Human Molecular Genetics, 2010, Vol. 19, No. 4 ***671–683***
doi:10.1093/hmg/ddp534
Advance Access published on December 3, 2009

Gain and loss of function of ALS-related mutations of *TARDBP* (TDP-43) cause motor deficits *in vivo*

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Received August 20, 2009; Revised and Accepted November 26, 2009

TDP-43 has been found in inclusion bodies of multiple neurological disorders, including amyotrophic lateral sclerosis, frontotemporal dementia, Parkinson's disease and Alzheimer's disease. Mutations in the TDP-43 encoding gene, *TARDBP*, have been subsequently reported in sporadic and familial ALS patients. In order to investigate the pathogenic nature of these mutants, the effects of three consistently reported *TARDBP* mutations (A315T, G348C and A382T) were tested in cell lines, primary cultured motor neurons and living zebrafish embryos. Each of the three mutants and wild-type (WT) human TDP-43 localized to nuclei when expressed in COS1 and Neuro2A cells by transient transfection. However, when expressed in motor neurons from dissociated spinal cord cultures these mutant *TARDBP* alleles, but less so for WT *TARDBP*, were neurotoxic, concomitant with perinuclear localization and aggregation of TDP-43. Finally, overexpression of mutant, but less so of WT, human *TARDBP* caused a motor phenotype in zebrafish (*Danio rerio)* embryos consisting of shorter motor neuronal axons, premature and excessive branching as well as swimming deficits. Interestingly, knock-down of zebrafisfh *tardbp* led to a similar phenotype, which was rescued by co-expressing WT but not mutant human *TARDBP*. Together these approaches showed that *TARDBP* mutations cause motor neuron defects and toxicity, suggesting that both a toxic gain of function as well as a novel loss of function may be involved in the molecular mechanism by which mutant TDP-43 contributes to disease pathogenesis.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) or Lou Gehrig's disease is a neurodegenerative disorder characterized by loss of upper and lower motor neurons. It is the third most common neurological disorder with an incidence of 1–2 people in 100 000, prevalence of 4–6 in 100 000 and a lifetime risk as high as 1 in 1000 (1,2). Generally, the onset of ALS occurs during mid-life (50–60 years) and death occurs generally within 1–5 years. Approximately 10% of ALS patients have a familial history for this disease (FALS), whereas the majority (90%) of cases appears to be of a sporadic nature (SALS) (3). *Copper/Zinc Superoxide Dismutase* (*SOD1*) was the first causative gene identified in ALS and its mutations underlie 10–20% of FALS (4). The *in vivo* expression of mutant SOD1 has been shown to cause a motor neuron disorder in several model organisms, including mouse (5), rat (6) and zebrafish (*Danio rerio*) (7). Experiments in these models jointly have helped to better understand the mechanisms of ALS pathogenesis caused by mutant SOD1 (2,8).

Over the years, a number of other genes have been linked to ALS, including *Alsin* (9), vesicle associated protein B (*VAPB)* (10), senataxin (11), vascular endothelial growth factor (*VEGF)* (12), angiogenin (13) and the most recently identified, fused in sarcoma/translated in liposarcoma (*FUS/TLS*) and TAR DNA Binding Protein (*TARDBP*). TDP-43, the protein coded by the *TARDBP* gene, was initially identified as a novel cellular protein that binds specifically to pyrimidine-rich

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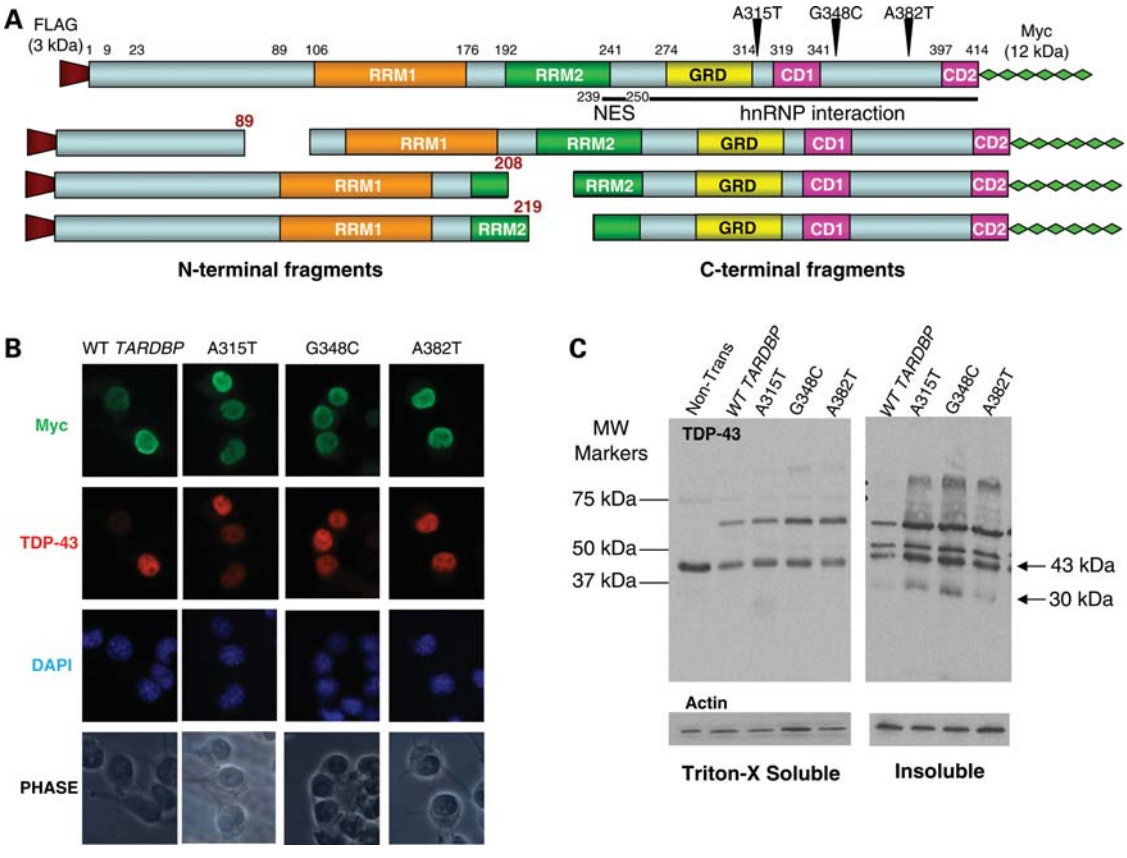

Figure 1. Schematic representation of TDP-43 and overexpression of WT and mutant *TARDBP* in cell lines. (**A**) A schematic representation of TDP-43 protein showing the functional domains, localization of FLAG (3 kDa) and myc tags (12 kDa), and sites where mutations found in ALS patients (A315T, G348C and A382T) were introduced by site-directed mutagenesis. (**B**) Triple immunocytochemical labeling using myc, TDP-43 and the nuclear marker, DAPI showing that TDP-43 were localized mainly in the nucleus. Cytosolic labeling and TDP-43-containing inclusions were very rare in cells overexpressing WT or mutant TDP-43 in cell lines. (**C**) Western blot analysis of cell lines overxpressing WT and mutant TDP-43 showed that the major band were the full-length of FLAG/*TARDBP*/myc (58 kDa) and endogeneous TDP-43 (43 kDa). Two minor bands at 45 and 35 were also observed.

motifs in the regulatory element of human immunodeficiency virus 1 (HIV-1) known as TAR DNA sequence motifs and to act as a transcriptional repressor of HIV-1 (14). TDP-43 has two RNA-binding motifs (RRM1 and RRM2) that bind to intronic TG repeats, recruit heterogeneous nuclear ribonucleoproteins (hnRNPs) (represented in Fig. 1A) (15) and promote exon splicing or exon inclusion of a number of mRNAs, including survival motor neuron 2 (*SMN2*) (16), cystic fibrosis transmembrane receptor (*CFTR*) (17) and apolipoprotein A-II (*Apo-AII*) (18), each of which has been previously implicated in neurodegenerative disorders; confirming the important role that TDP-43 plays in neuronal homeostasis and degeneration. In 2006, a breakthrough study by Neumann *et al*. (19) utilized proteomic methods to identify a serine phosphorylated C-terminal protein fragment of TDP-43 in preparations of detergent-insoluble inclusion bodies prepared from affected central nervous system tissue of ALS and FTD patients. Such inclusion bodies, which are ubiquitin-positive, have been the pathological hallmark of a broad range of neurodegenerative disorders for decades and multiple recent studies have confirmed the presence of TDP-43 within these inclusions (19–22). However, it is unclear whether TDP-43 is simply a pathological marker, or if mislocation and sequestration in inclusion bodies directly participates in the development of these diseases (23).

A new appreciation of the causative nature of TDP-43 in neurodegeneration was provided by several groups, including ours, who reported mutations in the *TARDBP* gene in FALS and SALS cases (24–26). To date, 14 reports have described 33 missense mutations and one truncating mutations in 29 FALS and 31 SALS cases (Supplementary Material, Table S1) (24–34). Except for a D169G mutation, all mutations are located in the glycine-rich domain (GRD) of the C-terminal region of the protein (25). While genetic and pathological evidence supports a role for TDP-43 in ALS, the underlying mechanisms of toxicity conferred by these mutations remain to be elucidated. An unusual amount of truncated TDP-43, reminiscent of the truncated fragment containing the C-terminus reported earlier in inclusion bodies (19,20), can be observed in whole cell homogenates derived from lymphoblasts of ALS patients with *TARDBP* mutations (25,27,29). In fact, truncated TDP-43 has a tendency to aggregate since it is selectively enriched in the detergent-insoluble fraction relative to the detergent-soluble fraction, and it further accumulates upon inhibition of proteasome activity (25,27). Additionally, levels of truncated TDP-43 containing the C-terminus accumulated when mutant M337V *TARDBP*, but less when WT *TARDBP*, were overexpressed in cell lines (26). Also, expression of two mutations (M3337V and

G294A), but not WT human *TARDBP*, in the spinal cord of chick embryos led to arrested development due to increased apoptosis (26). Further *in vitro* work has demonstrated that expression of progranulin, through caspase activation, also leads to the appearance of truncated TDP-43 products, leading to translocation of WT TDP-43 from the nucleus to the cytosol (35). Interestingly both progranulin (*PGRN*) and *TARDBP* mRNAs are up-regulated in the cytosol of mouse motor neurons following sciatic axotomies (36). However, the WT TDP-43 transported from the nucleus to the cytoplasm in these motor neurons is not truncated, suggesting that C-terminal truncation is not necessary for cytosolic translocation and subsequent toxicity (36). Altogether these studies indicate a toxic gain of function due to TDP-43 translocation from the nucleus to the cytosol may lead to formation of inclusion bodies. However, a possible loss of function phenotype (e.g. due to loss of expression in the nucleus) remains an untested possibility.

