to that of GFP.  $N \geq 60$  animals per genotype. \*\*\* $P < 0.001$  versus GFP; Days 1–4 H.S. (Log-rank test with Bonferroni adjusted P-value).

toxicity (34). In most systems, including our new *C. elegans* model, both (PR)<sub>50</sub> and (GR)<sub>50</sub> DPRs exhibit prominent nucleolar localization. However, notable non-nuclear localization is also observed, especially in the case of the (GR)<sub>50</sub> dipeptide. Our FRAP data suggest that while (GA)<sub>50</sub> dipeptides are clearly protein aggregates, nuclear (PR)<sub>50</sub> and (GR)<sub>50</sub> are not protein aggregates, since, unlike (GA)<sub>50</sub>, they exhibit significant recovery upon photobleaching. This localization pattern, as well as the pattern of genetic modifiers identified from yeast and *Drosophila* screens, has led to the hypothesis that (PR)<sub>50</sub> and (GR)<sub>50</sub> toxicity may occur through direct interactions within the nucleus. Alternatively, (PR)<sub>50</sub> and (GR)<sub>50</sub> could cause toxicity via non-nuclear interactions that then impinge on nuclear functions. Findings from our *C. elegans* model strongly support the former hypothesis, since we found that preventing nuclear localization completely abrogated the toxicity of the arginine-containing DPRs. We also found that forcing (PR)<sub>50</sub> into the nucleus maintained, and perhaps enhanced, toxicity. Our findings do not preclude the additional possibility that freely soluble cytoplasmic DPRs could also mediate toxicity, since our method of preventing nuclear accumulation restricted DPRs to cytosolic membranes, which would also remove freely soluble cytoplasmic DPRs. Moreover, our method for restricting DPRs to the nucleus could result in unusual toxicity mediated by charge interactions between positively charged DPRs and negatively charged DNA. Unfortunately, attempts at using traditional NLS/NES sequence to drive DPRs into or out of the nucleus were unsuccessful for unknown reasons. Nevertheless, our findings are consistent with recent work showing that these DPRs specifically interact with low-complexity nuclear and nucleolar proteins that shape the formation of non-membrane bound organelles within the nucleus (12).

Worms have been utilized to model other genetic forms of ALS, including those associated with mutant forms of proteins such as superoxide dismutase 1 (SOD1) (17,23,35–38), TAR DNA-binding protein 43 (TDP-43) (39–43) and fused in sarcoma (FUS) (39). In each of these cases, the worm model expresses a mutant protein that forms protein aggregates. The toxicity associated with the SOD1 and TDP-43 models is responsive to the activation state of the *daf-2/insulin* signaling pathway (41,44). One of the major mechanisms by which the *daf-2/insulin* signaling pathway is thought to oppose these proteotoxic disease models is via the upregulation of genes that maintain protein folding (i.e. chaperones) or enhance the clearance of misfolded/aggregated proteins (i.e. proteases) (21). The overall effect of these targets is to delay the proteotoxic consequences of aging, thus making mutants in the *daf-2/insulin* pathway tools for separating physiological aging from chronological aging. Like SOD1 and TDP-43, we found that (PR)<sub>50</sub> can also be suppressed by activating insulin signaling. However, unlike SOD1 and TDP-43, (PR)<sub>50</sub> did not form detectable protein aggregates. Given that mutations in genes comprising the *daf-2/insulin* signaling pathway can modify all three disease models, this suggests that (PR)<sub>50</sub> may share common toxicity mechanisms with SOD1 and TDP-43, for example, via disruptions in protein homeostasis. Interestingly, insulin signaling did not appear to alter DPR abundance or subcellular localization in our model, suggesting that this pathway may act downstream from DPR nuclear functions to provide toxicity protection. Future genetic modifier screens should reveal the identity of such mechanisms, as well as whether DPRs and other ALS disease genes share common disease mechanisms. The powerful phenotypes elicited by (PR)<sub>50</sub> in our *C. elegans* model, as well as the tractability of genome-wide RNAi approaches, will facilitate such screens.





