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TDP-43 Toxicity Proceeds via Calcium Dysregulation and Necrosis in Aging *Caenorhabditis elegans* Motor Neurons

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Amyotrophic lateral sclerosis (ALS) is a heterogeneous disease with either sporadic or genetic origins characterized by the progressive degeneration of motor neurons. At the cellular level, ALS neurons show protein misfolding and aggregation phenotypes. Transactive response DNA-binding protein 43 (TDP-43) has recently been shown to be associated with ALS, but the early pathophysiological deficits causing impairment in motor function are unknown. Here we used *Caenorhabditis elegans* expressing mutant TDP-43^{A315T} in motor neurons and explored the potential influences of calcium (Ca^{2+}). Using chemical and genetic approaches to manipulate the release of endoplasmic reticulum (ER) Ca^{2+} stores, we observed that the reduction of intracellular Ca^{2+} ($[Ca^{2+}]_i$) rescued age-dependent paralysis and prevented the neurodegeneration of GABAergic motor neurons. Our data implicate elevated $[Ca^{2+}]_i$ as a driver of TDP-43-mediated neuronal toxicity. Furthermore, we discovered that neuronal degeneration is independent of the executioner caspase CED-3, but instead requires the activity of the Ca^{2+}-regulated calpain protease TRA-3, and the aspartyl protease ASP-4. Finally, chemically blocking protease activity protected against mutant TDP-43^{A315T}-associated neuronal toxicity. This work both underscores the potential of the *C. elegans* system to identify key targets for therapeutic intervention and suggests that a focused effort to regulate ER Ca^{2+} release and necrosis-like degeneration consequent to neuronal injury may be of clinical importance.

Key words: ALS; *C. elegans*; calcium; ER; necrosis; TDP-43

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective loss of both upper and lower motor neurons in the cortex and spinal cord (Wijesekera and Leigh, 2009). ALS is an age-dependent, rapidly progressive disease with life expectancy typically being between 2 and 5 years after onset. Thanks to recent genetic advances, causative mutations for ALS have been discovered in over a dozen genes (Renton et al., 2014), including one encoding transactive response DNA-binding protein 43 (TDP-43; Kabashi et al., 2008) among others.

Evidence is mounting that pathways regulating protein degradation may influence ALS pathogenesis (Blokhuis et al., 2013). We previously reported that mutant TDP-43^{A315T} proteins expressed in *Caenorhabditis elegans* motor neurons are susceptible to misfolding, leading to insolubility, aggregation (Vaccaro et al., 2012a), and activation of the endoplasmic reticulum (ER) unfolded protein response (UPR^{ER}; Vaccaro et al., 2012b, 2013). Induction of the UPR^{ER} by mutant TDP-43 suggests that the capacity of the ER to properly fold proteins may be exceeded, leading to cellular dysfunction and death (Walker and Atkin, 2011).

The ER constitutes a Ca^{2+} store whose uptake and release are extensively regulated to maintain cellular Ca^{2+} homeostasis, and disrupted ER function can induce Ca^{2+} depletion (Burdakov and Verkhratsky, 2006). Altered Ca^{2+} homeostasis has been investigated as a mechanism to distinguish motor neurons that are vulnerable or resistant to degeneration in ALS (Palecek et al., 1999; Vanselow and Keller, 2000). Indeed, ALS-vulnerable motor neurons in mice display Ca^{2+} buffering capacities that are five to six times lower compared with those found in ALS-resistant oculomotor neurons (Vanselow and Keller, 2000), while a more recent study has shown that altered Ca^{2+} buffering may be a risk factor for SOD-1 toxicity (von Lewinski et al., 2008).

We investigated the role of cellular Ca^{2+} balance in our TDP-43 models to learn more about the mechanisms of Ca^{2+}-mediated cellular demise. We report that a null mutation in calreticulin (CRT-1), a central regulator of ER Ca^{2+} homeostasis, suppresses both paralysis and the neurodegeneration caused by mutant TDP-43^{A315T} in motor neurons. Furthermore, deletion of the Ca^{2+} binding ER protein calnexin (CNX-1), the ER Ca^{2+} release channels UNC-68 (ryanodine receptor), or ITR-1 (inositol 1,4,5 triphosphate receptor) suppressed TDP-43 toxicity. Consistently, pharmacological manipulations modulating ER Ca^{2+} release and/or uptake suppressed TDP-43 toxicity. Downstream from perturbed Ca^{2+} homeostasis, we discovered that mutations in the Ca^{2+}-regulated calpain protease TRA-3 and aspartyl protease ASP-4 also suppressed TDP-43 toxicity.

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Our findings suggest that the regulation, and possibly release, of ER Ca^{2+} stores are required for neurotoxicity of TDP-43 in *C. elegans*. It is generally believed that caspase-driven neuronal apoptosis is an underlying pathogenic mechanism in many late-onset neurodegenerative diseases including ALS (Martin, 1999), but the executioner caspase CED-3 is dispensable for neurodegeneration in our TDP-43 model. Instead, the involvement of calpain and aspartyl proteases is more similar to necrosis-mediated cell death (Syntichaki et al., 2002). We propose that misfolded mutant TDP-43 increases $[Ca^{2+}]_i$ by disrupting ER function and activates calpain proteases, which in turn activates killer aspartyl proteases, leading to cell destruction.

Materials and Methods

C. elegans *strains and methods.* Standard culturing and genetic methods were used (Stiernagle, 2006). Animals were maintained at 20°C unless otherwise indicated. Unless otherwise stated, the strains used in this study were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN) and include the following: *asp-4(ok2693)*, *ced-3(ok2734)*, *cnx-1(nr2009)*, *cnx-1(nr2010)*, *crt-1(bz30)*, *crt-1(jh101)*, *itr-1(sa73)*, *kbIs7 [nhx-2p::rde-1 + rol-6(su1006)]*, *kzIs20[pDM#715(hlh-1p::rde-1) + pTG95(sur-5p::nls::GFP)]*, *oxIs12 [unc-47p::GFP + lin-15(+)]*, *rde-1(ne219)*, *sid-1(pk3321)*, *tra-3(ok2207)*, *uIs69 [pCFJ90(myo-2p::mCherry) + unc-119p::sid-1]*, and *unc-68(e540)*. Genetic crosses generated transgenic/mutant combinations, and the presence of transgenes and mutations was confirmed by PCR, visible markers, sequencing, or a combination thereof.

Transgenic lines expressing mutant TDP-43^{A315T}, wild-type TDP-43 (TDP-43^{WT}), *unc-47p*::GFP; TDP-43^{A315T}, and *unc-47p*::GFP; TDP-43^{WT} were previously described (Vaccaro et al., 2012a) and created as follows: human cDNAs for TDP-43^{WT} and TDP-43^{A315T} (a gift from Dr. Guy Rouleau, McGill University, Montreal, QC, Canada) were amplified by PCR and cloned into the Gateway vector pDONR221 following the manufacturer's protocol (Invitrogen). Multisite Gateway recombination was performed with the pDONR TDP-43 clones along with clones containing the *unc-47* promoter (a gift from Dr. Erik Jorgensen, University of Utah, Salt Lake City, UT; and Dr. Marc Hammarlund, Yale University, New Haven, CT), the *unc-54* 3′ UTR plasmid pCM5.37 (Addgene plasmid 17253; a gift from Dr. Geraldine Seydoux, Johns Hopkins University, Baltimore, MD), and the destination vector pCFJ150 (Addgene plasmid 19329; a gift from Dr. Erik Jorgensen, University of Utah) to create *unc-47p::TDP-43* expression vectors. Transgenic lines were created by microinjection of *unc-119(ed3)* worms, multiple lines were generated, and strains behaving similarly were kept for further analysis. Transgenes were integrated by UV irradiation, and lines were outcrossed to wild-type N2 worms five times before use. Several strains showing comparable phenotypes and transgene expression levels were kept and the strains used in this study include the following: *xqIs132*[*unc-47p::TDP-43^{WT}*; *unc-119*(+)] and *xqIs133*[*unc-47p::TDP-43^{A315T};unc-119*(+)].

Pharmacological treatment. All chemicals were obtained from Sigma-Aldrich. Dantrolene and thapsigargin were dissolved in DMSO and added to agar plate to final concentrations of 10 μM and 3 μg/ml, respectively. EGTA was dissolved in 1N NaOH and was added to agar plates to a final concentration of 0.5 mM. MDL-28170 was dissolved in DMSO and added to agar plate to a final concentration of 20 μM.

Paralysis assay. Worms were grown at 20°C on standard Nematode Growth Media (NGM) plates with or without compounds (30 animals/plate, by triplicates) and scored daily for movement. Animals were scored as paralyzed if they failed to move upon prodding with a worm pick. Failure to move their head to touch and the absence of pharyngeal pumping was scored as dead. Statistical analysis was performed using GraphPad Prism software (log-rank Mantel–Cox test).

Neurodegeneration assay. Worms (*unc-47p*::GFP; TDP-43^{A315T} or *unc-47p*::GFP; TDP-43^{WT}, with or without additional mutations listed above) were grown at 20°C on standard NGM plates with or without compounds. Young adult worms were transferred onto seeded NGM plates (with or without compounds), and were selected at days 1, 5, and 9 of adulthood (100 animals/treatment). Live worms were placed on a 2% agarose pad containing 5 mM levamisole in M9 medium to immobilize the worms. Worms were observed under fluorescence microscopy (Leica 6000 microscope) and scored for gaps or breaks in the processes of GABAergic neurons. The mean and SEM were calculated, and ANOVA with Bonferroni correction were used for statistical analyses.