In this study we validated the pathogenicity of three recently described ALS-associated *TARDBP* mutations (A315T, G348C and A382T) in order to determine their pathogenicity. We first conducted experiments to test whether the expression of mutant TDP-43 in neuronal (Neuro2A) and non-neuronal (COS1) cell lines would lead to truncation, mislocalization or aggregation of TDP-43, or cell-specific toxicity. We also expressed mutant *TARDBP* specifically in motor neurons from primary dissociated spinal cord cultures to assess preferential toxicity in motor neurons by intranuclear injection of plasmid expression vector. This method has been used previously to create primary culture models of other motor neuron diseases, including FALS1 due to mutations in *SOD1* (37,38). Mutant human SOD1 preferentially aggregated into inclusions and induced cell death in these cultured motor neurons and this model has been utilized in multiple studies of pathogenesis and therapeutic assessment (37,38). Further, using an *in vivo* zebrafish (*Danio renio*) embryo model, we determined whether expression mutant human *TARDBP* or suppression of zebrafish *tardbp* is associated with selective motor neuron vulnerability early in development. Zebrafish are a well-established model used to study developmental biology because of their accessibility, optical transparency and rapid development (39,40). Over the past few years, this model organism has progressively become more common for the investigation of neurodegenerative disorders (41). A major advantage of this vertebrate model is the high homology to human genes. The zebrafish *tardbp* (NM_201476) has a 74% nucleotide and 72% amino acid identity with its human *TARDBP* orthologue (NM_007375) and contains both RNA binding motifs as well as a very high identity to the C-terminus region of TDP-43 (42). This genetic homology allows to effectively knock down (KD) specific mRNAs with antisense morpholino oligonucleotides (AMO) to test for loss of function or to overexpress mutant human *TARDBP* mRNAs to test for rescue or gain of function. We and others have used zebrafish to develop ALS models, including overexpression of mutant *SOD1* (7) and KD of *Alsin* (43) using AMOs. Thus, we established zebrafish models to examine whether *TARDBP* mutations directly cause a motor neuronal phenotype *in vivo* and to assess the potential involvement of gain and/or loss of function.

RESULTS

Characteristics of TARDBP mutations and cloning strategy

In order to evaluate the pathogenicity underlying *TARDBP* mutations found in ALS patients, three previously reported missense variants were selected (A315T, G348C and A382T) based on their being reported in more than one patient and by more than one group (Supplementary Material, Table S1) (24,25,28,29,31,34). The A315T variant was observed by our group in both a classic FALS case and by Gitcho *et al*. (24) in an individual affected by a familial lower motor neuron disease resembling ALS; in both cases the mutation segregated with the disease (24,25 #307). We observed the G348C mutation in four SALS cases from France (25), and recently it was also reported in German and Belgian FALS cases (31,34). Finally, our group identified the A382T mutation in two FALS cases from France (25), as well as it was reported in three more FALS and six more SALS from Italy (29,31).

To conduct tests for expression, mislocalization, aggregation and toxicity, FLAG and myc (six chains) were incorporated at the N- and C-termini of the WT *TARDBP* cDNA sequence, respectively (Fig. 1). A FLAG tag was fused to the N-terminus and six myc chains to the C-terminus portion of WT TDP-43 protein and the A315T, G348C and A382T mutations were generated subsequently by site-directed mutagenesis. Taken together, these two tags add only 15 kDa to the overall TDP-43 protein M.W. (FLAG $\sim$3 kDa and 6*myc $\sim$12 kDa) and therefore are expected to have minimal impact on the normal function of TDP-43. Both N- and C-termini tags were incorporated in order to identify both full length and cleaved species of TDP-43. In pathological inclusions from ALS and FTD patients, C-terminal fragments of TDP-43 were identified (with cleavage at amino acid 208) (47,48) (Fig. 1A). Furthermore, truncated forms cleaved at amino acids 89 and 219 (caspase 3 cleavage sites) have been described recently and associated with cell toxicity *in vitro* (49) (Fig. 1A). Similar to TDP-43 detected in inclusion bodies, these caspase-cleaved forms of TDP-43 are hyperphosphorylated, ubiquitinated and detergent-insoluble. (35). We therefore hypothesized that mutant TDP-43 would be cleaved, thus containing the myc chains, but not the FLAG tag. This cleavage would increase the cytosolic localization of TDP-43 and could induce cytotoxicity.

Neither WT nor TDP-43 mutants are toxic when expressed in Neuro2A or COS1 cell lines

Neuro2A cells were differentiated to develop neurites and neuronal-like properties through retinoic acid treatment. After transient transfection, expression of plasmid-derived WT human TDP-43 was similar to the endogenous TDP-43; the localization of WT or the three TDP-43 mutants was essentially nuclear. (Fig. 1B and Supplementary Material, Fig. S1). A similar pattern was also observed in COS1 cells transfected with WT and mutant *TARDBP* (Supplementary Material, Fig. S2). In rare occasions, WT and mutant TDP-43 were localized in the cytosol (Supplementary Material, Fig. S2; see arrows) and quantified in Supplementary

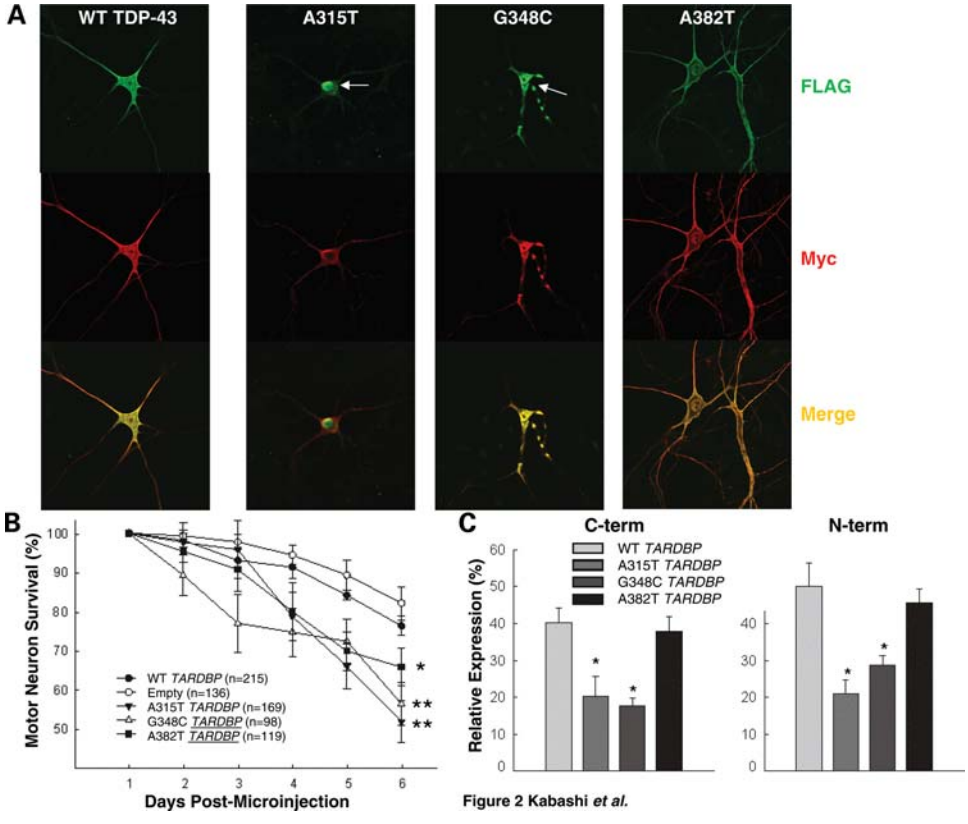

Figure 2. Mutant *TDP-43* expression in motor neurons leads to selective toxicity. Motor neurons from primary murine spinal cord-DRG cultures were microinjected (intranuclear) with the same vectors introduced in Figure 1 and 70 kDa dextran-FITC as a vital marker. (**A**) Immunocytochemical labeling revealed aggregation of TDP-43 in the perinuclear or neuritic compartments of neurons expressing in the more toxic mutants (A315T and G348C; see arrows) corresponding with a reduction of expression in dendritic and axonal processes in microinjected motor neurons with A315T and G348C mutants, but not WT *TARDBP*, or the least toxic, A382T mutant. (**B**) Viability of of motor neurons was measured over a period of 6 days by counting living cells containing the dextran-FITC and exhibiting an intact nucleus and neuritic processes. A315T, G348C and A382T were significantly more toxic compared with WT *TARDBP* cDNA injection. (**C**) Nuclear, axonal and dendritic expression of plasmid-derived TDP-43 was assessed following immunocytochemistry with antibodies to the C- (FLAG: green) and N- (myc:red) termini tags. Fluorescence was quantified using the Image J program. A significant decrease of both N- and C- termini labeling of TDP-43 was measured in dendrites and axonal processes, but not in nuclei (data not shown), of motor neurons overexpressing G348C and A315T mutants when compared with WT and the A382T *TARDBP* mutant ($^{*}P < 0.05$, $^{**}P < 0.01$).

Material, Figure S3A. Using immunoblotting analysis, the transient expression of mutant and WT TDP-43 was determined in the Triton-X 100 soluble and insoluble fractions of COS1 cells 72 h post-transfection (Fig. 1C) and in differentiated Neuro2A cells (data not shown). A major band of 58 kDa that represents full-length TDP-43 with both N- and C-termini tags was detected using antibodies to TDP-43 (Fig. 1C) as well as myc, and FLAG (Supplementary Material, Fig. S4; upper and middle panels) antibodies. Another major band of ~45 kDa was detected using the myc and TDP-43 antibodies (Fig. 1C and Supplementary Material, Fig. S4), but not FLAG antibody (Supplementary Material, Fig. S4), both in the soluble and insoluble fraction of WT and mutant TDP-43; this band may represent a truncated TDP-43 product of ~35 kDa lacking the N-terminus. Finally, a minor band immunodetected with myc and TDP-43, but not FLAG was enriched in the insoluble extract of mutant TDP-43 expressing cells at ~30–35 kDa, thus possibly representing the C-terminal region of TDP-43 at 18–25 kDa (Fig. 1C). Regardless, neither WT nor TDP-43 mutants appeared toxic to COS1 cells (Supplementary Material, Fig. S3B) or Neuro2A cells (data not shown) following cell counts and assessing nuclear envelope homogeneity by DAPI labeling.