**Figure 7.** Altering the rate of aging affects (PR)<sub>50</sub> but not (GR)<sub>50</sub> toxicity. (A) Paralysis assay of animals expressing GFP, (GR)<sub>50</sub> or (PR)<sub>50</sub> under the control of the *myo-3* promoter in the wild-type or *daf-2(e1370)* background. 'Day 0' animals were isolated as L4 stage animals. N = 48–50 animals per genotype. n.s., 'not significant'; \*\*\*P < 0.01 versus GFP control (Log-rank test with Bonferroni adjusted P-value). (B) Paralysis assay of animals expressing (PR)<sub>50</sub> under the control of the *myo-3* promoter in the wild-type, *daf-16(mu86)*, or *daf-2(e1370); daf-16(mu86)* background. N = 48–50 animals per genotype. n.s., 'not significant'; \*\*\*P < 0.001 versus (PR)<sub>50</sub> (Log-rank test with Bonferroni adjusted P-value). (C) Paralysis assay of animals expressing (PR)<sub>50</sub> under the control of the *myo-3* promoter in the wild-type or *hsf-1(sy441)* mutant background. N = 48–50 animals per genotype. \*\*\*P < 0.001 versus wild-type (Log-rank test with Bonferroni adjusted P-value). (D) Fluorescent microscopy images of day 1 adult worms expressing either (GR)<sub>50</sub> or (PR)<sub>50</sub> (green) and soluble muscle mCherry (red) in the wild-type or *daf-2(e1370)* mutant background. Arrow points to site of nuclear DPR accumulation. Scale bar = 10 μm. (E) Fluorescent microscopy images of Day 1 adult worms expressing (PR)<sub>50</sub> (green) and soluble muscle mCherry (red) in the *daf-16(mu86)* or *daf-2(e1370); daf-16(mu86)* mutant background. Arrow points to site of nuclear DPR accumulation. Scale bar = 10 μm. (F) Fluorescent microscopy images of day 1 adult worms expressing (PR)<sub>50</sub> (green) and soluble muscle mCherry (red) in the wild-type or *hsf-1(sy441)* mutant background. Arrow points to site of nuclear DPR accumulation. Scale bar = 10 μm.

Despite the fact that both (PR)<sub>50</sub> and (GR)<sub>50</sub> are required in the nucleus to exert their toxicity, we found important differences between their mechanisms of toxicity. For example, the toxicity associated with (PR)<sub>50</sub> was sensitive to manipulations in the activation state of the *daf-2*/insulin signaling pathway. In the *daf-2* mutant background, (PR)<sub>50</sub> toxicity was delayed, whereas in the *daf-16* and *hsf-1* mutant background, (PR)<sub>50</sub> toxicity was enhanced. Curiously, (PR)<sub>50</sub> toxicity in the *daf-2; daf-16* background was restored although not to the levels seen with *daf-16* alone. This could be due to variability in assay conditions. Alternatively, it could suggest that *daf-2* engages other factors,

such as *skn-1* (45) and/or *hsf-1* (28,46), in addition to *daf-16* to mediate protection against (PR)<sub>50</sub> toxicity. However, (GR)<sub>50</sub> toxicity was unaffected by the *daf-2*/insulin pathway activation state. Additionally, acute induction of (PR)<sub>50</sub> using a heat shock-inducible promoter led to toxicity, while acute induction of (GR)<sub>50</sub> did not produce toxicity. These observations suggest that (PR)<sub>50</sub> and (GR)<sub>50</sub> exert different types of toxicity and that only (PR)<sub>50</sub> toxicity is susceptible to the consequences of *daf-2*/insulin pathway activation. The ability of insulin/IGF signaling to modify toxicity of one dipeptide but not another may partially explain the failure of insulin/IGF therapies in ALS clinical trials

among patients with C9orf72 mutations (47–49). Perhaps additional therapies targeting insulin/IGF-independent toxicity like that associated with (GR)<sub>50</sub> could be used in combinatorial therapies to protect against the multiple forms of toxicity associated with C9orf72 mutations. Comparative genetic modifier screens, as well as comparative RNAseq studies, should be useful in determining how cells differentially respond to these two DPRs.