RNA interference experiments. RNA interference (RNAi)-treated strains were fed *Escherichia coli* (HT115) containing an empty vector (EV) or an RNAi clone corresponding to the gene of interest indicated above. All RNAi clones were from the ORFeome RNAi library (Open Biosystems). RNAi experiments were performed at 20°C. Worms were grown on NGM enriched with 1 mM isopropyl-β-D-thiogalactopyranoside. All RNAi paralysis tests were performed using a TDP-43^{A315T}; *unc-47p*::GFP in conjunction with the appropriate mutation and transgenes for tissue-specific silencing in neurons, intestine, or muscle cells based on strains TU3401, VP303, or NR350, respectively. To minimize developmental effects, L4 worms were grown on plates with RNAi bacteria and assayed for paralysis as adults. Worms were transferred every 2 d.

Western blot analysis. Synchronized populations of worms expressing TDP-43 were grown at 20°C on standard NGM plates with or without compounds (15 plates/treatment). Immunoblot analysis of protein levels was performed on whole-animal extracts prepared by washing animals in M9 medium to remove adherent bacteria. The pellets were placed at −80°C overnight and homogenized in 1 ml/g RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) plus 0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml chymostatin; 1:1000). Pellets were sonicated and centrifuged at 16,000 × *g*. Supernatants were collected and were saved as the total fraction. Protein concentrations were determined by the Quick Start Bradford Protein Assay (Bio-Rad). Supernatants, 50 μg/well, were subjected to SDS-PAGE (10%) and transferred to nitrocellulose membranes (Bio-Rad). The immunoblotting analyses were performed using the following antibodies: rabbit anti-TDP-43 (1:1000; Proteintech) and mouse anti-actin (1:20,000 for worms; MP Biomedicals). Proteins were visualized using peroxidase-conjugated secondary antibodies and ECL Western Blotting Substrate (Thermo Scientific). Densitometry was performed with Photoshop (Adobe).

Results

Genetic manipulation of $[Ca^{2+}]$ suppresses TDP-43 toxicity in motor neurons

CRT-1 is a Ca^{2+} binding/storing protein of the ER that serves both as a molecular chaperone and as a central regulator of Ca^{2+} homeostasis (Michalak et al., 1999). Worms expressing mutant TDP-43 in their motor neurons show age-dependent motility defects, leading to paralysis and neurodegeneration (Vaccaro et al., 2012a). To investigate the role of Ca^{2+} balance in TDP-43 neuronal toxicity, we constructed *crt-1(bz30);* TDP-43^{A315T} and *crt-1(jh101);* TDP-43^{A315T} strains, and scored them for paralysis. We observed a significant reduction in the rate of paralysis for *crt-1(bz30);* TDP-43^{A315T} and *crt-1(jh101);* TDP-43^{A315T} animals compared with control TDP-43^{A315T} transgenics (Fig. 1*A*). Focusing on *crt-1(bz30);* TDP-43^{A315T}, we also observed a significant rate of motor neuron degeneration compared with control TDP-43^{A315T} transgenics (Fig. 1*B*).

In the ER, calreticulin works in conjunction with CNX-1 to execute chaperone functions and mediate cellular Ca^{2+} homeostasis (Krause and Michalak, 1997). Given the functional and structural similarities between the two proteins, we tested whether calnexin, encoded by *cnx-1* in *C. elegans*, also influenced TDP-43 toxicity. We observed that introduction of the loss of function mutations *cnx-1(nr2009)* or *cnx-1(nr2010)* into mutant TDP-43^{A315T} transgenics led to a significant decrease in paralysis compared with control TDP-43^{A315T} transgenics (Fig. 1*A*). Focusing on *cnx-1(nr2010);* TDP-43^{A315T}, we also observed a sig-

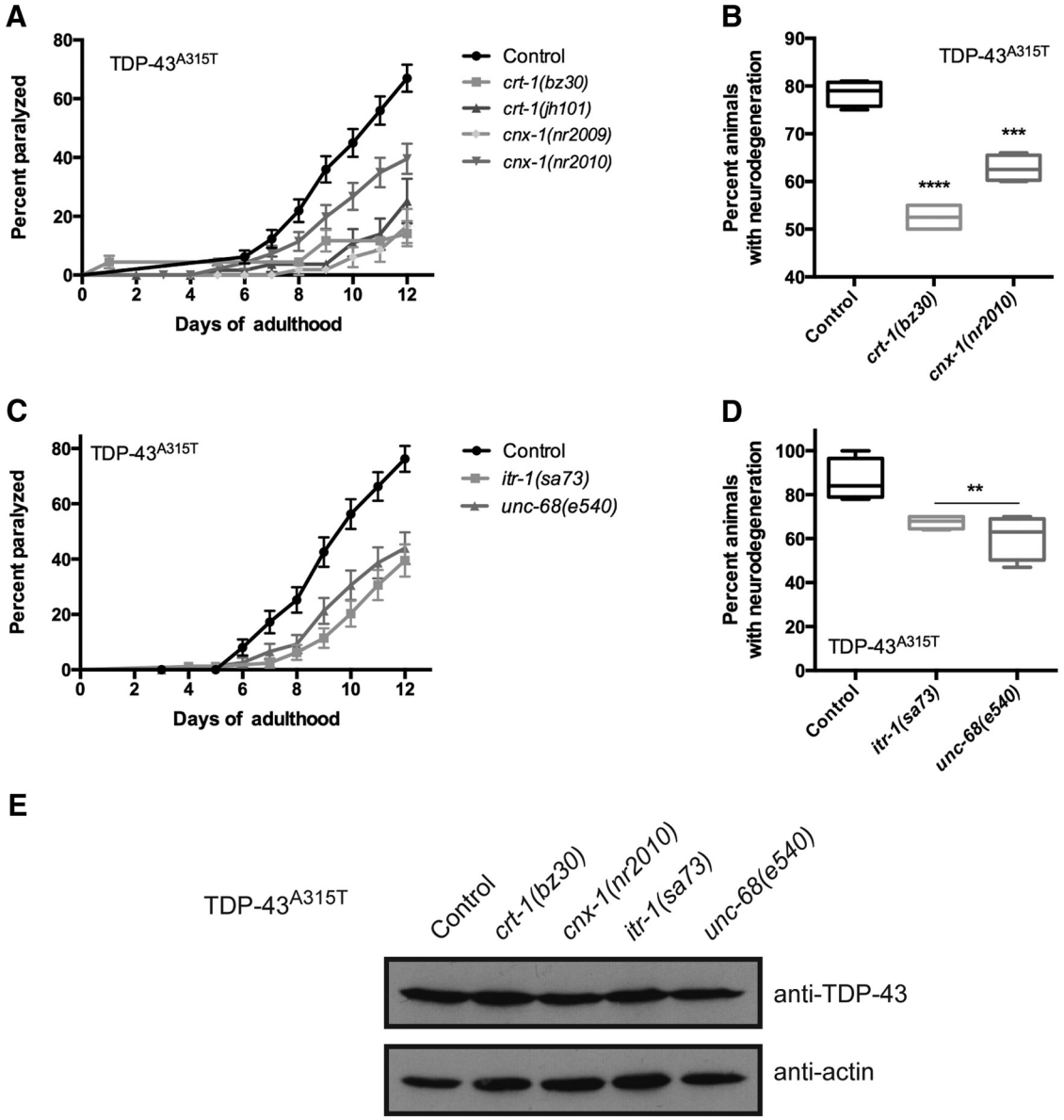

Figure 1. Genes regulating ER calcium release promote TDP-43 neuronal toxicity. ***A***, Null mutations in *cnx-1* or *crt-1* suppress age-dependent paralysis caused by TDP-43^{A315T} compared with transgenic TDP-43^{A315T} controls. $p < 0.0001$ for TDP-43^{A315T}; *cnx-1(nr2009)* versus TDP-43^{A315T}; $p = 0.0002$ for TDP-43^{A315T}; *cnx-1(nr2010)* versus TDP-43^{A315T}; $p < 0.0001$ for TDP-43^{A315T}; *crt-1(bz30)* versus TDP-43^{A315T}; $p < 0.0001$ for TDP-43^{A315T}; *crt-1(jh101)* versus TDP-43^{A315T}. TDP-43^{A315T}, $n = 114$; TDP-43^{A315T}; *cnx-1(nr2009)*, $n = 76$; TDP-43^{A315T}; *cnx-1(nr2010)*, $n = 98$; TDP-43^{A315T}; *crt-1(bz30)*, $n = 90$; and TDP-43^{A315T}; *crt-1(jh101)*, $n = 63$. ***B***, Mutations in *cnx-1* or *crt-1* reduce age-dependent neurodegeneration in TDP-43^{A315T} transgenics compared with TDP-43^{A315T} control animals. ***$p < 0.001$ versus TDP-43^{A315T} at day 9; ****$p < 0.0001$ versus TDP-43^{A315T} at day 9. ***C***, Null mutations in *unc-68* and *itr-1* reduce TDP-43^{A315T}-mediated paralysis compared with control TDP-43^{A315T} transgenics. $p < 0.0001$ for either for TDP-43^{A315T}; *unc-68(e540)* or for TDP-43^{A315T}; *itr-1(sa73)* versus TDP-43^{A315T}. TDP-43^{A315T}, $n = 90$; TDP-43^{A315T}; *itr-1(sa73)*, $n = 88$; and TDP-43^{A315T}; *unc-68(e540)*, $n = 84$. ***D***, Degeneration of motor neurons is reduced in adult day 9 TDP-43^{A315T} transgenics compared with controls. **$p < 0.01$ versus TDP-43^{A315T} at day 9. ***E***, Western blotting with a human anti-TDP-43 antibody revealed comparable levels of protein expression in all strains.

nificant decrease of motor neuron degeneration compared with control TDP-43^{A315T} transgenics (Fig. 1*B*).