Mutant TARDBP expression leads to selective toxicity in motor neurons

The WT and mutant *TARDBP* constructs described in Figure 1 were expressed in motor neurons of primary murine spinal cord-DRG cultures by intranuclear microinjection, with dextran-FITC being included in the injectate to follow these neurons in the living state by epifluorescence microscopy (37,38). In contrast to findings in cell lines (*vide supra*), the A315T, G348C and A382T *TARDBP* mutations were toxic to motor neurons relative to WT *TARDBP* (Fig. 2A) At 6 days post-microinjection the percentage of motor neurons lost relative to the number counted on day 1, was A315T: 48%, G348C: 44%, A382T: 31% and WT *TARDBP*: 23% of motor neurons dead (A315T versus WT $P = 0.001$; G348C versus WT $P = 0.01$; A382T versus WT $P = 0.03$) (Fig. 2B). In order to determine whether the 23% toxicity resulting from overexpression of WT *TARDBP* was significant, an empty plasmid containing both FLAG and myc tags

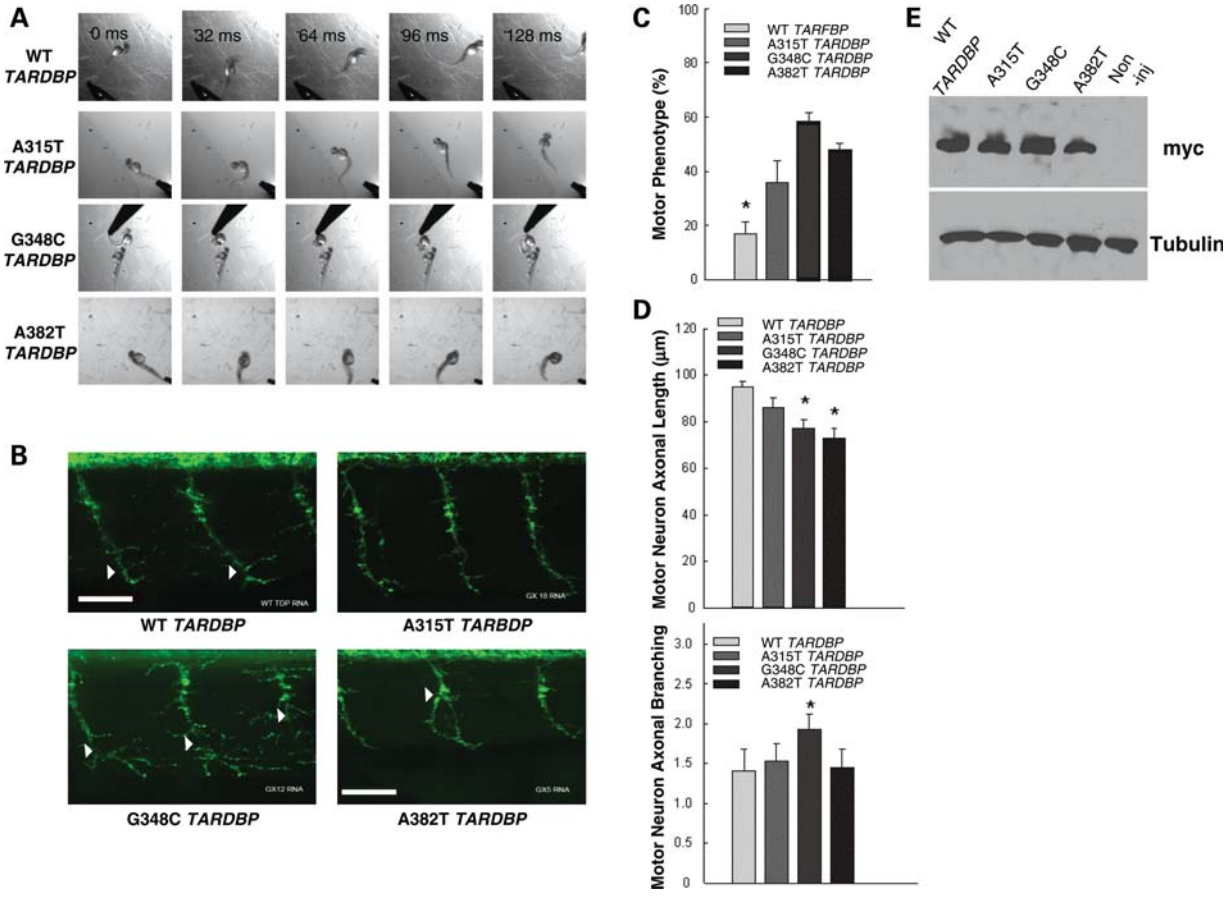

Figure 3. Expression of mutant *TARDBP* leads to a similar motor phenotype. (**A**) Deficit in the swimming response evoked by touch when 48 hpf zebrafish were injected with A315T (2nd panel), G348C (3rd panel) and A382T (4th panel) mutant *TARDBP* RNAs compared with zebrafish injected with WT *TARDBP* RNA (1st panel). (**B**) Expression of G348C and A382T mutant *TARDBP* RNA also led to excessive and premature branching and shorter motor axons (see arrows), whereas this phenotype was not as obvious in larvae expressing the A315T mutation. (**C**) The touch-evoked swimming phenotype was significantly increased when the A315T, G348C and A382T mutant RNAs were injected. (**D**) Motor axons of zebrafish injected with G348C and A382T *TARDP* RNA were significantly shorter than in zebrafish inected with WT and A315T *TARDBP* RNAs. Axon branching of embryos injected with G348C mutant *TARDBP* RNA, but not A315T or A382T, was significantly increased when compared with axonal branching in zebrafish expressing WT *TARDBP* RNA (*significantly different from WT *TARDBP* RNA; **significantly different from WT and G348C *TARDBP* RNAs).

was injected in primary motor neurons at the same concentration (25 ng/μl). A similar ($P = 0.61$) level of motor neuron death (18%) was measured at this concentration (Fig. 2B), indicating a non-specific basal lethality due to injection of constructs.

The intracellular distribution of plasmid-derived TDP-43 was determined by immunolabeling cultures with antibodies against the C-terminal myc and N-terminal FLAG tags. Total TDP-43 was visualized using TDP-43-specific antibodies. As previously reported in cultured hippocampal neurons (50), TDP-43 was detected in both nuclear and cytosolic compartments of the perikaryon as well as in dendritic and axonal processes (Fig. 2A). Cytosolic TDP-43 is thought to play an important role in RNA transport, formation of RNA granules and as an activity-dependent signaling factor in dendrites (50). Upon expression of WT *TARDBP* or the least toxic mutant A382T, both C- (FLAG: green) and N-termini (myc: red) tags were diffusely localized in the nucleus, cytosol and neuronal processes (Fig. 2A). On the other hand, perinuclear localization and aggregation of TDP-43 was observed in motor neurons injected with A315T and G348C mutant cDNA (Fig. 2A; see arrows), reminiscent of TDP-43 inclusions in motor neurons of ALS patients. Green (FLAG) and red (myc) fluorescence was quantified in nuclei, axonal and dendritic processes of these motor neurons as described in Materials and Methods. Though no change in fluorescence was observed in nuclear expression (data not shown), dendritic and axonal expression of N- and C-termini were significantly reduced in motor neurons injected with A315T and G348C mutants when compared with WT, and the least toxic mutant A382T (Fig. 2C). Finally, whereas for WT and the A382T mutant, labeling is similar with FLAG and myc antibodies, overexpression of the A315T and G348C *TARDBP* mutations induces a different distribution of FLAG and myc suggesting cleavage of TDP-43 could confer motor neuron toxicity. Thus, expression of mutant TDP-43 in motor neurons causes increased motor neuron death, and this cellular toxicity is associated with decreased expression in neurites, perinuclear accumulation and aggregation of mutant TDP-43.

Mutant TDP-43 overexpression causes a motor phenotype in zebrafish

The data from experiments in culture pointed to preferential vulnerability of motor neurons to toxicity of TDP-43 mutants. This was validated *in vivo* using the zebrafish model. The embryos normally respond to a touch with a vigorous contraction that initiates a bout of swimming. Expression of mutated human *TARDBP* resulted in embryos with a curly tail, their spontaneous coiling ability at 24 hpf was severely impaired (data not shown) and at 48 hpf were unable and/or were delayed to swim away following a touch (Fig. 3A; second row), indicating that there was a motor but not a sensory deficit. The phenotype was dose-dependent and most consistent, with minimal lethality, at a concentration of 25 ng/μl and at this concentration we observed the phenotype (Fig. 3A; third row) within 24 hpf in 57% of zebrafish embryos injected with G348C *TARDBP* mutant RNA, 47% of those injected with the A315T mutation and in 39% of those injected with the A382T mutation but only in 17% of those injected with WT *TARDBP* mRNA (A315T versus WT $P = 0.01$; A382T versus WT $P = 0.03$; Fig. 3C and Table 1). No significant ($P = 0.22$) motor phenotype was observed upon injection of a mismatched morpholino (11%) when compared with WT *TARDBP* mRNA (17%) (Table 1). No changes in TDP-43 protein expression were observed upon injection of WT and mutant TARDBP RNAs as determined by western blot analysis (Supplementary Material, Fig. S5). This phenotype was not observed in zebrafish embryos injected with dye only or non-injected (Table 1). We also observed shorter and disorganized axons with excessive branching from motor neurons as measured by labeling with SV2 antibody, a marker of synaptic vesicles (Fig. 3B) or with acetylated tubulin, a marker growing axons (data not shown). The length of the motor axons ($P < 0.001$) was decreased and motor axon branching ($P = 0.02$) was increased when fish injected solely with G348C and A315T *TARDBP* RNA were compared with fish injected with WT *TARDBP* RNA (Fig. 3B and D), whereas injection of A382T *TARDBP* caused a significant change in motor axon length ($P = 0.01$), but no significant changes in axon branching when compared with fish injected with WT *TARDBP* RNA ($P = 0.36$).

