ALS mutations in FUS cause neuronal dysfunction and death in *Caenorhabditis elegans* by a dominant gain-of-function mechanism

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It is unclear whether mutations in fused in sarcoma (FUS) cause familial amyotrophic lateral sclerosis via a loss-of-function effect due to titrating FUS from the nucleus or a gain-of-function effect from cytoplasmic overabundance. To investigate this question, we generated a series of independent *Caenorhabditis elegans* lines expressing mutant or wild-type (WT) human FUS. We show that mutant FUS, but not WT-FUS, causes cytoplasmic mislocalization associated with progressive motor dysfunction and reduced lifespan. The severity of the mutant phenotype in *C. elegans* was directly correlated with the severity of the illness caused by the same mutation in humans, arguing that this model closely replicates key features of the human illness. Importantly, the mutant phenotype could not be rescued by overexpression of WT-FUS, even though WT-FUS had physiological intracellular localization, and was not recruited to the cytoplasmic mutant FUS aggregates. Our data suggest that FUS mutants cause neuronal dysfunction by a dominant gain-of-function effect related either to neurotoxic aggregates of mutant FUS in the cytoplasm or to dysfunction in its RNA-binding functions.

INTRODUCTION

Mutations in the fused in sarcoma (FUS; ALS6) genes (1–3) cause familial forms of amyotrophic lateral sclerosis (ALS), and FUS may contribute to sporadic cases of frontotemporal lobar degeneration (FTLD). FUS encodes an RNA-binding protein that, under physiological conditions, is almost exclusively expressed in the nucleus (4–6). However, in patients with pathogenic FUS mutations and in patients with sporadic forms of FTLD-FUS, the FUS protein accumulates in the cytoplasm of neurons in the spinal cord and brain (7–10). The majority of mutations occur in the C-terminus of the FUS protein, which is thought to contain a nuclear targeting signal (11). This has led to the conclusion that mislocalization of mutant FUS causes either a loss-of-function effect (because of titration of FUS from the nucleus) or a gain-of-function effect (arising from aberrant accumulation of FUS in the cytoplasm) (12,13). The fact that mutations in the Tar DNA-binding
protein 43 (TDP-43), another RNA-binding protein, also cause familial ALS has led to the hypothesis that FUS and TDP-43 might work by the same mechanism (13). To further explore the mechanism by which mutant FUS protein causes ALS, we chose to create transgenic models bearing wild-type (WT) or mutant human FUS in Caenorhabditis elegans. We chose this model for three reasons. First, C. elegans has an orthologue of human FUS, encouraging the notion that models built in this animal would have biological validity. Secondly, the genetics of C. elegans provides a powerful tool to seek upstream and downstream partners via enhancer and suppressor screens. Thirdly, the optical transparency of C. elegans allows the application of biophysical techniques to monitor the distribution and folding state of proteins of interest (14).

RESULTS
A series of WT and mutant human FUS transgenes were generated and were then expressed in C. elegans under the control of a pan-neuronal promoter (Fig. 1). These transgenes included: (i) full-length (FL) WT human FUS (WT-FUS); (ii) four different missense mutations associated with varying clinical severity (as defined by age at onset and by disease duration) of human ALS (R514G and R521G = mild; R522G = moderate and P525L = severe) (1–3) (Supplementary Material, Table S1) and (iii) two different C-terminal-truncated FUS constructs (FUS513 and FUS501—lacking the C-terminal 13 and 25 amino acids of FUS, respectively). While the C-terminal-truncated constructs are artificial, they are very similar to several human C-terminal FUS, respectively). While the C-terminal-truncated constructs (FUS513 and FUS501) were generated and injected into C. elegans. GFP, green fluorescent protein; QGSY-rich, glutamine–glycine–serine–tyrosine-rich region; G-rich, glycine-rich region; RRM, RNA recognition motif; RGG-rich, arginine–glycine–glycine-rich region.