To complete the genetic investigation of cellular Ca^{2+} balance, we tested the following two other genes involved in ER regulation of $[Ca^{2+}]_i$: the ER Ca^{2+} release channel inositol triphosphate receptor channel InsP3R, encoded by *itr-1* (Dal Santo et al., 1999), and the ER Ca^{2+} release channel ryanodine receptor channel RyR, encoded by *unc-68* (Maryon et al., 1996). We then investigated the effects of mutations in the InsP3R and RyR genes on TDP-43^{A315T}-mediated paralysis and motor neuron degeneration. Similar to the disruption of calnexin and calreticulin function, *itr-1(sa73);* TDP-43^{A315T} and *unc-68(e540)*; TDP-43^{A315T} strains displayed significantly reduced paralysis and motor neuron degeneration phenotypes compared with TDP-43^{A315T} controls (Fig. 1*C*,*D*). To confirm that the suppression of TDP-43^{A315T} neuronal toxicity was not due to transgene effects, we quantified the level of TDP-43 protein expression by immunoblotting and observed no difference in protein levels for TDP-43 in combination with any of the mutations (Fig. 1*E*).

Pharmacological modulation of $[Ca^{2+}]_i$ regulates TDP-43^{A315T} toxicity in motor neurons

To confirm that altering $[Ca^{2+}]_i$ levels in turn regulates TDP-43 toxicity, we turned to a complementary approach by using chemical reagents to manipulate ER Ca^{2+} release and/or uptake. We first treated TDP-43^{A315T} mutants with EGTA, a Ca^{2+}-specific

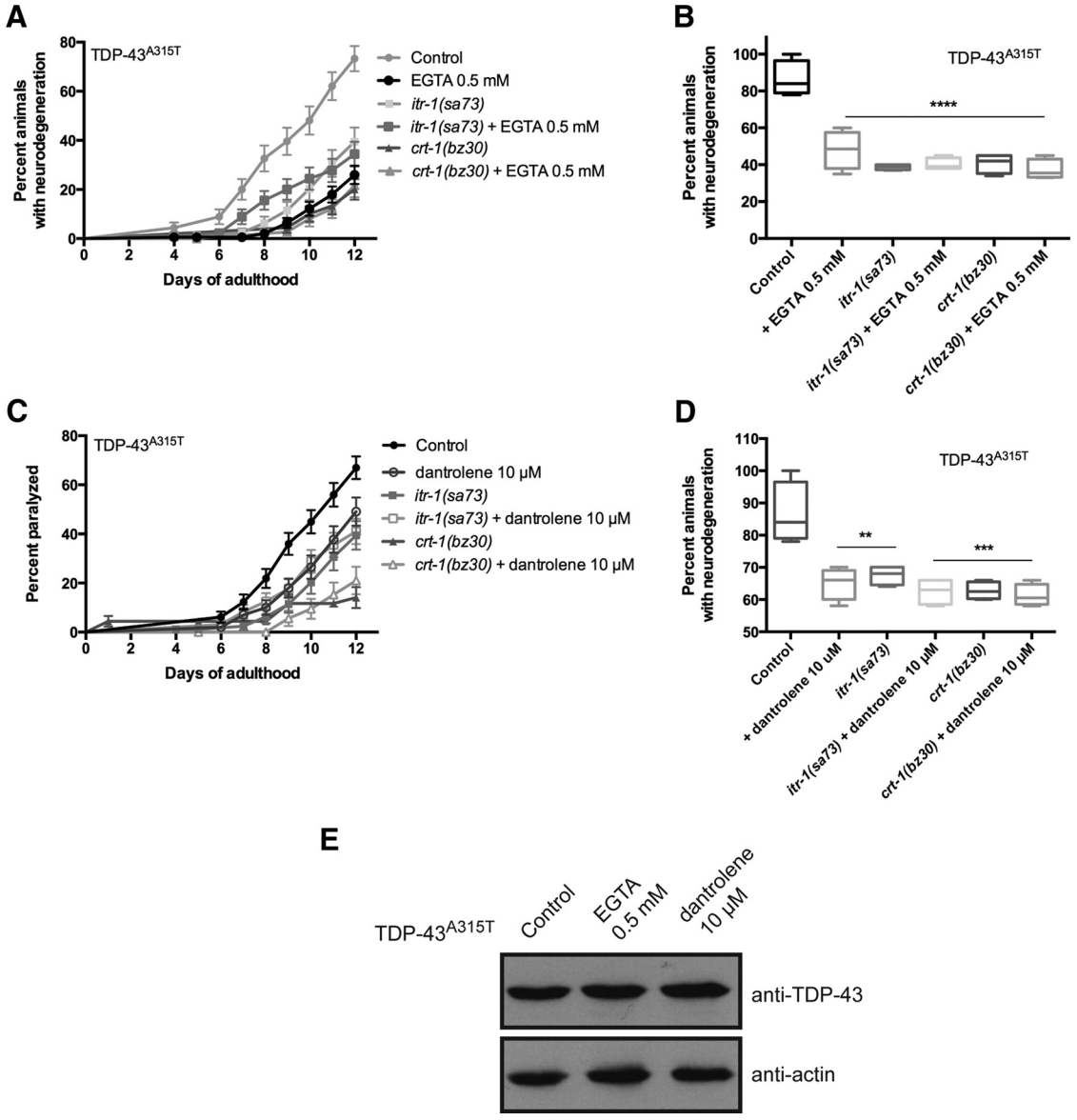

Figure 2. Pharmacological manipulation of $[Ca^{2+}]_i$ reduces TDP-43 neuronal toxicity. ***A***, TDP-43^{A315T} transgenics treated with EGTA showed significantly less paralysis compared with untreated controls ($p < 0.0001$ versus TDP-43^{A315T}). The protective effect of EGTA was not additive to the suppression of TDP-43-mediated paralysis by mutation in either *crt-1* or *itr-1*. $p = 0.8470$ versus TDP-43^{A315T}; *crt-1(bz30)*; and $p = 0.7817$ versus TDP-43^{A315T}; *itr-1(sa73)*. TDP-43^{A315T}, $n = 90$; TDP-43^{A315T} + EGTA, $n = 181$; TDP-43^{A315T}; *itr-1(sa73)*, $n = 90$; TDP-43^{A315T}; *itr-1(sa73)* + EGTA, $n = 90$; TDP-43^{A315T}; *crt-1(bz30)*, $n = 90$; TDP-43^{A315T}; *crt-1(bz30)* + EGTA, $n = 90$. ***B***, Degeneration of motor neurons in TDP-43^{A315T} animals at day 9 of adulthood was reduced to comparable levels in TDP-43^{A315T} transgenics treated with EGTA alone or in combination with mutations in *crt-1* or *itr-1*. ****$p < 0.0001$ versus TDP-43^{A315T} at day 9. ***C***, Treatment with dantrolene suppressed TDP-43^{A315T}-mediated paralysis compared with untreated control animals ($p = 0.0031$ versus TDP-43^{A315T}). Suppression of TDP-43^{A315T}-mediated paralysis by *crt-1* or *itr-1* was not significantly different from these same mutant strains treated with dantrolene. TDP-43^{A315T}, $n = 114$; TDP-43^{A315T} + dantrolene, $n = 100$; TDP-43^{A315T}; *itr-1(sa73)*, $n = 88$; TDP-43^{A315T}; *itr-1(sa73)* + dantrolene, $n = 96$; TDP-43^{A315T}; *crt-1(bz30)*, $n = 90$; TDP-43^{A315T}; *crt-1(bz30)* + dantrolene, $n = 90$. ***D***, Degeneration of motor neurons, in TDP-43^{A315T} animals at day 9 of adulthood was reduced to similar levels in TDP-43^{A315T} transgenics treated with dantrolene alone or in combination with mutations for *crt-1* or *itr-1*. **$p < 0.01$ versus TDP-43^{A315T} at day 9; ***$p < 0.001$ versus TDP-43^{A315T} at day 9. ***E***, TDP-43 protein expression was unchanged by culture conditions with EGTA or dantrolene.

chelator, and observed a clear reduction in the rate of paralysis and neurodegeneration compared with untreated TDP-43^{A315T} transgenics (Fig. 2*A*,*B*). Additionally, EGTA did not further suppress paralysis and neurodegeneration in *crt-1(bz30);* TDP-43^{A315T} or *itr-1(sa73);* TDP-43^{A315T} strains (Fig. 2*A*,*B*), suggesting that *crt-1*, *itr-1*, and EGTA use a common mechanism to reduce TDP-43 toxicity, namely reduced $[Ca^{2+}]_i$.