Knockdown of *tardbp* causes a loss of function motor phenotype in zebrafish

We designed an AMO to specifically bind and block translation of *tardbp* (NM_201476). Microinjection of this AMO caused a dose-dependent phenotype and at a relatively low concentration (0.1 mM) there was minimal lethality and a motor phenotype similar to that described earlier for overexpression of mutant human *TARDBP* was observed (delay or absence of touch-evoked escape response). Specifically, more than 60% of embryos injected with *tardbp* AMO had a curly tail phenotype and their coiling ability was severely impaired (Fig. 4A and Table 1). We observed shorter and disorganized motor neuronal axons following labeling with antibody against the synaptic vesicle marker, SV2, but no changes in the number of interneurons labeled by Pax2 (Fig. 4B). No significant changes in the total number of motor axons and their respective somites were measured in embryos injected with TDO-43 AMO and/or overexpressing mutant *TARDBP*, indicating that there was no motor neuron death in these embryos. The effectiveness of the AMO was confirmed via western blotting (Fig. 4C). As a control, a mismatch AMO of the *tardbp* AMO with four nucleotide substitutions was injected at the same concentration that did not affect TDP-43 protein levels (Fig. 4C) and no coiling defects were observed. No coiling defects were also observed upon injection of an AMO that would bind and block the translation of the *tardbp-like* gene (NM_198364) (data not shown), which has high homology to the N-terminus portion (including the RRM1 and 2), but has very little homology to the portion that encodes the C-terminus of TDP-43. These data suggest that the phenotype observed in zebrafish where *tardbp* was KD is specific to the motor behavior and could be due to down-regulation of the C-terminal region of TDP-43.

At 48 hpf, zebrafish injected with *tardbp* AMO were unable to swim away following a touch, even though they were sensitive to touch (Fig. 5A; second row). This phenotype was not observed in zebrafish embryos injected with dye only or non-injected (Fig. 5A; first row) and was rarely observed in embryos injected with a mismatched AMO (Table 1). The motor neuron specific phenotype caused by *tardbp* AMO was significantly reduced by co-injecting WT human *TARDBP* RNA (Fig. 5A; third row) from 58 to 13% ($P < 0.001$) (Table 1 and Fig. 5C). Similarly, a small percentage of fish (17%; $P < 0.001$) had this phenotype in zebrafish embryos upon microinjection of WT *TARDBP* RNA (Fig. 5A; fourth row); thus both co-injection of *tdp-43* AMO and WT *TARDBP* RNA as well as injection of WT *TARDBP* RNA alone led to virtually indistinguishable percentages with over 70% of the fish injected appearing completely normal (Table 1). Further, labeling of motor neurons with antibodies to SV2 (Fig. 5B) and acetylated-tubulin (data not shown) showed premature, disorganized and excessive branching of motor axons and slightly shorter motor axons at 24 and 48 hpf. The motor axon length was significantly reduced by ~30% and axonal branching was significantly increased by almost 2-fold in *tardbp* AMO embryos when compared with non-injected embryos at 48 h hpf ($P < 0.001$) (Fig. 5D).

Table 1. Profile of the phenotype profile in zebrafish injected with *tardbp* AMO and/or *TARDBP* RNA

Condition	Motor phenotype	Normal	Delayed (Monster)
Non-injected (680; 21)	0	94.1	5.3
Mismatch AMO (109; 3)	11.3	84.1	4.6
TDP-43 AMO (638; 15)	63.7	24.6	9.4
Rescue wthTDP-43 (205; 5)	13	68.8	20.7
Rescue A315T TDP-43 (149; 4)	55.6	22.7	22.4
Rescue G348C TDP-43 (155; 4)	73.3	8.8	18
Rescue A382T TDP-43 (167; 5)	46.5	32.7	12.4
wthTDP-43 (163; 5)	17.2	68.9	13.9
A315T TDP-43 (161; 5)	35.4	37.6	26.5
G348C TDP-43 (153; 5)	57.3	27.9	14.8
A382T TDP-43 (152; 4)	47.2	29.9	22.7

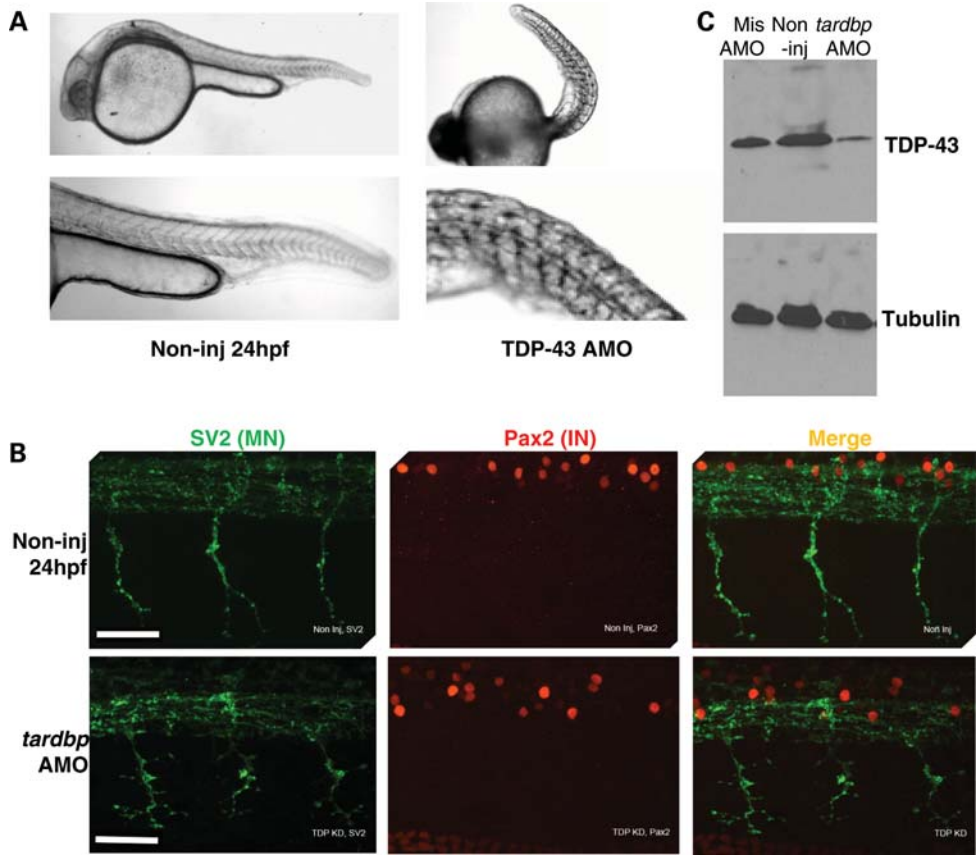

Figure 4. KD of *tardbp* causes developmental defects in zebrafish. A specific AMO was designed to specifically bind and inhibit the translation of *tardbp* RNA. (**A**) Zebrafish injected with *tardbp* AMO 24 h post-fertilization have deficits in the coiling behavior as compared with non-injected fish (Non-inj). (**B**) These defects were accompanied by a decrease in length and an increase in branching of axons from motor neurons as measured by labeling with an SV2 antibody, whereas the number of interneurons as labeled by Pax2 was not affected. (**C**) Immunoblot analysis of protein extracts from zebrafish injected with *tardbp* AMO, mismatch AMO and non-injected demonstrates that TDP-43 expression was dramatically reduced specifically in the fish that were injected with *tardbp* AMO.

Co-injection of WT *TARDBP* RNA as well as the injection of WT *TARDBP* RNA alone rescued significantly the length of the motor axons ($P < 0.001$) and the branching pattern in zebrafish (WT *TARDBP* Rescue $P = 0.003$; WT *TARDBP* $P = 0.02$) (Fig. 5D) Therefore, expression of WT human *TARDBP* RNA is able to partially rescue the swimming and coiling phenotype as well as the axonal length and branching from zebrafish motor neurons induced by *tdp-43* AMO. These results indicate a specific loss of function phenotype upon KD of *tardbp*.

Mutant TARDBP fails to rescue the KD phenotype

In contrast to the rescue by WT *TARDBP*, the motor neuron phenotype caused by KD of *tardbp* was not rescued by co-injection of A315T mutant *TARDBP* RNA (Fig. 6A; second row), G348C mutant *TARDBP* RNA (Fig. 6A; third row) or A382T mutant *TARDBP* RNA (Fig. 6A; fourth row). The phenotype caused by KD of *tardbp* expression in 65% of fish injected was comparable to the 56, 73 and 47% phenotype observed following co-injection of *tardbp* AMO with A315T, G348C and A382T mutant human *TARDBP* RNAs, respectively (Table 1; Fig. 6C). For each of the three mutants RNAs co-injected with *tardbp* AMO, the phenotype was significantly different from embryos co-injected with WT *TARDBP* RNA and *tardbp* AMO (A315T versus WT $P < 0.001$; G348C versus WT $P < 0.001$; A382T versus WT $P = 0.01$). In fact, co-injection of *tardbp* AMO and G348C *TARDBP* RNA caused a slightly more pronounced phenotype in zebrafish embryo (Table 1 and Fig. 6C). In the case of each mutant, the motor axon and branching phenotype was not significantly altered from the embryos only injected with *tardbp* AMO, but the length of the motor axons was significantly shorter (A315T versus WT $P < 0.001$; G348C versus WT $P < 0.001$; A382T versus WT $P < 0.001$) and the branching significantly increased (A315T versus WT $P < 0.001$; G348C versus WT $P < 0.001$; A382T versus WT $P = 0.03$) (Fig. 6D) when each of the three mutant *TARDBP* RNAs co-injected with *tardbp* AMO (Fig. 6B; second to fourth image) was compared with fish co-injected with WT *TARDBP* RNA and *tardbp* AMO (Fig. 6B; first image). Overall, these results suggest that TDP-43 expression plays an important role in motor neuron development. Furthermore, only WT, but not mutant TDP-43, is able to functionally replace *tardbp* KD in zebrafish. These data indicate that a loss of function might contribute to the motor disorder induced by the mutants as a result of impaired function of the endogenous WT protein.