RAN translation products are an emerging component of several nucleotide repeat expansion disorders, including C9orf72-associated FTD/ALS and CAG-associated Huntington's disease (50). *Caenorhabditis elegans* is a particularly attractive model for identifying the mechanisms underlying the toxicity of these various RAN proteins. Given the strong phenotypes described here for the C9orf72-associated (PR)<sub>50</sub> and (GR)<sub>50</sub> RAN products, genetic, and pharmacological screens should prove to be a valuable approach for identifying the genes and pathways that are required for these and perhaps other RAN protein-mediated toxicity. In addition to delineating mechanisms of DPR toxicity, *C. elegans* may also prove useful in defining the requirements for RAN translation. Worms expressing pure GGGGCC repeats were recently described (51,52). These studies suggest that G<sub>4</sub>C<sub>2</sub> repeat expression produces RNA foci, dipeptide protein expression and toxicity in the form of a shortened lifespan and paralysis, although whether such toxicity was due to the expression of dipeptides was not determined. If such RAN products were tagged with GFP and these RAN-GFP products were easily detectable, high-throughput genetic approaches could be used to decipher the genetic, cellular and age-dependent requirements for RAN translation *in vivo*.

In conclusion, we developed the first *C. elegans* model for C9orf72-associated DPR toxicity. Our findings reveal prominent roles for protein localization and aging in the toxicity of arginine-containing dipeptides and suggest significant differences in the response of the DPRs to protective genetic pathways. Using these phenotypes, our future studies will utilize the genetic and pharmacological accessibility of worms to better define the mechanisms of DPR toxicity. Given the conserved nature of worm genetics, we anticipate that our findings may guide new ideas for treatments or biomarkers in C9orf72 associated diseases.

## Materials and Methods

### *Caenorhabditis elegans* strains and culture

Strains were cultured on standard NGM media with *E. coli* OP50 bacteria. Strains expressing (PR)<sub>50</sub> and (GR)<sub>50</sub> DPRs were cultured on *gfp(RNAi)* bacteria at 20 °C until the experiment, when they were shifted to OP50. The following strains were used; *daf-2(e1370)*, *daf-16(mu86)*, *hsf-1(sy441)*. Standard genetic approaches were utilized to cross mutants into the DPR backgrounds. The homozygous genotype of every strain was confirmed by DNA sequencing of the mutant lesion. Wild-type animals were re-isolated in every cross and utilized as the DPR only control in resulting experiments. DPR toxicity studies were carried out using animals grown at 25 °C, unless otherwise noted.

### Molecular biology and transgenics

Codon-varied dipeptide sequences were isolated from previously described plasmids (6) (nucleotide sequences can be found in [Supplementary Material](#), Table S3). (PR)<sub>5</sub>, (PR)<sub>15</sub> and (PR)<sub>25</sub> codon-varied plasmids were synthesized (GeneArt,