We next tested whether Ca^{2+} derived from ER stores might contribute to the progressive paralysis caused by mutant TDP-43. We treated TDP-43^{A315T} mutants with dantrolene, a reagent that specifically inhibits Ca^{2+} release from ER stores (Song et al., 1993). In dantrolene-treated animals, paralysis and neurodegeneration were markedly reduced consistent with the hypothesis that ER Ca^{2+} stores contribute to TDP-43 neuronal toxicity (Fig. 2*C*,*D*). Dantrolene treatment did not further suppress paralysis and neurodegeneration phenotypes caused by TDP-43^{A315T} in *crt-1(bz30)* and *itr-1(sa73)* mutants, suggesting a shared mechanism of action (Fig. 2*C*,*D*). The suppression of TDP-43 toxicity by EGTA and dantrolene were not due to reduced transgene expression since similar levels of TDP-43 protein expression were detected by immunoblotting in treated and untreated TDP-43 transgenics (Fig. 2*E*).

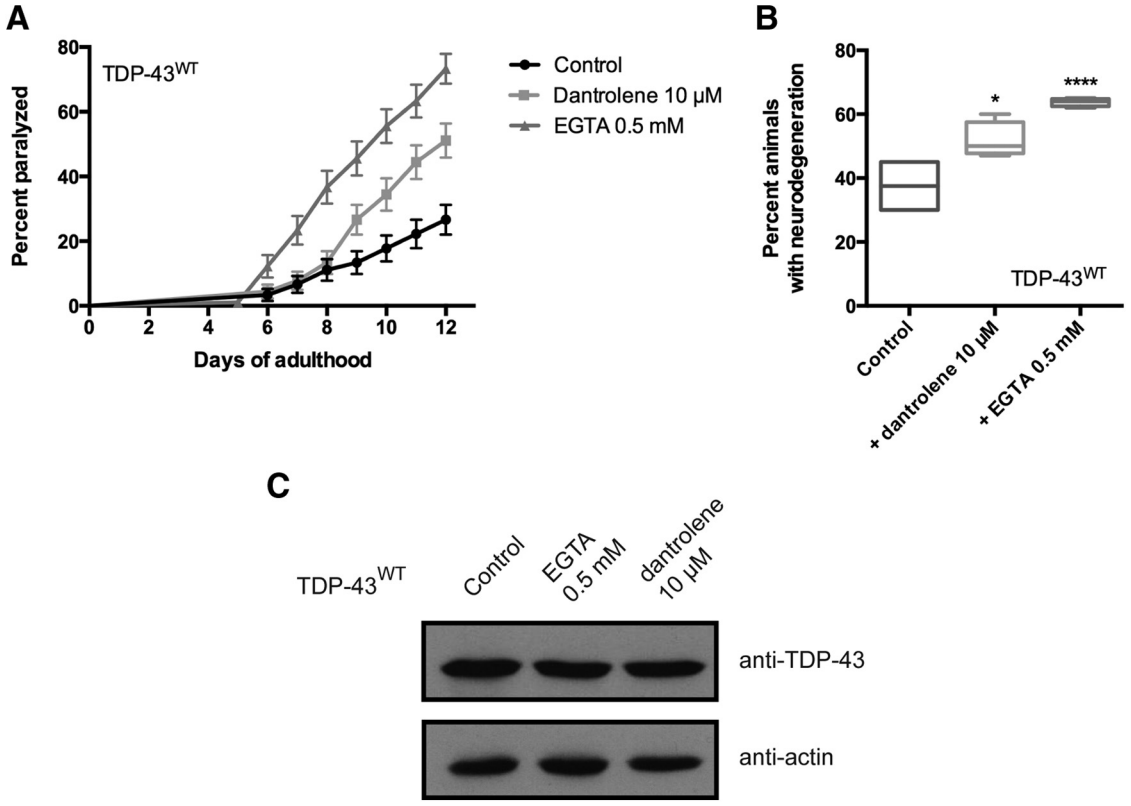

Figure 3. Disrupted Ca^{2+} homeostasis enhances wild-type TDP-43 toxicity. ***A***, Transgenic worms expressing TDP-43 ^{WT} treated with either EGTA or dantrolene had increased rates of paralysis compared with untreated controls ($p < 0.01$ for dantrolene-treated worms versus untreated TDP-43 ^{WT} controls; $p < 0.0001$ for EGTA-treated worms versus untreated TDP-43 ^{WT} controls). TDP-43 ^{WT}, $n = 90$; TDP-43 ^{WT} + dantrolene, $n = 90$; TDP-43 ^{WT} + EGTA, $n = 90$. ***B***, Treatment with dantrolene or EGTA increased neurodegeneration in adults on day 9; TDP-43 ^{WT} transgenics compared with untreated TDP-43 ^{WT} transgenics. *$p < 0.05$ versus TDP-43 ^{WT} at day 9, ****$p < 0.0001$ versus TDP-43 ^{WT} at day 9. ***C***, Similar levels of TDP-43 proteins were detected by Western blotting in untreated TDP-43 ^{WT} transgenics compared with animals treated with EGTA or dantrolene.

We previously reported that worms expressing mutant TDP-43, but not wild-type TDP-43, show elevated ER and oxidative stress (Vaccaro et al., 2013). Ca^{2+} release and uptake is essential for normal cellular function, and we hypothesized that chemically manipulating $[Ca^{2+}]_i$ may only be beneficial to neurons expressing mutant TDP-43 if disrupted Ca^{2+} homeostasis is indeed an underlying mechanism of mutant TDP-43 toxicity. To explore this possibility, we tested transgenics expressing TDP-43 ^{WT} with EGTA or dantrolene, and observed that exposure to either compound greatly enhanced paralysis and neurodegeneration (Fig. 3). Thus, chemically manipulating $[Ca^{2+}]_i$ is beneficial only to neurons expressing mutant TDP-43 proteins.

Thapsigargin-induced ER Ca^{2+} release restores TDP-43 ^{A315T}-dependent cell death in the absence of calreticulin

We next explored whether forced ER Ca^{2+} release might overcome the *crt-1*-induced block on TDP-43-mediated paralysis and neurodegeneration. Here, we sought to reverse *crt-1*-dependent suppression of TDP-43 neuronal toxicity by driving release of the remaining ER Ca^{2+} stores in TDP-43 ^{A315T}*; crt-1(bz30)* animals. We treated TDP-43 ^{A315T}*; crt-1(bz30)* animals, which are fully suppressed for paralysis and neurodegeneration, with thapsigargin, a compound that inhibits the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) ER Ca^{2+} reuptake pump and induces the release of ER Ca^{2+} via the InsP3 receptor channel (Takemura et al., 1989). We found that thapsigargin treatment significantly restored paralysis and neurodegeneration in TDP-43 ^{A315T}*; crt-1(bz30)* mutants compared with chemically untreated controls (Fig. 4*A*,*B*). As a positive control, we exposed transgenics expressing TDP-43 ^{WT} to thapsigargin, and we observed the enhancement of paralysis and neurodegeneration phenotypes (Fig. 4*A*,*B*), suggesting that increased $[Ca^{2+}]_i$ induces cellular damage in *C. elegans*. Finally, using immunoblotting we confirmed that treatment with thapsigargin did not affect the expression of TDP-43 transgenes (Fig. 4*C*). In summary, these data suggest that the elevation of $[Ca^{2+}]_i$ itself may be cytotoxic in nematode motor neurons, supporting a model in which a critical rise in $[Ca^{2+}]_i$ is a causative factor in neurotoxicity, and that CRT-1 is not required for thapsigargin-induced cell death.

Calpain and aspartyl proteases are required for neurodegeneration in a *C. elegans* ALS model

Caspase-dependent apoptosis is a major mechanism promoting cell death in neurodegenerative diseases (Fuchs and Steller, 2011). To determine whether apoptosis was involved in TDP-43 toxicity, we disrupted the main executioner protease, caspase CED-3, which mediates programmed cell death in *C. elegans* (Ellis and Horvitz, 1986). We observed that *ced-3* was not required for paralysis or degenerative phenotypes induced by mutant TDP-43 ^{A315T} in motor neurons (Fig. 5*A*,*B*). Thus, having shown that genetic and pharmacological manipulation of $[Ca^{2+}]_i$ suppresses neurotoxic effects of TDP-43 ^{A315T} in motor neurons, and based on the "calpain–cathepsin" hypothesis described previously (Yamashima et al., 1998), we investigated aspartyl and calpain protease function in paralysis and neurodegeneration. There are 17 genes with similarity to calpain, 7 of which show significant identity to mammalian calpains (Syntichaki et al., 2002), and 7 aspartyl protease genes encoded in the *C. elegans*