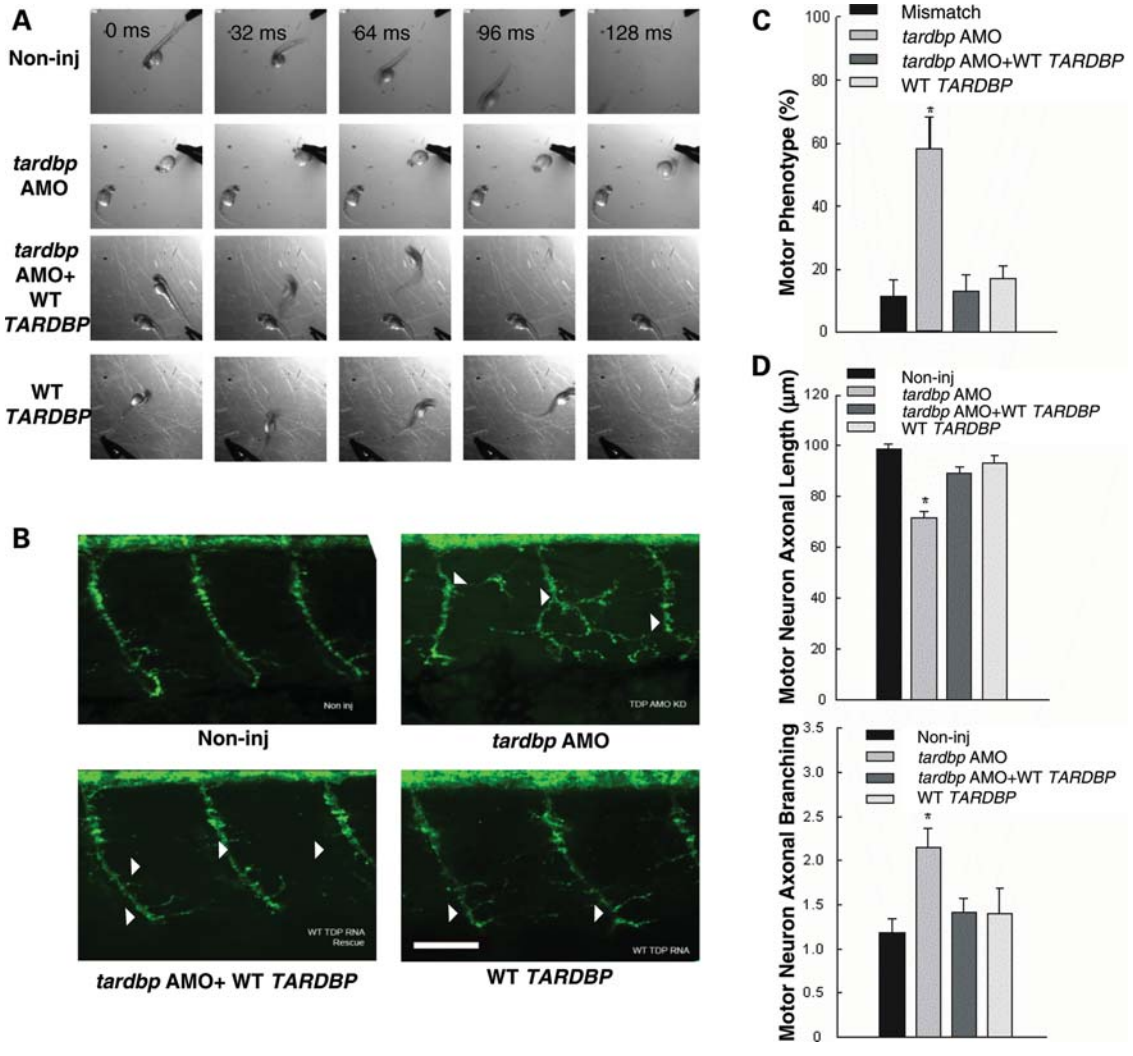

Figure 5. KD of *tardbp* leads to a motor phenotype in zebrafish. (**A**) Zebrafish develop a touch-evoked escape response by 48 hpf as observed in non-injected larvae (1st panel). This swim escape response was severely impaired in zebrafish injected with *tardbp* AMO (2nd panel). This phenotype could be rescued by co-injection of WT *TARDBP RNA* and *tardbp* AMO (3rd panel) and was not impaired by injection of WT *TARDBP* RNA alone (4th panel). (**B**) Immunocytochemical labeling of motor axons by SV2 antibody revealed a premature and excessive branching and shortening of the motor axons in fish injected with *tardbp* AMO. (**C**) A significant increase in the swimming phenotype was only observed when *tardbp* AMO was injected, which was observed to a much lesser extent in zebrafish co-injected with *TARDBP* RNA and *tardbp* AMO or injected with WT *TARDBP* RNA alone. (**D**) Quantification of the motor axon length and branching demonstrated that only fish injected with *tardbp* AMO had a significant shortening of the motor axons and a significant increase of axonal branching ($^{*}P < 0.05$).

DISCUSSION

Several dominantly inherited mutations in *TARDBP* have been reported in more than one patient and by more than one group, corroborates their causative role in disease pathogenesis (Supplementary Material, Table S1) (24,25,28,29,31,34), but it remains to be proved whether these mutants can induce a motor neuronal phenotype and to determine the mechanism of toxicity. In this study, the role of three new *TARDBP* mutations (A315T, G348C and A382T) was investigated using well-established *in vitro* (primary motor neurons and cell lines) and *in vivo* (zebrafish) experimental models. The data validate that these mutations confer toxic properties to the protein, TDP-43, of which motor neurons are particularly vulnerable, and that their expression causes a motor phenotype in zebrafish embryos. In neuronal (Neuro2A) and non-neuronal (COS1) cell lines expression of mutant TDP-43 did not induce toxicity and aggregation was not observed. In contrast, in primary motor neurons derived from murine spinal cord cultures, expression of A315T and G348C *TARDBP* mutations caused toxicity and abnormal redistribution relative to the similarly expressed WT TDP-43 protein. Finally, overexpression of the *TARDBP* mutations or KD of the native *tardbp* in zebrafish embryos led to similar swimming and motor neuron axonal phenotypes. However, the embryos remained sensitive to touch, and the number of interneurons in the spinal cord was not affected upon KD, suggesting that motor neurons are specifically affected. Both the swimming and motor axon phenotypes were partially rescued upon KD by expression of WT *TARDBP* RNA, but not when each of the three *TARDBP* mutant RNAs were expressed. These results indicate that mutant TDP-43 caused motor neuron

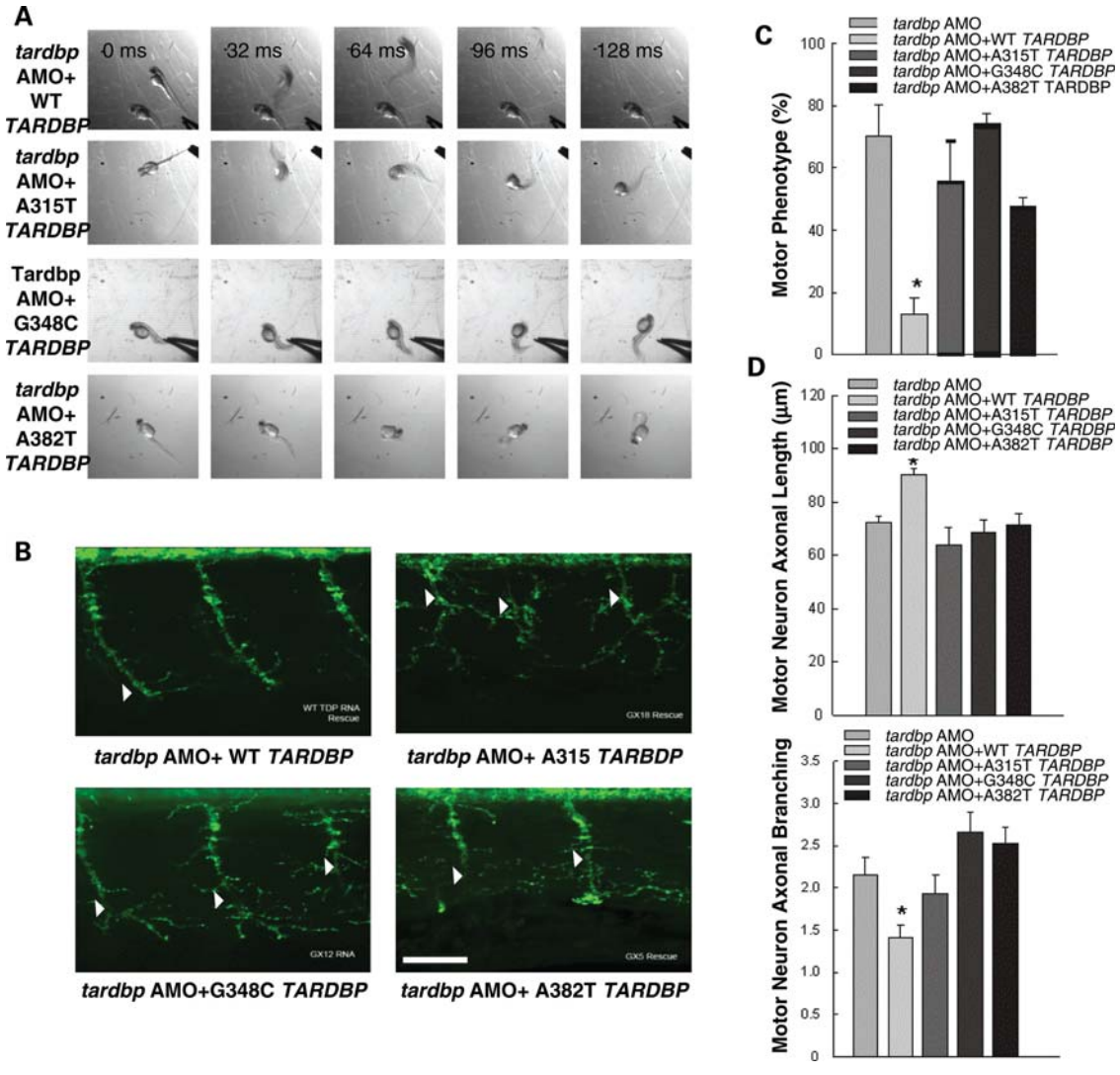

Figure 6. Mutant TARDBP RNA does not rescue the *tardbp* KD motor specific phenotype. (**A**) The touch-evoked swim-escape response of zebrafish at 48 hpf could be rescued when *tardbp* AMO was co-injected with WT *TARDBP* RNA (1st panel). However, the A315T (2nd panel), G348C (3rd panel) and A382T (4th panel) were unable to rescue the phenotype as quantified in (**D**). (**B**) The excessive and premature branching as well as the shortening of the motor axon observed in zebrafish injected with *tardbp* AMO was also evident in the embryos co-injected with all three mutants (A315T, G348C and A382T) RNAs, but was rescued upon co-injection of WT *TARDBP* RNA as quantified in (**D**) ($^*P < 0.05$).

abnormalities in zebrafish through molecular mechanisms involving simultaneously a toxic gain and a novel loss of function.