Mutations in FUS cause its accumulation in cytoplasmic inclusions
Transgenic animals expressing only GFP under the control of the same pan-neuronal Pr gef-1 promoter showed diffuse nuclear and cytoplasmic signals in neurons (data not shown). In contrast, the GFP-tagged FL WT-FUS was detected only in the nuclei of neurons (nuclear FUS/total FUS ratio = 93 ± 3%, n = 20 animals per transgenic strain) (Figs 2A and B and 3A). The nuclear localization of WT-FUS is consistent with the physiological subcellular localization of FUS in healthy human neurons (1,2). Immunostaining with anti-FUS antibody revealed that the FUS immunoreactivity fully replicated the subcellular distribution of the GFP signal in the same animals, indicating that the GFP tag was a reliable indicator of FUS localization in vivo (Supplementary Material, Fig. S2).

In animals expressing R514G and R521G mutations, which have a mild ALS phenotype in human ALS cases (1), the pattern of GFP-FUS was identical to WT-FUS at all ages (nuclear FUS/total FUS ratio = 92 ± 3% and 96 ± 3%, respectively) (Figs 2A and B and 3A). In animals expressing the R522G mutation, which has a moderate human ALS phenotype (1), FUS was also present in the nuclei of neurons, but there was some FUS diffusely present in the cytoplasm (nuclear FUS/total FUS ratio = 59 ± 10%, P < 0.001 compared with WT-FUS) (Figs 2A and B and 3A). In animals expressing the clinically severe P525L mutation, nearly half of the total neuronal FUS was located in the cytoplasm in an uneven, clumpy distribution (nuclear FUS/total FUS ratio = 52 ± 9%, P < 0.001 compared with WT-FUS) (Figs 2A and B and 3A). Both truncated FUS constructs also showed strong, clumpy, cytoplasmic FUS with less nuclear FUS (nuclear FUS/total FUS ratio = 44 ± 4 and 43 ± 5% for FUS513 and FUS501, respectively) (Figs 2A and B and 3A).

Mutant FUS accumulates and aggregates in neuronal cytoplasm in vivo
To determine whether the cytoplasmic inclusions contained insoluble aggregates of FUS, we undertook sequential extraction of animals with RIPA and then with urea buffer. This analysis revealed that there were increased steady-state levels of FUS
in animals that had cytoplasmic FUS accumulation (Fig. 3B) and that some of this accumulated protein was in the form of sodium dodecyl sulphate-insoluble, urea-soluble aggregates (Fig. 3C). All animals had similar levels of transgene mRNA expression, and there was no correlation between the level of transgene expression and the abundance of FUS (Supplementary Material, Fig. S1). The differences in steady-state levels of FUS protein, therefore, likely reflect differences in the amount of cytoplasmic aggregated FUS, which in turn could reflect differences in the intrinsic aggregation propensity of the various mutant FUS proteins.

**Animals with FUS aggregates show progressive motor defects and die prematurely**

To determine whether the cytoplasmic aggregation of FUS was accompanied by functional deficits, we assessed the motor activity of the transgenic animals by employing a standard thrashing assay (http://www.wormbook.org/chapters/www_behavior/behavior.html#sec7). This assay measured motor activity by counting the frequency of body bends when animals were placed in liquid M9 buffer. At 3 days of age (i.e. young adults), animals expressing only a GFP transgene had a slight motor disturbance (100.7 ± 5.6/min) compared with non-transgenic N2 animals of the same age (n = 20, 115.7 ± 6.3/min, P < 0.05, Fig. 3D). Animals expressing WT-FUS had a similar minor impairment of motor function (104.8 ± 5.0/min), indicating that expression of WT-FUS per se has little or no toxicity. The motor function of animals expressing the clinically mild R514G and R521G mutations, which had predominantly nuclear FUS, was indistinguishable from that of WT-FUS and GFP-only animals (R514G 103.5 ± 6.0/min and R521G = 103.8 ± 5.9/min). In contrast, animals expressing either the moderate R522G (96.3 ± 4.9/min) or the severe P525L mutation (91.8 ± 5.2/min) showed significantly impaired motor function compared with WT-FUS (P < 0.05 and P < 0.001, respectively). Animals expressing C-terminal-truncated FUS513 (69.7 ± 8.9/min) or FUS501 (54.6 ± 8.7/min) also had significantly impaired motor function compared with WT-FUS (P < 0.001). Representative locomotor behaviours of the animals are shown in Supplementary Material, Movies SM1–SM5.