genome (Tcherepanova et al., 2000). Based on previous work linking some of these genes to neurodegeneration in *C. elegans* (Syntichaki et al., 2002), as well as the availability of viable loss-of-function mutant strains, we focused on the Ca^{2+}-regulated calpain protease TRA-3 and the aspartyl protease ASP-4. Furthermore, *asp-4* has previously been identified as a modifier of α-synuclein toxicity in *C. elegans* (Qiao et al., 2008). Using null mutants for *tra-3* and *asp-4*, we scored for paralysis and neurodegeneration in strains expressing TDP-43^{A315T}. We observed a significant reduction in the rate of paralysis and the progressive degeneration of motor neurons for TDP-43^{A315T}; *tra-3(ok2207)* or TDP-43^{A315T}; *asp-4(ok2693)* animals compared with control TDP-43^{A315T} strains (Fig. 5*A*,*B*). To confirm that these calpain and aspartyl proteases acted downstream of elevated $[Ca^{2+}]_i$ to regulate mutant TDP-43 toxicity, we treated TDP-43^{A315T}; *tra-3(ok2207)* and TDP-43^{A315T}; *asp-4(ok2693)* strains with thapsigargin. We observed that thapsigargin treatment failed to restore TDP-43^{A315T}-induced paralysis and neurodegeneration when the calpain or aspartyl proteases were absent (Fig. 5*C*,*D*). These data suggest that *tra-3* and *asp-4* are essential for calcium-mediated neurotoxicity associated with mutant TDP-43, and that these proteases may be a terminal effector of neurodegeneration. Furthermore, TDP-43 transgene expression was not affected by mutations in *ced-3*, *tra-3*, or *asp-4* (Fig. 5*E*). We next wanted to determine whether the calpain–aspartyl protease pathway could be a target for small-molecule intervention against TDP-43 toxicity. Z-Val-Phe-CHO (MDL-28170) is a calpain inhibitor previously shown to suppress necrosis in *C. elegans* (Syntichaki et al., 2002). We observed a significant reduction of TDP-43^{A315T}-mediated paralysis in worms treated with MDL-28170 (Fig. 5*F*). These data suggest that calpain and aspartyl proteases may be targeted for preventing neurodegeneration associated with mutant ALS proteins.

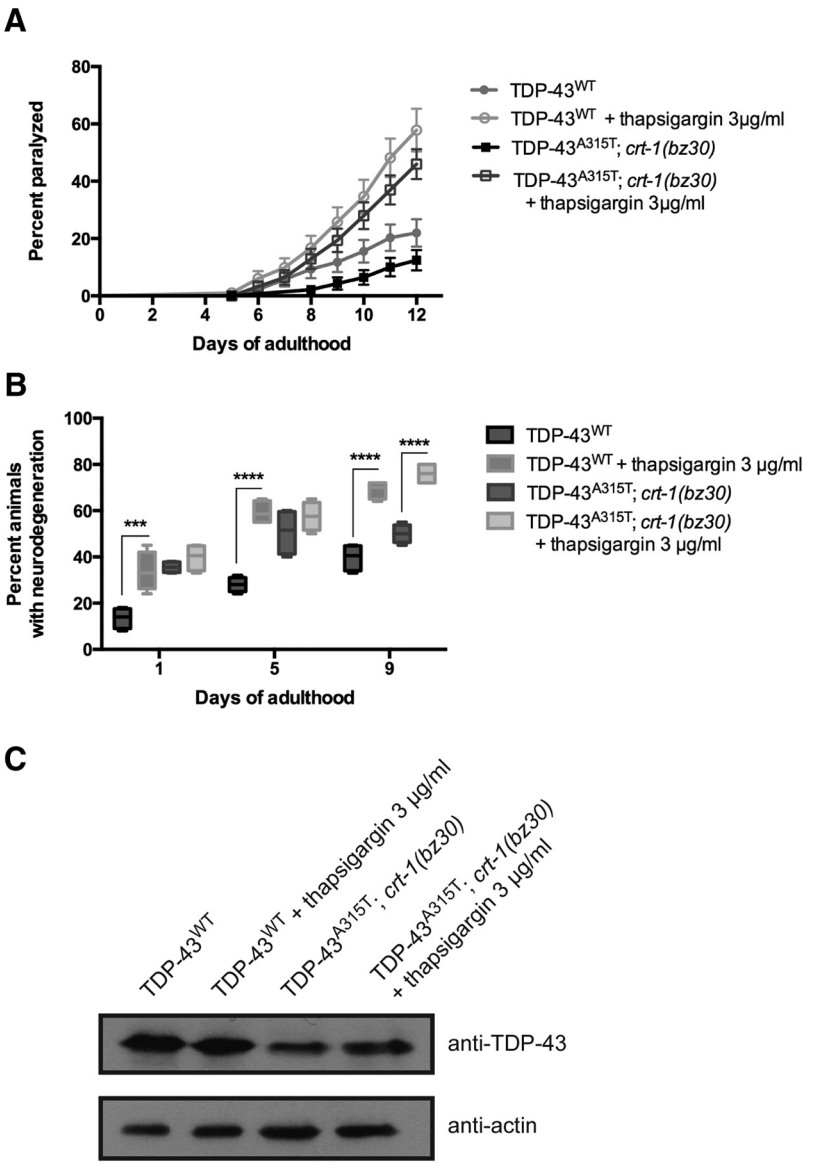

Figure 4. Forced release of ER Ca^{2+} stores enhances TDP-43 neuronal toxicity. ***A***, Paralysis was enhanced in TDP-43^{A315T}; *crt-1(bz30)* and TDP-43^{WT} transgenics treated with thapsigargin compared with untreated transgenic controls. $p < 0.001$ for TDP-43^{A315T}; *crt-1(bz30)* animals versus those treated with thapsigargin; $p < 0.001$ for TDP-43^{WT} versus those treated with thapsigargin. TDP-43^{WT}, $n = 98$; TDP-43^{WT} + thapsigargin, $n = 95$; TDP-43^{A315T}; *crt-1(bz30)*, $n = 96$; TDP-43^{A315T}; *crt-1(bz30)* + thapsigargin, $n = 100$. ***B***, Thapsigargin enhanced neurodegeneration transgenics expressing TDP-43^{WT} at days 1, 5, and 9 of adulthood compared with untreated controls. The suppression of neurodegeneration in TDP-43^{A315T}; *crt-1(bz30)* animals was lost by thapsigargin treatment in adult day 9 transgenics. ***$p < 0.001$ versus TDP-43^{WT} at day 1; ****$p < 0.0001$ versus TDP-43^{WT} or TDP-43^{A315T}; *crt-1(bz30)*. ***C***, Thapsigargin did not affect TDP-43 protein expression in TDP-43^{WT} or TDP-43^{A315T} worms.

Cell-autonomous suppression of TDP-43 toxicity by *tra-3* and *asp-4* in motor neurons

We wondered whether the regulation of TDP-43 toxicity was specific to *tra-3* and *asp-4*, or involved additional calpain and aspartyl proteases. Using RNAi, we conducted a blind screen of five calpain (*clp-1*, *clp-2*, *clp-4*, *clp-7*, and *tra-3*) and seven aspartyl (*asp-1*, *asp-3*, *asp-4*, *asp-6*, *asp-7*, *asp-10*, and *asp-13*) protease genes against TDP-43^{A315T} transgenics engineered for RNAi sensitivity only within the nervous system (Calixto et al., 2010). We observed that RNAi against only *tra-3* or *asp-4* significantly suppressed the paralysis phenotype of TDP-43^{A315T} transgenics (Fig. 6*A*,*B*). In conjunction with our experiments using null mutants, our RNAi experiments suggest that the regulation of TDP-43^{A315T} toxicity is specific to *tra-3* and *asp-4*. We wondered whether the effects of *tra-3* and *asp-4* in mediating TDP-43^{A315T} motor defects were cell autonomous or cell nonautonomous. We conducted *tra-3* or *asp-4* RNAi experiments in TDP-43^{A315T} transgenics sensitized to RNAi only within intestinal cells or body wall muscle cells. We observed no significant reduction of paralysis by *tra-3* or *asp-4* RNAi targeted to intestinal or body wall muscle cells (Fig. 6*C*,*D*). These data suggest that motor defects and degenerative phenotypes caused by TDP-43^{A315T} require the activity of *tra-3* and *asp-4* in the nervous system, and are not likely to be influenced by protease activity in other tissues. Unfortu-