TDP-43 acts as a transcription repressor and recruits hnRNP to bind and splice the RNA of a variety of genes linked to neurodegenerative disorders, including *SMN2* (16), *CFTR* (17) and *Apo-AII* (18). Thus it is thought to play an important role in neuronal developmental and degenerative processes. In concordance to this, expression of mutant TDP-43 in chick embryos led to arrested development and knock-out of the *Drosophila* homologue of TDP-43 caused deficient locomotive behaviors, reduced life span and defects at the neuromuscular junctions of a variety of neurons, including motor neurons (51). TDP-43 is involved in nuclear-cytoplasmic shuttling of RNA granules including NFL, NFH and β-actin mRNAs to the neurites (52). Furthermore, TDP-43 translocates to these RNA granules in dendrites following neuronal depolarization via KCl stimuli (50). Our results using both motor neuron cultures and zebrafish embryos indicate that mutant TDP-43 expression could genuinely cause the loss of these neuronal specific functions. Indeed, expression of mutant TDP-43 was selectively reduced in the axonal and dendritic processes of cultured motor neuron cultures. Further, mutant TDP-43 expression in zebrafish embryos led to a shortening of the motor axon with increased, premature and disorganized branching. Very recently, two groups reported that mutations of the *FUS/TLS* gene were found in FALS (53,54). Interestingly, similar to TDP-43, FUS/TLS plays an important role in transcriptional regulation as a protein that interacts with several hnRNP, and is known to bind, splice, translate and transport RNAs (55). Further studies are needed to determine whether TDP-43 and FUS/TLS play a

complementary role in RNA granules and nucleo-cytoplasmic RNA shuttling and whether these functions are affected upon expression of mutant TDP-43 and FUS/TLS.

Here we show for the first time that mutant TDP-43 can cause motor neuron toxicity in primary motor neuron in culture as well as both a loss and gain of function motor phenotype and abnormal development of motor axons in zebrafish. The molecular mechanism through which mutant TDP-43 is toxic to motor neuron is still not understood and a better understanding of the molecular partners of TDP-43 as well as its function in motor neurons is required. Expression of the G348C mutant alone caused a strong motor phenotype, whereas expression of A315T and A382T led to a less pronounced, but still significant phenotype when compared with zebrafish injected with WT *TARDBP* RNA. This toxic gain of function associated with mutant TDP-43 expression could be similar to the role that mutant SOD1 plays in ALS pathogenesis. Thus, several of the pathogenic functions associated with mutant SOD1, including protein misfolding (56–58), glutamate excitoxicity (59), oxidative stress (56) as well as axonal transport deficits (60) could participate in mutant TDP-43 pathogenic mechanisms. However, unlike *TARDBP*, it is commonly assumed that a loss of function is not implicated in dominantly inherited FALS because of expression of a normal allele. This is particularly so in the case of FALS1 caused by mutant *SOD1*, since knock-out of *Sod1* in mice does not lead to a motor neuron phenotype (61). However KD of *tardbp* did lead to a deficit in motor neuron development and a motor deficit in zebrafish embryos, supporting the possibility of a loss of function mechanism of toxicity. Similar to the results presented in this report, *huntingtin*, the gene responsible for HD through an expansion of polyglutamine repeats leads to pathogenic mechanisms through a toxic gain of function or loss of function depending on the location of the stretch of polyglutamine repeats (62). In fact similar to TDP-43 results reported here, both KD of *huntingtin* (63) as well as overexpression of expanded polyglutamine repeats (64) in zebrafish embryos lead to impaired neuronal development arguing that both loss and toxic gain of function may be involved in the molecular pathways that lead to neurodegeneration in these diseases. Furthermore, a number of biochemical and genetic studies suggest that other genes that cause neurodegeneration through polyglutamine expansion, including Ataxin 1 (65,66) and androgen receptor (67), contribute to disease by both gain of function and a partial loss of function mechanisms.

The knowledge obtained from the zebrafish embryo is useful to understand pathogenic mechanisms of mutant TDP-43, but certain limitations arise from the use of this developmental model in the case of ALS which has an apparent mid-life onset in patients. It is interesting to note that altering expression in zebrafish of several genes related to early-onset [*SMN1* (68) and *ALS2* (43)] as well as late-onset motor degeneration [*SOD1* (7), *HSP8* (45), *TAU* (69)] and here *TARDBP* all result in shorter, more highly branched motor axons and similar deficits in early swimming. This evidence indicates that a common developmental and possibly degenerative pathway is affected. It will be important to eventually study adult zebrafish expressing mutant TDP-43 and follow these fish throughout their lifespan for a motor neuron disorder in order to further understand pathogenic processes of motor neuron degeneration. Our transient transgenic *in vivo* model nonetheless suggests the preferential targeting of motor neurons by mutant TDP-43 is consistent with other ALS-causing mutant proteins and a role in disease pathogenesis.

MATERIALS AND METHODS

Plasmids and vectors

Human TARDBP cDNA (untagged) was obtained from Open Biosystems. Site-directed mutagenesis to introduce mutations in the appropriate vectors was performed using a QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene, #200516) as previously described (44,45). To evaluate the consequences of *TARDBP* mutations, we have generated cDNA constructs encoding N- (FLAG) and C-termini (myc) tagged WT and three *TARDBP* mutations, A315T, G348C and A382T subcloned into pCS2+ plasmid vectors. These constructs were used in all the experiments described here.

Cell lines

Neuro2A and Cos1 cell lines were maintained in Dulbecco's minimal essential medium (DMEM) enriched with 10% fetal calf serum (FCS) and 1% penicillin/ streptomycin/neomycin (PSN). To differentiate into neuronal-like cells and to extend neurites, 50% confluent Neuro2A cells were treated with retinoic acid by replacing the feeding medium with DMEM supplemented with 2% FCS and 20 μM all-*trans*-retinoic acid (Sigma) as it has been previously reported (46). Transient transfection experiments were performed in 60 mm plates using 1 μg of DNA combined with DNA Plus and Lipofectamine reagents. Cells were fixed with 4% paraformaldehyde and/or lysed with PBS supplied with 0.2% Triton-X 100 36, 48 and 72 h post-transfection. For immunocytochemical labeling, cells were cultured on glass coverslips and processed as previously described (44). Lysed cells were maintained on ice and sonicated, centrifuged for 15 min at 13 000 rpm and separated into soluble and insoluble fractions. SDS/PAGE western blotting of both fractions were carried out as previously described (25), using monoclonal antibodies against myc (Invitrogen), FLAG (Clone M2; Sigma) and actin (Clone C4; ICN BIOMEDICALS, Inc.), as well as a polyclonal antibodies against TDP-43 (ProteinTech).

Motor neuron cultures

Cultures were prepared from embryonic day 13 CD1 mice (Charles River Laboratories) as previously described (37,38). In brief, spinal cords were removed from the embryos and dissociation by mincing and incubating with trypsin (Invitrogen), murine spinal cord cells were plated onto 18-mm glass coverslips in 12-well culture dishes at a density of 350 000–400 000. Coverslips were pre-coated with 10 μg/ml poly-D-lysine (Sigma). The culture medium was composed of MEM supplemented with 5 g/l glucose, 10 ng/ml nerve growth factor (BD Biosciences Clontech), 10 μg/ml bovine serum albumin [(BSA), Invitrogen], 26 ng/ml selenium

(Sigma), 20 μg/ml triiodothyronine (Sigma), 10 μg/ml insulin (Sigma), 200 μg/ml apo-transferrin (Sigma), 32 μg/ml putrescine (Sigma), 9.1 ng/ml hydrocortisone (Sigma), 13 ng/ml progesterone (Sigma) and 2% horse serum (Invitrogen). The plating medium also contained 5% FCS and 1% PSN. When the cells reached ~90% confluency (4–6 days from initial plating), cultures were treated with 1.4 μg/ml cytosine-β-D-arabinoside (Calbiochem) for 4–5 days to arrest division of nonneuronal cells. Cultures were used in experiments at 3–6 weeks after plating. Plasmids were expressed in motor neurons by intranuclear microinjection. Motor neurons were identified based on size and other morphological properties as described previously (37,38). Both WT *TARDBP* and three ALS-causing mutants (A315T, G348C, A382T) were microinjected at the final concentrations of 25 μg/ml in 5 mM Tris/0.5 mM EDTA and immunocytochemical analysis was performed to ascertain that similar levels of mutant and WT TDP-43 were expressed. 70 kDa dextran-fluorescein isothiocyanate (Molecular Probes, Invitrogen) was included in the injectate at 20 μg/μl to identify microinjected cells in living cultures. Plasmid-derived protein expression derived from each was detected by 72 h by immunolabeling with the FLAG and myc antibodies. The intensity of immunolabeling of cells for mutant and WT TDP-43 indicated similar levels of expression. Z-stacks images of labeled motor neurons were obtained using a Leica confocal TCS SP5 and images were converted to 16-bit grayscale. The nucleus and 3–5 neurites (including several dendrites and the axon) of each immunolabeled motor neuron were traced to their first branching point and their pixilated volume was measured and quantified using the Image J program in relation to background.

Zebrafish (*Danio renio*)

Zebrafish were raised from a colony maintained according to established procedures and all procedures described here were carried out in compliance with the Canadian Council for Animal Care.