ThermoFisher Scientific, Waltham, MA, USA). Dipeptide sequences were isolated as a *HindIII/BamHI* fragment and subcloned into the *C. elegans* expression vector pPD95.79 to generate dipeptide with a C-terminal GFP tag. Promoters were PCR amplified, incorporating both a 3XFLAG epitope immediately downstream from the start ATG and *HindIII* sites flanking the fragment. Promoters were subcloned into the DPR-pPD95.79 vectors as *HindIII* fragments. To make N-terminal GFP-(PR)<sub>50</sub> and GFP-(GR)<sub>50</sub> fusions, GFP was PCR amplified and cloned in frame with the start codon of the *myo-3* promoter. The dipeptide sequences were subcloned as a *HindIII/BamHI* fragment into the pPD49.26 expression vector. The *myo-3-GFP* fragment was then subcloned into the DPR-pPD49.26 as a *HindIII* fragment. The membrane-bound DPRs were generated by a PCR fusion method. We first amplified the signal sequence and transmembrane domain from the *pat-3* gene as supplied in vector pPD122.39. The membrane domain was then fused to the *myo-3* promoter in-frame with the start ATG and then subcloned as a *HindIII* fragment into the DPR-pPD95.79 vectors. The resulting clones produce a membrane-localized DPR with the DPR facing the cytoplasm. To make the *his-58-DPR* fusions, the *his-58* genomic sequence was PCR amplified and Gibson cloned (New England Biolabs, Ipswich, MA, USA) into the *myo-3p::GFP* sequence downstream of and in-frame with the GFP sequence. The resulting *myo-3p::GFP-his-58* fragment was subcloned as a *HindIII* fragment into the DPR-pPD49.26 vectors. Primer sequences and plasmids utilized for this study are available upon request. A list of nucleotide sequences for promoters used in this study can be found in [Supplementary Material](#), Table S4.

Transgenic worms were generated by injecting the DPR construct (20 ng/μl) and the *myo-3p::mCherry* pCFJ104 or *myo-3p::dsRed2* marker plasmid (100 ng/μl) into the gonad of wild-type animals. Transgenes were integrated using a standard gamma-ray (Cs<sup>137</sup>) mutagenesis, followed by selection of animals exhibiting 100% transmission of the mCherry marker. Integrated strains were outcrossed six times to wild-type animals. In the case of (PR)<sub>50</sub> and (GR)<sub>50</sub>, injected animals were maintained on *gfp(RNAi)* plates until the experimental assay was performed. All procedures involving recombinant or synthetic nucleic acid molecules and materials were approved by the University of Pittsburgh Institutional Biosafety Committee.

### Microscopy

Worms were anesthetized (10 mM levamisole) and mounted on agar pads for fluorescence microscopy. Images were collected on either a Leica MZ16FA stereo dissecting scope or a Leica DMI4000 inverted microscope and a Leica DFC 340Fx digital camera (Leica Microsystems, Wetzlar, Germany). Z-stack images were deconvolved using Leica AF6000 software. Unless noted, images within an experiment were collected using the same exposure settings and processed with identical deconvolution parameters.

FRAP studies were carried out on levamisole anesthetized day 1 adult hermaphrodites using a Leica DMI8 confocal microscope at the University of Pittsburgh Center for Biologic Imaging. Images were captured at 20–30 Hz. Following imaging of baseline fluorescence, a region of interest corresponding to a portion of the nuclear or cytoplasmic foci was photobleached and fluorescence recovery within the photobleached area was monitored over at least 60 s. Data were normalized so that the image preceding the photobleach was set to 100% and the first image following the photobleach was set to zero percent.

Imaging conditions over the time course of the experiment caused minimal loss of signal, suggesting an absence of photobleaching during the monitoring period. Photobleaching of the entire foci led to no recovery of signal over the imaging time period, suggesting that any resulting new signal was owing to diffusion of molecules within the foci rather than exchange of new molecules outside of the foci.

To quantify DPR subcellular localization of the DPRs, we co-expressed each DPR with a *myo-3p::tagRFP-his-58* transgene to mark the nucleus. Z-stack images were collected for 1–21 nuclei across 5–6 individual animals, looking at at least 55 nuclei per DPR. We scored each nucleus for DPR expression in the histone-free region of the nucleus (Nucleolar), histone positive region of the nucleus (Nuclear) and outside of the histone marker (non-nuclear). The percentage of each category in each worm was determined and used to calculate and mean  $\pm$  SD for each category.