Because human FUS mutations differ both in their age of onset and rates of progression, we next assessed rates of progression by comparing motor performance of 3-day-old and 6-day-old adult animals (n = 20 for each strain). The 6-day-old non-transgenic WT (N2), WT-FUS, R514G and R521G animals showed minor degrees of age-related decline compared with the 3-day-old N2 animals (−11.0 ± 0.8, −9.2 ± 1.3, −10.8 ± 1.7 and −7.5 ± 1.4%, respectively; P = NS) (Fig. 3E and F). However, the decline in R522G, P525L, FUS513 and FUS501 animals showed marked cytoplasmic GFP signals (A and B, arrowheads), which are rather stronger than those in nuclei (A and B, arrows). Staining patterns with anti-GFP and anti-FUS antibodies are almost the same (B; Supplementary Material, Fig. S2). There is no difference in the distribution of GFP-positive neurons in each line. Scale bars: 20 μm (A) and 2.0 μm (B).

Figure 2. Representative images of transgenic FUS worms. (A) Live fluorescent images of neurons in the head and body and (B) confocal microscopic images of immunostained animals. Non-transgenic N2 animals show no fluorescent signals with either wide-field (A) or confocal (B) microscope. GFP signals are observed almost exclusively in the nuclei in WT-FUS (WT), R514G and R521G (A, arrow). GFP signals in WT and R514G and DAPI perfectly overlap, suggesting that FUS in these two strains is exclusively expressed in the nuclei (B, WT, arrow). R522G animals show both nuclear (A and B, R522G, arrow) and cytoplasmic FUS (A and B, arrowhead), but nuclear signal is still stronger (A and B, P525L, FUS513 and FUS501 animals show marked cytoplasmic GFP signals (A and B, arrowheads), which are rather stronger than those in nuclei (A and B, arrows). Staining patterns with anti-GFP and anti-FUS antibodies are almost the same (B; Supplementary Material, Fig. S2). There is no difference in the distribution of GFP-positive neurons in each line. Scale bars: 20 μm (A) and 2.0 μm (B).
analyses (*n* = 20 animals/transgenic strain) revealed that under our culture conditions, the mean lifespans of animals expressing WT-FUS or the mild ALS mutations (R514G and R521G) were not different from those of N2 animals (Fig. 4B and C). In contrast, the mean lifespans of animals expressing the R522G, P525L, FUS513 and FUS501 mutants were reduced to 9.7 ± 0.4, 9.3 ± 0.3, 8.9 ± 0.3 and 8.1 ± 0.2 days, respectively, and were significantly shorter than...
those of non-transgenic N2 (12.8 ± 0.3 days, P < 0.001) and WT-FUS animals (12.1 ± 0.4 days, P < 0.001). Neurons from 6–8-day-old R522G, P525L, FUS513 and FUS501 animals also had abundant FUS-positive granules in the cytoplasm that were not present at younger ages (Fig. 4D).