Injections in 1–4 cell stage blastulae were performed as previously described (42). *TARDBP* WT and mutant mRNAs were transcribed from *Not*I-linearized pCS2+ using SP6 polymerase with the mMESSAGE Machine Kit (Ambion). This was followed by a phenol-chloroform purification and ethanol precipitation, and diluted in nuclease-free water (Ambion). The mRNA was diluted in nuclease free water (Ambion) with 0.05% Fast Green vital dye (Sigma, St Louis, MO) at a concentration of 25 ng/μl and was pulse-injected into early embryos using a Picospritzer III (General Valve, Fairfield, NJ) pressure ejector. The zebrafish *TARDBP* gene orthologue, *tardbp* (NM_201476) was identified using the Ensembl's gene homology prediction program (http://www.ensembl.org) (42). An AMO (**TGC**ACTCCTCCATCCTCCGTCACGA) (Gene Tools, OR, USA) sequence was designed complimentary to the region of translational initiation of the *tardbp* (ggaaacagttagcacagctcgcgcattcggtgtaatc [(ATG)**ACG**GAGTGCTATATTCGTGTGG]) in order to inhibit protein translation. We also tested, as a control, a mismatched AMO with the sequence GTAGATCTCCGCGATCTTTGCTGAG. An AMO was also designed that would bind and block the translation *tardbp*-like *(tardbpl)* gene (42). A dose-dependence curve of AMO toxicity was performed and AMOs were injected at a concentration of 0.1 mM to minimize morpholino-induced developmental delay and toxicity and to yield a consistent phenotype. Morphology and behavioral touch responses were assessed with a stereomicroscope (Zeiss, Oberkochen, Germany). For escape swimming at 48 hpf, embryos were touched lightly at the tails with a pair of blunt forceps and their responses were recorded using a Photron (San Diego, CA) Fastcam PCI high-speed video camera at 125 frames/s. The sensory response was measured using the mustard oil test as it has been previously described. For immunohistochemical analysis of axonal projections of motor neurons, a monoclonal antibody anti-SV2 (Developmental Studies Hybridoma) was used.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We are grateful to G. Laliberté, R. Milette and M. Drits for animal care, N. Champagne and M. Liao for technical assistance, S. Minotti for primary spinal cord cultures and F. Barralle for comments on the manuscript.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Canadian Institute of Health Research (to E.K., H.D.D., C.V.V., G.A.R. and P.D.), by the Fonds de la Recherche en Sante du Quebec (to P.D.), by Genome Canada/Genome Quebec (to G.A.R. and P.D.) and by Amyotrophic Lateral Sclerosis Canada and Muscular Dystrophy Association (to E.K.).

REFERENCES

1. Nelson, L.M. (1995) Epidemiology of ALS. *Clin. Neurosci.*, **3**, 327–331.
2. Boillee, S., Vande Velde, C. and Cleveland, D.W. (2006) ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron*, **52**, 39–59.
3. Gros-Louis, F., Gaspar, C. and Rouleau, G.A. (2006) Genetics of familial and sporadic amyotrophic lateral sclerosis. *Biochim. Biophys. Acta*, **1762**, 956–972.
4. Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X. *et al.* (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, **362**, 59–62.
5. Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H.X. *et al.* (1994) Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science*, **264**, 1772–1775.
6. Howland, D.S., Liu, J., She, Y., Goad, B., Maragakis, N.J., Kim, B., Erickson, J., Kulik, J., DeVito, L., Psaltis, G. *et al.* (2002) Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc. Natl Acad. Sci. USA*, **99**, 1604–1609.
7. Lemmens, R., Van Hoecke, A., Hersmus, N., Geelen, V., D'Hollander, I., Thijs, V., Van Den Bosch, L., Carmeliet, P. and Robberecht, W. (2007) Overexpression of mutant superoxide dismutase 1 causes a motor axonopathy in the zebrafish. *Hum. Mol. Genet.*, **16**, 2359–2365.

8. Julien, J.P. and Kriz, J. (2006) Transgenic mouse models of amyotrophic lateral sclerosis. *Biochim. Biophys. Acta*, **1762**, 1013–1024.
9. Hadano, S., Hand, C.K., Osuga, H., Yanagisawa, Y., Otomo, A., Devon, R.S., Miyamoto, N., Showguchi-Miyata, J., Okada, Y., Singaraja, R. *et al.* (2001) A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2. *Nat. Genet.*, **29**, 166–173.
10. Nishimura, A.L., Mitne-Neto, M., Silva, H.C., Richieri-Costa, A., Middleton, S., Cascio, D., Kok, F., Oliveira, J.R., Gillingwater, T., Webb, J. *et al.* (2004) A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am. J. Hum. Genet.*, **75**, 822–831.
11. Chen, Y.Z., Bennett, C.L., Huynh, H.M., Blair, I.P., Puls, I., Irobi, J., Dierick, I., Abel, A., Kennerson, M.L., Rabin, B.A. *et al.* (2004) DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am. J. Hum. Genet.*, **74**, 1128–1135.
12. Lambrechts, D., Storkebaum, E., Morimoto, M., Del-Favero, J., Desmet, F., Marklund, S.L., Wyns, S., Thijs, V., Andersson, J., van Marion, I. *et al.* (2003) VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nat. Genet.*, **34**, 383–394.
13. Greenway, M.J., Andersen, P.M., Russ, C., Ennis, S., Cashman, S., Donaghy, C., Patterson, V., Swingler, R., Kieran, D., Prehn, J. *et al.* (2006) ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. *Nat. Genet.*, **38**, 411–413.
14. Ou, S.H., Wu, F., Harrich, D., Garcia-Martinez, L.F. and Gaynor, R.B. (1995) Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. *J. Virol.*, **69**, 3584–3596.
15. Buratti, E., Brindisi, A., Giombi, M., Tisminetzky, S., Ayala, Y.M. and Baralle, F.E. (2005) TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing. *J. Biol. Chem.*, **280**, 37572–37584.
16. Bose, J.K., Wang, I.F., Hung, L., Tarn, W.Y. and Shen, C.K. (2008) TDP-43 overexpression enhances exon 7 inclusion during the survival of motor neuron pre-mRNA splicing. *J. Biol. Chem.*, **283**, 28852–28859.
17. Buratti, E., Dork, T., Zuccato, E., Pagani, F., Romano, M. and Baralle, F.E. (2001) Nuclear factor TDP-43 and SR proteins promote *in vitro* and *in vivo* CFTR exon 9 skipping. *EMBO J.*, **20**, 1774–1784.
18. Mercado, P.A., Ayala, Y.M., Romano, M., Buratti, E. and Baralle, F.E. (2005) Depletion of TDP 43 overrides the need for exonic and intronic splicing enhancers in the human apoA-II gene. *Nucleic Acids Res.*, **33**, 6000–6010.
19. Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.T., Bruce, J., Schuck, T., Grossman, M., Clark, C.M. *et al.* (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*, **314**, 130–133.
20. Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., Mann, D., Tsuchiya, K., Yoshida, M., Hashizume, Y. *et al.* (2006) TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem. Biophys. Res. Commun.*, **351**, 602–611.
21. Amador-Ortiz, C., Lin, W.L., Ahmed, Z., Personett, D., Davies, P., Duara, R., Graff-Radford, N.R., Hutton, M.L. and Dickson, D.W. (2007) TDP-43 immunoreactivity in hippocampal sclerosis and Alzheimer's disease. *Ann. Neurol.*, **61**, 435–445.
22. Nakashima-Yasuda, H., Uryu, K., Robinson, J., Xie, S.X., Hurtig, H., Duda, J.E., Arnold, S.E., Siderowf, A., Grossman, M., Leverenz, J.B. *et al.* (2007) Co-morbidity of TDP-43 proteinopathy in Lewy body related diseases. *Acta Neuropathol.*, **114**, 221–229.
23. Rothstein, J.D. (2007) TDP-43 in amyotrophic lateral sclerosis: pathophysiology or patho-babel? *Ann. Neurol.*, **61**, 382–384.
24. Gitcho, M.A., Baloh, R.H., Chakraverty, S., Mayo, K., Norton, J.B., Levitch, D., Hatanpaa, K.J., White, C.L. III, Bigio, E.H., Caselli, R. *et al.* (2008) TDP-43 A315T mutation in familial motor neuron disease. *Ann. Neurol.*, **63**, 535–538.
25. Kabashi, E., Valdmanis, P.N., Dion, P., Spiegelman, D., McConkey, B.J., Vande Velde, C., Bouchard, J.P., Lacomblez, L., Pochigaeva, K., Salachas, F. *et al.* (2008) TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat. Genet.*, **40**, 572–574.
26. Sreedharan, J., Blair, I.P., Tripathi, V.B., Hu, X., Vance, C., Rogelj, B., Ackerley, S., Durnall, J.C., Williams, K.L., Buratti, E. *et al.* (2008) TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science*, **319**, 1668–1672.
27. Rutherford, N.J., Zhang, Y.J., Baker, M., Gass, J.M., Finch, N.A., Xu, Y.F., Stewart, H., Kelley, B.J., Kuntz, K., Crook, R.J. *et al.* (2008) Novel mutations in TARDBP (TDP-43) in patients with familial amyotrophic lateral sclerosis. *PLoS Genet.*, **4**, e1000193.
28. Daoud, H., Valdmanis, P.N., Kabashi, E., Dion, P., Dupre, N., Camu, W., Meininger, V. and Rouleau, G.A. (2009) Contribution of TARDBP mutations to sporadic amyotrophic lateral sclerosis. *J. Med. Genet.*, **46**, 112–114.
29. Corrado, L., Ratti, A., Gellera, C., Buratti, E., Castellotti, B., Carlomagno, Y., Ticozzi, N., Mazzini, L., Testa, L., Taroni, F. *et al.* (2009) High frequency of TARDBP gene mutations in Italian patients with amyotrophic lateral sclerosis. *Hum. Mutat.*, **30**, 688–694.
30. Van Deerlin, V.M., Leverenz, J.B., Bekris, L.M., Bird, T.D., Yuan, W., Elman, L.B., Clay, D., Wood, E.M., Chen-Plotkin, A.S., Martinez-Lage, M. *et al.* (2008) TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis. *Lancet Neurol.*, **7**, 409–416.
31. Del Bo, R., Ghezzi, S., Corti, S., Pandolfo, M., Ranieri, M., Santoro, D., Ghione, I., Prelle, A., Orsetti, V., Mancuso, M. *et al.* (2009) TARDBP (TDP-43) sequence analysis in patients with familial and sporadic ALS: identification of two novel mutations. *Eur. J. Neurol.*, **16**, 727–732.
32. Yokoseki, A., Shiga, A., Tan, C.F., Tagawa, A., Kaneko, H., Koyama, A., Eguchi, H., Tsujino, A., Ikeuchi, T., Kakita, A. *et al.* (2008) TDP-43 mutation in familial amyotrophic lateral sclerosis. *Ann. Neurol.*, **63**, 538–542.
33. Lemmens, R., Race, V., Hersmus, N., Matthijs, G., Van Den Bosch, L., Van Damme, P., Dubois, B., Boonen, S., Goris, A. and Robberecht, W. (2009) TDP-43 M311V mutation in familial amyotrophic lateral sclerosis. *J. Neurol. Neurosurg. Psychiatry*, **80**, 354–355.
34. Kuhnlein, P., Sperfeld, A.D., Vanmassenhove, B., Van Deerlin, V., Lee, V.M., Trojanowski, J.Q., Kretzschmar, H.A., Ludolph, A.C. and Neumann, M. (2008) Two German kindreds with familial amyotrophic lateral sclerosis due to TARDBP mutations. *Arch. Neurol.*, **65**, 1185–1189.
35. Zhang, Y.J., Xu, Y.F., Dickey, C.A., Buratti, E., Baralle, F., Bailey, R., Pickering-Brown, S., Dickson, D. and Petrucelli, L. (2007) Progranulin mediates caspase-dependent cleavage of TAR DNA binding protein-43. *J. Neurosci.*, **27**, 10530–10534.
36. Moisse, K., Volkening, K., Leystra-Lantz, C., Welch, I., Hill, T. and Strong, M.J. (2009) Divergent patterns of cytosolic TDP-43 and neuronal progranulin expression following axotomy: implications for TDP-43 in the physiological response to neuronal injury. *Brain Res.*, **1249**, 202–211.
37. Roy, J., Minotti, S., Dong, L., Figlewicz, D.A. and Durham, H.D. (1998) Glutamate potentiates the toxicity of mutant Cu/Zn-superoxide dismutase in motor neurons by postsynaptic calcium-dependent mechanisms. *J. Neurosci.*, **18**, 9673–9684.
38. Durham, H.D., Roy, J., Dong, L. and Figlewicz, D.A. (1997) Aggregation of mutant Cu/Zn superoxide dismutase proteins in a culture model of ALS. *J. Neuropathol. Exp. Neurol.*, **56**, 523–530.
39. Grunwald, D.J. and Eisen, J.S. (2002) Headwaters of the zebrafish—emergence of a new model vertebrate. *Nat. Rev. Genet.*, **3**, 717–724.
40. Lieschke, G.J. and Currie, P.D. (2007) Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genet.*, **8**, 353–367.
41. Beattie, C.E., Carrel, T.L. and McWhorter, M.L. (2007) Fishing for a mechanism: using zebrafish to understand spinal muscular atrophy. *J. Child Neurol.*, **22**, 995–1003.
42. Shankaran, S.S., Capell, A., Hruscha, A.T., Fellerer, K., Neumann, M., Schmid, B. and Haass, C. (2008) Missense mutations in the progranulin gene linked to frontotemporal lobar degeneration with ubiquitin-immunoreactive inclusions reduce progranulin production and secretion. *J. Biol. Chem.*, **283**, 1744–1753.
43. Gros-Louis, F., Kriz, J., Kabashi, E., McDearmid, J., Millecamps, S., Urushitani, M., Lin, L., Dion, P., Zhu, Q., Drapeau, P. *et al.* (2008) Als2 mRNA splicing variants detected in KO mice rescue severe motor dysfunction phenotype in Als2 knock-down zebrafish. *Hum. Mol. Genet.*, **17**, 2691–2702.
44. Messaed, C., Dion, P.A., Abu-Baker, A., Rochefort, D., Laganiere, J., Brais, B. and Rouleau, G.A. (2007) Soluble expanded PABPN1 promotes cell death in oculopharyngeal muscular dystrophy. *Neurobiol. Dis.*, **26**, 546–557.