### Paralysis and thrashing assays

For all assays except for that shown in Figure 5D and E, gravid DPR expressing transgenic animals were moved from *gfp(RNAi)* to OP50 plates and allowed to lay eggs for 24 h. The resulting progeny were allowed to grow up on OP50, permitting DPR accumulation. Ten L4 animals were placed on each of three to five plates ( $N = 30$ –50 per assay). Each day, animals that failed to move at least half a body length in response to manual stimulation with a platinum wire but were still alive (pharyngeal pumping, movement of less than half a body length) were scored as paralyzed. Animals that died, desiccated on the plate edges or exhibited internal hatching of progeny were censored from the assay. Each day, mobile animals were transferred to a new plate and paralyzed, dead and censored animals were removed from the assay. For the assay in Figure 5D and E, *his-58-(PR)<sub>50</sub>* progeny from animals moved from *gfp(RNAi)* to OP50 were not viable, presumably owing to the enhanced toxicity of the nuclear localized (PR)<sub>50</sub> protein. Therefore, for these assays, animals were removed from *gfp(RNAi)* as L4 stage animals, placed on OP50 at 25 °C, and motility in the animals moved from *gfp(RNA)* was monitored as described above. Since animals are removed from the assay once they are scored as paralyzed, it is not appropriate to utilize statistical approaches that compare means and errors between time points (i.e. T-tests, ANOVA tests). Instead, we utilized the Log-rank statistical method, a cumulative statistical approach that compares changes in population sizes over time for a specified endpoint (53). Because this end-point assay is cumulative and not replicative, data points do not contain error bars. For each assay, 2–3 independent trials with 45–50 animals per assay were performed and the results from one representative trial are shown.

To measure thrashing, animals were maintained at 25 °C on OP50, and transgenic animals for each strain were picked as L4's the day before the experiment. The following day, worms were placed on clean NGM plates and allowed to move freely for 10 min so that most of the bacteria came off the animal before the experiment. Worms were then placed individually into 3 cm petri dishes containing M9 buffer and allowed to adjust to the new environment for 5 min. The worms were then observed for 30 s and the number of thrashes (reversal of body bend that crosses the midline) was counted. We were unable to score thrashing in *daf-2(e1370)* animals since this mutation on its own causes significant defects in liquid thrashing.

### Brood size assays

For brood size assays, at least 10 L4 stage animals were individually picked to OP50-containing NGM plates at 25 °C and transferred to a new plate every 24 h until cessation of egg laying. Each plate was allowed to age for 48–72 h and the number of animals  $\geq$  L4 stage of development was counted. Brood sizes were normalized within each strain to the mean brood size of animals grown on *gfp(RNAi)* bacteria.

### Neurodegeneration assays

L4 animals of the indicated genotype were isolated at 25 °C and imaged 24 h later as 'Day 1 adults'. All of the strains contained an *unc-47p::GFP* marker to reveal GABA motor neuron morphology. Animals were anesthetized in levamisole and Z-series images of GABAergic commissures were collected. Commissure breaks were identified as interruptions in the GFP signal surrounded by dorsal and ventral GFP in the commissures. Blebbing was scored only in the commissures and was identified by the presence of one or more GFP varicosities. For assays involving *daf-2*, we were unable to score either membrane blebbing or commissure breakage in (GR)<sub>50</sub> expressing animals because the neuronal GFP marker used to score such events was undetectable, likely due to significant neurodegeneration that continued to occur in (GR)<sub>50</sub>; *daf-2* animals.

### Statistical analysis

Paralysis assays were analyzed using the Kaplan–Meier log-rank function (OASIS) (54,55). Comparisons of means were analyzed with either a two-tailed Student's t-test (two groups) or ANOVA (three or more groups) using the Tukey's or Dunn's post-test analysis as indicated in GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). P-values of  $<0.05$  were considered significant.

### Supplementary Material

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

### Funding

This work was supported by grants from the NIH [NS094921 (U.B.P. and T.L.), 1S10OD021540 (S.C.W.), R01NS081303, R21NS100055, R21NS098379, R21NS101661 (U.B.P.), and R01GM105655 and NS096319 (T.L.)], the Robert Packard Center for ALS at Johns Hopkins (U.B.P.), the Muscular Dystrophy Association (U.B.P.), and the Israeli Binational Science Foundation Grant [2011323 (T.L.)]. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Funding to pay the Open Access publication charges for this article was provided by NIH NS094921 and NS096319.

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