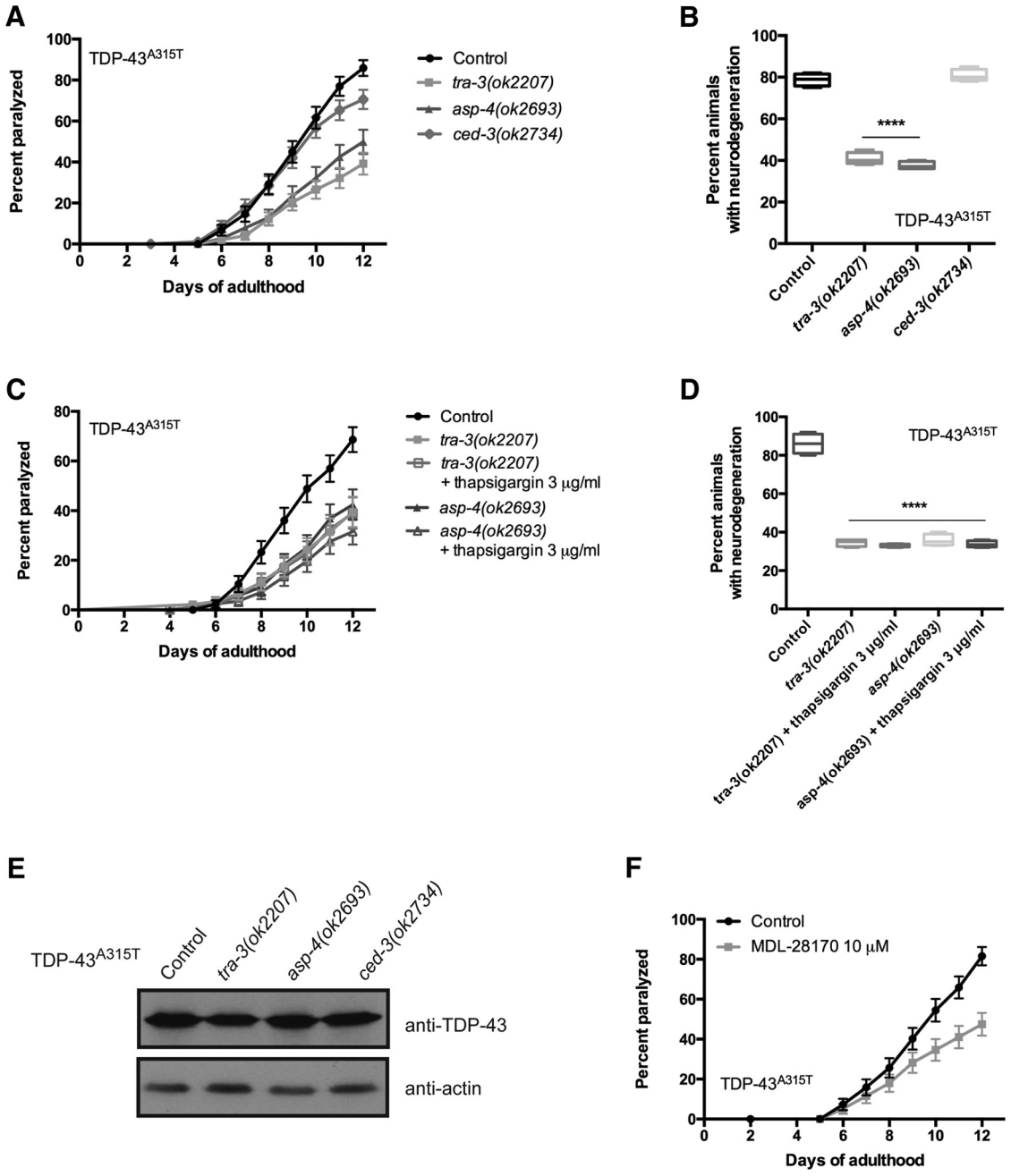

Figure 5. Calpain and aspartyl proteases facilitate TDP-43 neuronal toxicity. ***A***, Null mutations in *tra-3* or *asp-4* suppress age-dependent paralysis in TDP-43^{A315T} transgenics compared with TDP-43^{A315T} controls. Mutation in *ced-3* had no significant effect on paralysis phenotypes compared with TDP-43^{A315T}. $p < 0.0001$ for TDP-43^{A315T}; *tra-3(ok2207)* or TDP-43^{A315T}; *asp-4(ok2693)* versus TDP-43^{A315T} transgenic controls. TDP-43^{A315T}, $n = 90$; TDP-43^{A315T}; *tra-3(ok2207)*, $n = 102$; TDP-43^{A315T}; *asp-4(ok2693)*, $n = 79$; TDP-43^{A315T}; *ced-3(ok2734)*, $n = 96$. ***B***, Neurodegeneration was significantly reduced in adult, day 9, TDP-43^{A315T} transgenics by *tra-3* or *asp-4* null mutations compared with TDP-43^{A315T} alone. A null mutation of *ced-3* failed to suppress TDP-43^{A315T} neurodegeneration. ****$p < 0.0001$ versus TDP-43^{A315T} at day 9. ***C***, The suppression of TDP-43^{A315T}-mediated paralysis by *tra-3* or *asp-4* was unaffected by the addition of thapsigargin. $p < 0.0001$ for TDP-43^{A315T} versus TDP-43^{A315T}; *tra-3(ok2207)* or TDP-43^{A315T}; *asp-4(ok2693)* with or without thapsigargin treatment. TDP-43^{A315T}, $n = 90$; TDP-43^{A315T}; *tra-3(ok2207)*, $n = 90$; TDP-43^{A315T}; *tra-3(ok2207)* + thapsigargin, $n = 90$; TDP-43^{A315T}; *asp-4(ok2693)*, $n = 90$; TDP-43^{A315T}; *asp-4(ok2693)* + thapsigargin, $n = 90$. ***D***, Suppression of age-dependent neurodegeneration in TDP-43^{A315T} transgenics by *tra-3* or *asp-4* mutations was unchanged by thapsigargin treatment. ****$p < 0.0001$ versus TDP-43^{A315T} at day 9. ***E***, Null mutations of *tra-3*, *asp-4*, or *ced-3* did not affect TDP-43 protein expression. ***F***, The calpain inhibitor MDL-28170 reduced paralysis in TDP-43^{A315T} transgenics. $p < 0.001$ for treated versus untreated TDP-43^{A315T} animals. TDP-43^{A315T}, $n = 92$; TDP-43^{A315T} + MDL-28170, $n = 82$.

nately, we could not extend this analysis to Ca^{2+} homeostasis genes since *crt-1* and *itr-1* RNAi were ineffective in our assays.

Ca^{2+} homeostasis and protease genes do not suppress TDP-43^{WT} motor defects

Since our primary assay to identify neuroprotective agents depends on a behavioral assay to detect improved motility of TDP-43^{A315T} transgenics, there is the possibility that we may have identified mutants that nonspecifically augment motility phenotypes. To rule this out, we turned to our TDP-43^{WT} strains that show limited toxicity, with paralysis phenotypes comparable to the expression of GFP alone (Vaccaro et al., 2012a). We crossed *itr-1(sa73)*, *unc-68(e540)*, *crt-1(bz30)*, *cnx-1(nr2010)*, *tra-3(ok2207)*, or *asp-4(ok2693)* mutations into the