45. Valdmanis, P.N., Meijer, I.A., Reynolds, A., Lei, A., MacLeod, P., Schlesinger, D., Zatz, M., Reid, E., Dion, P.A., Drapeau, P. *et al.* (2007) Mutations in the KIAA0196 gene at the SPG8 locus cause hereditary spastic paraplegia. *Am. J. Hum. Genet.*, **80**, 152–161.
46. Mao, A.J., Bechberger, J., Lidington, D., Galipeau, J., Laird, D.W. and Naus, C.C. (2000) Neuronal differentiation and growth control of neuro-2a cells after retroviral gene delivery of connexin43. *J. Biol. Chem.*, **275**, 34407–34414.
47. Igaz, L.M., Kwong, L.K., Chen-Plotkin, A., Winton, M.J., Unger, T.L., Xu, Y., Neumann, M., Trojanowski, J.Q. and Lee, V.M. (2009) Expression of TDP-43 C-terminal fragments *in vitro* recapitulates pathological features of TDP-43 proteinopathies. *J. Biol. Chem.*, **284**, 8516–8524.
48. Igaz, L.M., Kwong, L.K., Xu, Y., Truax, A.C., Uryu, K., Neumann, M., Clark, C.M., Elman, L.B., Miller, B.L., Grossman, M. *et al.* (2008) Enrichment of C-terminal fragments in TAR DNA-binding protein-43 cytoplasmic inclusions in brain but not in spinal cord of frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Am. J. Pathol.*, **173**, 182–194.
49. Zhang, Y.J., Xu, Y.F., Cook, C., Gendron, T.F., Roettges, P., Link, C.D., Lin, W.L., Tong, J., Castanedes-Casey, M., Ash, P. *et al.* (2009) Aberrant cleavage of TDP-43 enhances aggregation and cellular toxicity. *Proc. Natl Acad. Sci. USA*, **106**, 7607–7612.
50. Wang, I.F., Wu, L.S., Chang, H.Y. and Shen, C.K. (2008) TDP-43, the signature protein of FTLD-U, is a neuronal activity-responsive factor. *J. Neurochem.*, **105**, 797–806.
51. Feiguin, F., Godena, V.K., Romano, G., D'Ambrogio, A., Klima, R. and Baralle, F.E. (2009) Depletion of TDP-43 affects Drosophila motoneurons terminal synapsis and locomotive behavior. *FEBS Lett.*, **583**, 1586–1592.
52. Strong, M.J., Volkening, K., Hammond, R., Yang, W., Strong, W., Leystra-Lantz, C. and Shoesmith, C. (2007) TDP43 is a human low molecular weight neurofilament (hNFL) mRNA-binding protein. *Mol. Cell. Neurosci.*, **35**, 320–327.
53. Kwiatkowski, T.J. Jr, Bosco, D.A., Leclerc, A.L., Tamrazian, E., Vanderburg, C.R., Russ, C., Davis, A., Gilchrist, J., Kasarskis, E.J., Munsat, T. *et al.* (2009) Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science*, **323**, 1205–1208.
54. Vance, C., Rogelj, B., Hortobagyi, T., De Vos, K.J., Nishimura, A.L., Sreedharan, J., Hu, X., Smith, B., Ruddy, D., Wright, P. *et al.* (2009) Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science*, **323**, 1208–1211.
55. Lagier-Tourenne, C. and Cleveland, D.W. (2009) Rethinking ALS: the FUS about TDP-43. *Cell*, **136**, 1001–1004.
56. Kabashi, E., Valdmanis, P.N., Dion, P. and Rouleau, G.A. (2007) Oxidized/misfolded superoxide dismutase-1: the cause of all amyotrophic lateral sclerosis? *Ann. Neurol.*, **62**, 553–559.
57. Kabashi, E. and Durham, H.D. (2006) Failure of protein quality control in amyotrophic lateral sclerosis. *Biochim. Biophys. Acta.*, **1762**, 1038–1050.
58. Wang, Q., Johnson, J.L., Agar, N.Y. and Agar, J.N. (2008) Protein aggregation and protein instability govern familial amyotrophic lateral sclerosis patient survival. *PLoS Biol.*, **6**, e170.
59. Cleveland, D.W. and Rothstein, J.D. (2001) From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat. Rev. Neurosci.*, **2**, 806–819.
60. De Vos, K.J., Grierson, A.J., Ackerley, S. and Miller, C.C. (2008) Role of axonal transport in neurodegenerative diseases. *Annu. Rev. Neurosci.*, **31**, 151–173.
61. Reaume, A.G., Elliott, J.L., Hoffman, E.K., Kowall, N.W., Ferrante, R.J., Siwek, D.F., Wilcox, H.M., Flood, D.G., Beal, M.F., Brown, R.H. Jr *et al.* (1996) Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet.*, **13**, 43–47.
62. Di Prospero, N.A. and Fischbeck, K.H. (2005) Therapeutics development for triplet repeat expansion diseases. *Nat. Rev. Genet.*, **6**, 756–765.
63. Diekmann, H., Anichtchik, O., Fleming, A., Futter, M., Goldsmith, P., Roach, A. and Rubinsztein, D.C. (2009) Decreased BDNF levels are a major contributor to the embryonic phenotype of huntingtin knockdown zebrafish. *J. Neurosci.*, **29**, 1343–1349.
64. Schiffer, N.W., Broadley, S.A., Hirschberger, T., Tavan, P., Kretzschmar, H.A., Giese, A., Haass, C., Hartl, F.U. and Schmid, B. (2007) Identification of anti-prion compounds as efficient inhibitors of polyglutamine protein aggregation in a zebrafish model. *J. Biol. Chem.*, **282**, 9195–9203.
65. Lim, J., Crespo-Barreto, J., Jafar-Nejad, P., Bowman, A.B., Richman, R., Hill, D.E., Orr, H.T. and Zoghbi, H.Y. (2008) Opposing effects of polyglutamine expansion on native protein complexes contribute to SCA. *Nature*, **452**, 713–718.
66. Zoghbi, H.Y. and Orr, H.T. (2009) Pathogenic mechanisms of a polyglutamine mediated neurodegenerative disease, spinocerebellar ataxia type. *J. Biol. Chem.*, **284**, 7425–7429.
67. Thomas, P.S. Jr, Fraley, G.S., Damian, V., Woodke, L.B., Zapata, F., Sopher, B.L., Plymate, S.R. and La Spada, A.R. (2006) Loss of endogenous androgen receptor protein accelerates motor neuron degeneration and accentuates androgen insensitivity in a mouse model of X linked spinal and bulbar muscular atrophy. *Hum. Mol. Genet.*, **15**, 2225–2238.
68. McWhorter, M.L., Monani, U.R., Burghes, A.H. and Beattie, C.E. (2003) Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. *J. Cell. Biol.*, **162**, 919–931.
69. Paquet, D., Bhat, R., Sydow, A., Mandelkow, E.M., Berg, S., Hellberg, S., Falting, J., Distel, M., Koster, R.W., Schmid, B. *et al.* (2009) A zebrafish model of tauopathy allows in vivo imaging of neuronal cell death and drug evaluation. *J. Clin. Invest.*, **119**, 1382–1395.