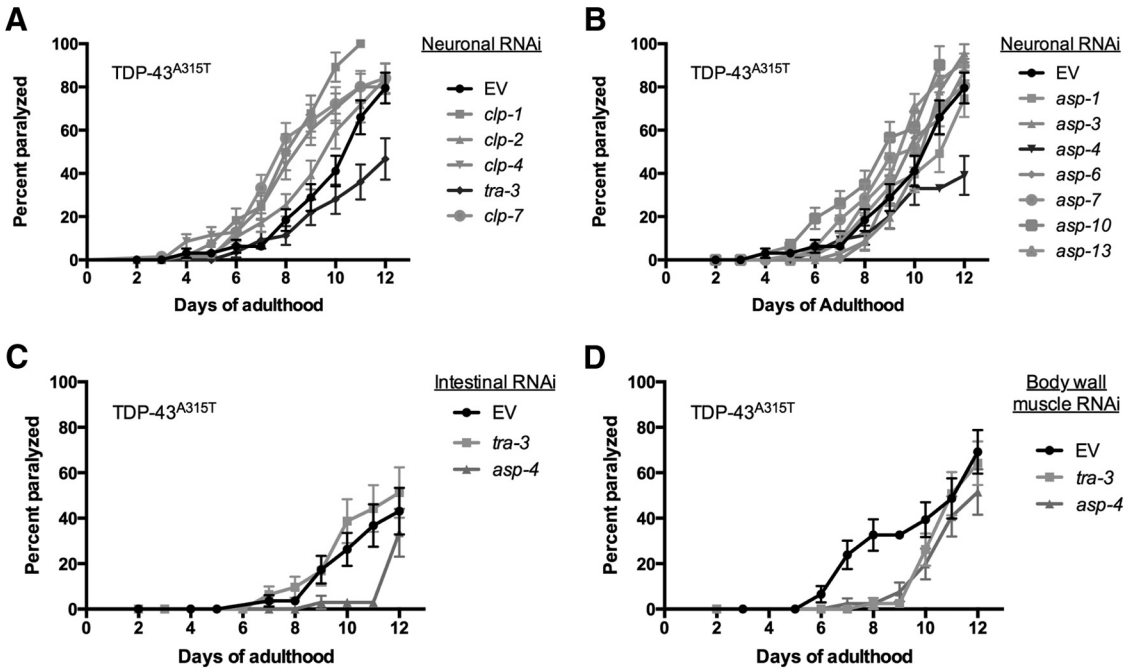

Figure 6. TDP-43-mediated motility defects require *tra-3* and *asp-4* in the nervous system. ***A***, RNAi against *tra-3* suppressed TDP-43^{A315T}-mediated paralysis. $p < 0.05$ for TDP-43^{A315T} treated with *tra-3*(RNAi) versus TDP-43^{A315T} treated with EV control RNAi. TDP-43^{A315T} + EV, $n = 71$; TDP-43^{A315T} + *clp-1*(RNAi), $n = 71$; TDP-43^{A315T} + *clp-2*(RNAi), $n = 84$; TDP-43^{A315T} + *clp-4*(RNAi), $n = 79$; TDP-43^{A315T} + *tra-3*(RNAi), $n = 68$; TDP-43^{A315T} + *clp-7*(RNAi), $n = 69$. ***B***, RNAi against *asp-4* suppressed TDP-43^{A315T}-mediated paralysis. $p < 0.05$ for TDP-43^{A315T} treated with *asp-4*(RNAi) versus TDP-43^{A315T} treated with EV control RNAi. TDP-43^{A315T} + EV, $n = 71$; TDP-43^{A315T} + *asp-1*(RNAi), $n = 54$; TDP-43^{A315T} + *asp-3*(RNAi), $n = 66$; TDP-43^{A315T} + *asp-4*(RNAi), $n = 67$; TDP-43^{A315T} + *asp-6*(RNAi), $n = 59$; TDP-43^{A315T} + *asp-7*(RNAi), $n = 62$; TDP-43^{A315T} + *asp-10*(RNAi), $n = 68$; TDP-43^{A315T} + *asp-13*(RNAi), $n = 58$. ***C***, There were no significant differences in the rates of paralysis for TDP-43^{A315T} sensitized for intestine-specific RNAi by treatment with EV(RNAi), *tra-3*(RNAi), or *asp-4*(RNAi). TDP-43^{A315T} + EV, $n = 77$; TDP-43^{A315T} + *tra-3*(RNAi), $n = 54$; TDP-43^{A315T} + *asp-4*(RNAi), $n = 59$. ***D***, There were no significant differences in the rates of paralysis for TDP-43^{A315T} sensitized for body wall muscle-specific RNAi by treatment with EV(RNAi), *tra-3*(RNAi), or *asp-4*(RNAi). TDP-43^{A315T} + EV, $n = 64$; TDP-43^{A315T} + *tra-3*(RNAi), $n = 51$; TDP-43^{A315T} + *asp-4*(RNAi), $n = 60$.

TDP-43^{WT} strain, but none of the mutations suppressed the paralysis phenotype caused by TDP-43^{WT} (Fig. 7). However, TDP-43^{WT}; *unc-68(e540)* animals had a higher rate of paralysis compared with TDP-43^{WT} controls ($p < 0.05$), suggesting that a general impairment of UNC-68 function may negatively impact the neuronal function and motility observed in TDP-43^{WT} worms. Thus, we conclude that neuroprotective effects of these mutants against TDP-43^{A315T} toxicity are not due to a general improvement of motor function.

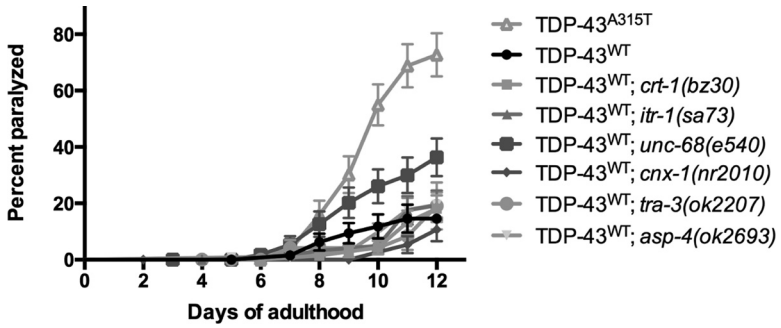

Figure 7. Calcium homeostasis and protease genes do not suppress motility defects in TDP-43^{WT} animals. There was no significant suppression of TDP-43^{WT} motility defects by Ca^{2+} or protease gene mutations. $p < 0.001$ for TDP-43^{A315T} versus TDP-43^{WT}. TDP-43^{A315T}, $n = 81$; TDP-43^{WT}, $n = 65$; TDP-43^{WT}; *crt-1(bz30)*, $n = 71$; TDP-43^{WT}; *itr-1(sa73)*, $n = 71$; TDP-43^{WT}; *unc-68(e540)*, $n = 60$; TDP-43^{WT}; *cnx-1(nr2010)*, $n = 61$; TDP-43^{WT}; *tra-3(ok2207)*, $n = 71$; TDP-43^{WT}; *asp-4(ok2693)*, $n = 115$.

Discussion

Ca^{2+} homeostasis and ALS

Ca^{2+} is an intracellular signaling molecule that regulates many mechanisms in the nervous system (Nikoletopoulou and Tavernarakis, 2012). Cells maintain a tightly controlled resting cytosolic free calcium concentration of ~100 nM by extruding excess Ca^{2+} by pumps and exchangers, and by compartmentalizing Ca^{2+}. The ER is involved in many critical processes, including being a specialized Ca^{2+}-storing organelle (100–800 μM range). The ER is closely involved in the regulation of Ca^{2+} flow within cells and is found in all neurons, occupying cell bodies, and extending toward axons, dendrites, and dendritic spines. In the context of ALS, evidence is mounting that the capacity of the cellular machinery of the ER to properly fold proteins is exceeded (Hetz and Mollereau, 2014), leading cells to react with the UPR^{ER} and that a perturbation of the ER function can induce Ca^{2+} depletion. Studies investigating Ca^{2+} homeostasis in motor neurons have shown that ALS-vulnerable motor neurons in mice display low endogenous Ca^{2+} buffering capacities (Lips and Keller, 1998; Palecek et al., 1999). We hypothesized that dysregulated $[Ca^{2+}]_I$, possibly from the release of ER Ca^{2+} stores, may contribute to mutant TDP-43 neuronal toxicity.

We tested for potential influences of Ca^{2+} in mutant TDP-43^{A315T}-induced degeneration in the following two ways: with genetic mutations that alter release of ER Ca^{2+} stores; and by using chemical reagents to manipulate ER Ca^{2+} release (Fig. 8). We found that null mutations in *crt-1*, a molecular chaperone that plays a critical and complex role in cellular calcium homeostasis as a major site for stored Ca^{2+} (Michalak et al., 1999), suppressed both paralysis and neurodegeneration induced by

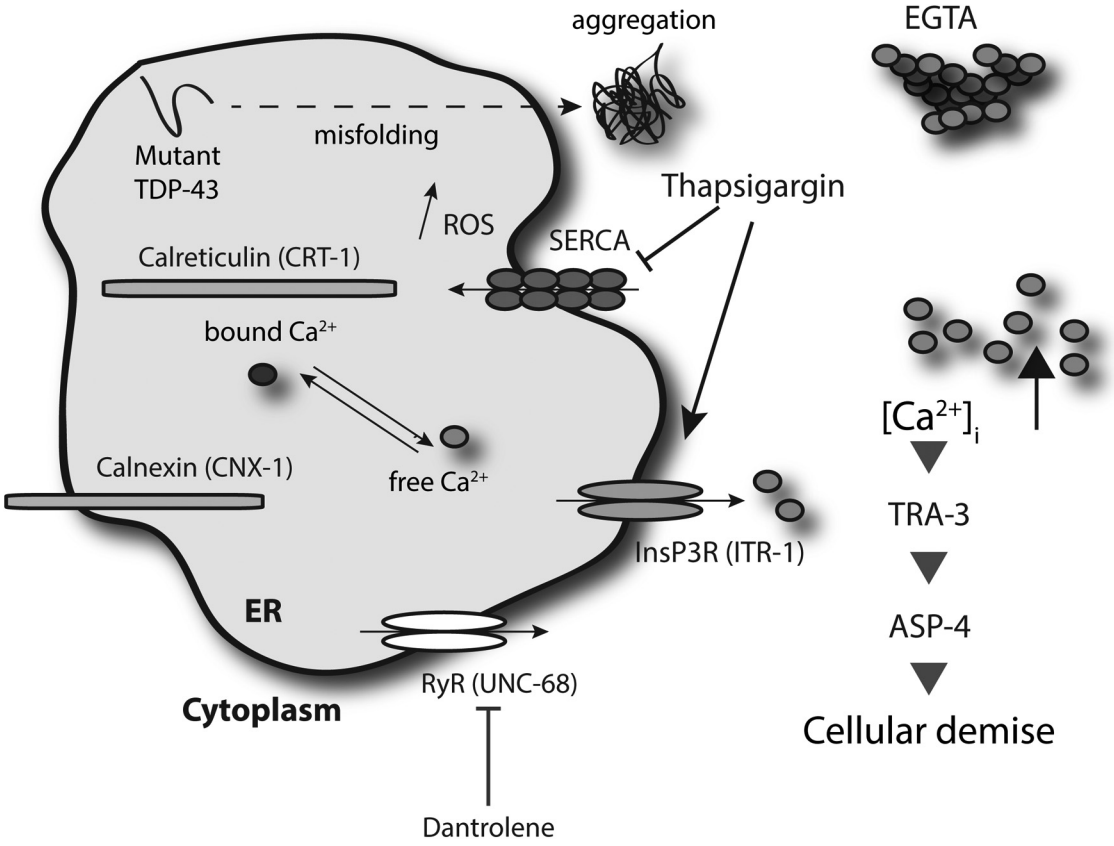

Figure 8. Working model for TDP-43 and Ca^{2+}-dependent necrosis-like neuronal toxicity. The chronic stress induced by protein misfolding and aggregation of mutant TDP-43 proteins may lead to inappropriate release of Ca^{2+} from ER stores into the cytoplasm. The resultant $[Ca^{2+}]_i$ increase is essential for downstream events, including activation of the Ca^{2+}-regulated TRA-3 calpain protease, which in turn mediates leakage of killer aspartyl proteases (ASP-4), leading to neuronal dysfunction and cell death. Mutations affecting ER Ca^{2+} storage (calreticulin and calnexin) or ER calcium release (InsP3R and RYR calcium release channels) disrupt release and are therefore neuroprotective. Pharmacological reduction of $[Ca^{2+}]_i$ by EGTA or dantrolene is also neuroprotective, while a forced increase of $[Ca^{2+}]_i$ by thapsigargin enhances neuronal toxicity. Disabling the activity of calpain or aspartyl proteases also protects against TDP-43-associated neuronal dysfunction and degeneration.

mutant TDP-43. Dysregulation of the ER-resident Ca^{2+} binding protein calreticulin may directly contribute to motor neuron death in ALS models (Bernard-Marissal et al., 2012). Our data showing that calreticulin contributes to neurodegeneration is somewhat at odds with findings for an SOD-1 model showing that reduced calreticulin levels activate the FAS/CD95 pathway to trigger cell death (Bernard-Marissal et al., 2012). However, no clear ortholog of FAS/CD95 exists in the *C. elegans* genome, suggesting that the role of calreticulin in mediating TDP-43 neuronal toxicity in our study may follow different cellular mechanisms. Alternatively, the mechanisms governing the degradation of SOD1 proteins may be distinct from TDP-43 (Mulligan and Chakrabartty, 2013).

However, ER stress has emerged as a mechanism in ALS (Matus et al., 2013; Tadic et al., 2014), and has been linked to the motor neuron vulnerability observed in SOD-1 mouse models (Nishitoh et al., 2008; Saxena et al., 2009), but it remains to be seen whether the ER is a site of action for other ALS mouse models. Furthermore, many aspects of the genetic signaling pathways controlling ER stress response were discovered in *C. elegans* and are conserved in mammalian systems (Mori, 2009). Encouragingly, we previously linked the ER stress response to TDP-43 toxicity (Vaccaro et al., 2013), and identified a number of small molecules that reduce neurodegeneration in *C. elegans* and zebrafish TDP-43 motor neuron models. Further insights into the induction of the ER stress response and neurodegeneration come from a recent report using a *C. elegans* model of SOD1 neuronal toxicity (Thompson et al., 2014). Linking the model organism studies to mammals are the ER stress-protective compounds salubrinal (Saxena et al., 2009) and guanabenz (Jiang et al., 2014), both of which show neuroprotective activity in mouse SOD-1 models. Thus, work from *C. elegans* models may be predictive for mechanisms of motor neuron degeneration in mammalian systems.

Because luminal calreticulin works in conjunction with calnexin to effectuate chaperone functions and mediate cellular Ca^{2+} homeostasis (Krause and Michalak, 1997), we also disrupted calnexin function using loss-of-function mutations and confirmed the suppression of TDP-43 neuronal toxicity. It is known that Ca^{2+} is released from ER stores into the cytoplasm via the InsP3R and the RyR Ca^{2+} channels. We blocked the RyR function by using a null mutation in *unc-68* or by treatment with dantrolene, a reagent that specifically inhibits Ca^{2+} release from ER stores (Song et al., 1993), and InsP3R by using a null mutation in *itr-1*, and we showed the same suppression of TDP-43 toxicity. Thus, our data extend upon and are consistent with recent work showing that inositol triphosphate receptors regulate neurotoxicity in *Drosophila* and cell culture TDP-43 models (Kim et al., 2012). Further highlighting the role of Ca^{2+} homeostasis, treatment of TDP-43^{A315T} mutants with EGTA, a Ca^{2+}-specific chelator, produced a clear reduction of paralysis and neurodegeneration phenotypes. Importantly, the fact that nei-

ther dantrolene nor EGTA enhanced TDP-43 toxicity in TDP-43^{A315T}; *crt-1* or TDP-43^{A315T}; *itr-1* double mutants suggests that the inappropriate release of Ca^{2+} from ER stores may be a common mechanism of TDP-43-mediated neuronal toxicity. Conversely, thapsigargin-induced ER Ca^{2+} release, by activating the InsP3R function and blocking the Ca^{2+} return to the ER from the cytoplasm via the SERCA Ca^{2+} pump, can restore TDP-43^{A315T}-induced cell death in the absence of calreticulin, indicating that Ca^{2+} release and uptake are essential for TDP-43^{A315T} neuronal toxicity, and can in fact also enhance the toxicity of worms expressing TDP-43^{WT} in motor neurons.

The role of endogenous TDP-1/TDP-43 in neurodegeneration

Our group (Vaccaro et al., 2012b), plus another research team (Zhang et al., 2012), previously reported that endogenous TDP-1 (*C. elegans* ortholog of TDP-43) is required for toxicity caused by the transgenic expression of mutant TDP-43 in the *C. elegans* nervous system. Additionally, we also showed that TDP-1/TDP-43 is required for the toxicity of mutant polyglutamine proteins in *C. elegans* and mammalian cell culture models of Huntington's disease (Tauffenberger et al., 2013). These findings suggest that wild-type TDP-1/TDP-43 may actively contribute to neurodegeneration beyond ALS. Our previous work also showed that *tdp-1* is ubiquitously expressed, and is mainly a nuclear protein. Under stress conditions, including ER stress, TDP-1 expression is increased, and this chronic, elevated expression is cytotoxic, leading to decreased lifespan in worms (Vaccaro et al., 2012b). These observations are consistent with the function of TDP-43 as a stress-inducible protein, as is seen in many systems (Janssens and Van Broeckhoven, 2013). Another conserved phenotype of TDP-43 is its cytotoxicity when overexpressed, suggesting that its expression levels are tightly regulated (Buratti and Baralle, 2011). A caveat of our data interpretation in this current study is that a role for TDP-1 in the necrosis-like degeneration of TDP-43^{A315T} motor neurons was not examined. Part of the cascade of molecular events described here leading to neurodegeneration may involve endogenous TDP-1. The cytotoxicity of TDP-1 itself may depend on Ca^{2+} homeostasis and/or protease genes. Additionally, since TDP-1 is a DNA/RNA binding protein, another possibility is that TDP-1 may regulate the expression of Ca^{2+} and protease genes under stress conditions, including for proteotoxicity and ER stress. Future studies will explore the contribution of TDP-1 to these molecular mechanisms.

Necroptosis as a key mechanism of neurodegeneration in ALS

The perturbation of cytosolic Ca^{2+} homeostasis has been implicated in necrotic cell death both in mammals and in *C. elegans* (Sattler and Tymianski, 2000; Xu et al., 2001), but the mechanism by which Ca^{2+} triggers cellular demise remains unclear; so, we investigated relevant signaling pathways based upon the "calpain–cathepsin hypothesis." In 1998, Yamashima et al. formulated the calpain–cathepsin hypothesis, according to which Ca^{2+}-activated cysteine proteases compromise the integrity of the lysosome and cause leakage of acidic hydrolases (Yamashima et al., 1998). We tested the requirement for calpain and aspartyl protease activity in neurodegeneration inflicted by the expression of TDP-43^{A315T} in GABAergic motor neurons, and showed that null mutations in the calcium-regulated *tra-3* calpain protease and aspartyl protease *asp-4* suppress both paralysis and neurodegeneration. Interestingly, TDP-43^{A315T} toxicity was unaffected by a null mutation in the cysteine–aspartate protease CED-3, a protein central to apoptosis in *C. elegans*, and in agreement with previous studies of TDP-43 toxicity in *C. elegans* (Liachko et al., 2010). Thus, in terms of genetic signaling pathways the neuronal toxicity caused by TDP-43^{A315T} in *C. elegans* more closely resembles necrosis than classic apoptosis (Troulinaki and Tavernarakis, 2012).

Recent work has suggested that motor neuron death in models of both sporadic and familial ALS occurs through necroptosis (Re et al., 2014), a form of programmed necrosis that does not require caspases (Ofengeim and Yuan, 2013). Our work is in agreement with this notion as inactivation of a *C. elegans* key executioner caspase, *ced-3*, had no effect on motor neuron dysfunction in our TDP-43 models. Key molecules regulating necroptosis in ALS models include the receptor-interacting serine/threonine-protein kinase 1 and mixed-lineage kinase domain-like, but whether orthologs of these proteins regulate TDP-43 toxicity in our *C. elegans* ALS models requires further investigation. Importantly, work from *C. elegans* detailing programmed necrosis may shed light on mechanisms relevant to neurodegeneration in mammalian settings and perhaps specifically ALS.

Abnormal Ca^{2+} signaling has been linked to multiple neurological disorders, but the challenge remains in identifying disease-specific mechanisms (Bezprozvanny, 2009). We propose that the chronic stress induced by misfolded mutant TDP-43 proteins induces the inappropriate release of Ca^{2+} from ER stores into the cytoplasm is a trigger for subsequent neurodegeneration (Fig. 8). Disrupted Ca^{2+} homeostasis may have multiple downstream, effector mechanisms promoting neuronal dysfunction and cell death, including the inappropriate activation of the Ca^{2+}-dependent proteases identified here, disrupting mitochondrial activity (Tradewell et al., 2011; Parone et al., 2013), or altered Ca^{2+} signaling at the neuromuscular junction (Armstrong and Drapeau, 2013). Thus, restoration of Ca^{2+} homeostasis in ALS motor neurons and/or limiting programmed necrosis may be pursued as potential therapeutic interventions.

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