Cellular stress exacerbates both cytoplasmic mislocalization of FUS and motor dysfunction

Because heat stress has been previously shown to cause mislocalization of FUS, we applied brief periods of thermal stress (35°C for 30 min) and then monitored both the subcellular distribution of FUS and the severity of motor dysfunction. Because FUS is an RNA-binding protein, we were particularly interested to determine whether it might co-localize with cytoplasmic stress granules, which can be monitored by poly (A)-binding protein (PAB-1) (17). At all ages, animals over-expressing PAB-1 tagged at the N-terminus with mCherry showed only diffuse cytoplasmic fluorescent signals with no apparent stress granules under normal conditions (Fig. 5A, no HS). However, 30 min after application of 35°C heat shock, PAB-1 coalesced into numerous cytoplasmic granules (Fig. 5A). These PAB-1-positive stress granules slowly disappeared, and PAB-1 staining reverted to the basal diffuse cytoplasmic distribution by 6 h after heat shock (Fig. 5A).

When PAB-1 was co-expressed with either WT-FUS or mutant FUS, a small amount of PAB-1-positive stress granules was observed under basal conditions (Fig. 5B). In the double transgenic animals expressing WT or mutant FUS and PAB-1, under the same basal conditions, the distribution and morphology of FUS were identical to those in single transgenic FUS animals, indicating that PAB-1 does not affect localization of either WT-FUS or mutant FUS animals per se.
Following the heat shock, WT-FUS animals exhibited a small increment in the number of stress granules in the cytoplasm, but there was no corresponding cytoplasmic accumulation of FUS (Fig. 5B). In sharp contrast, when the same heat shock stimulus was applied to the R522G, P525L or FUS501 animals, all three mutant FUS lines showed more stress granules and obvious recruitment of the mutant FUS to the heat-stress-induced granules. Concomitantly, we assessed motor function in these transgenic animals. All animals showed impaired motility immediately after the heat shock. However, animals expressing WT-FUS and animals expressing the very mild ALS mutants (R514G and R521G) all rapidly recovered to baseline motor function within 30 min. In sharp contrast, all of the transgenic animals expressing the moderate and severe ALS mutants that cause cytoplasmic aggregates of FUS showed persistent motor deficits at 30 min after the heat shock (Fig. 5C, *P* < 0.001, compared with WT-FUS animals). Other assays of neuronal activity, such as escaping behaviour following body touch, also showed a persistent post-heat-shock motor defect in the animals with persistent cytoplasmic FUS (Supplementary Material, Fig. S3). These results support the notion that cytoplasmic accumulation of FUS is neurotoxic, but does not exclude the possibility that mutant FUS binds WT-FUS and titrates it from the nucleus.

**WT-FUS does not rescue mutant FUS and is not recruited to mutant FUS aggregates**

To test the hypothesis that mutant FUS might remove WT-FUS from the nucleus, and thereby cause a loss-of-function effect, we generated transgenic animals expressing WT-FUS labelled with TagRFP (TagRFP-WT-FUS) under the same pan-neuronal promoter. TagRFP-WT-FUS, like the previously described GFP-WT-FUS, was expressed in the nucleus, but was not recruited onto stress granules following heat shock and was not associated with significant neurological deficits (Fig. 6A and B, *n* = 10 each). In sharp contrast, the double transgenic animals (TagRFP-WT-FUS and GFP-FUS-P525L) showed the presence of TagRFP-WT-FUS in the nucleus and GFP-FUS-P525L in the nucleus and cytoplasm under basal conditions (Fig. 6A). Following heat shock, significant quantities of GFP-FUS-P525L were recruited to stress granules, but the TagRFP-
WT-FUS remained in the nucleus (Fig. 6A). Importantly, these double transgenic animals and the single transgenic GFP-FUS-P525L animals had equivalent degrees of impaired motor activity and equivalent degrees of sensitivity to heat stress (Fig. 6B). Mutant FUS is therefore likely to cause ALS through a dominant gain-of-function effect, instead of a loss-of-function effect through titration and reducing the WT-FUS function.

**DISCUSSION**

The experiments reported here demonstrate that pathogenic missense mutations in FUS that cause ALS in humans also cause neuronal dysfunction and death of *C. elegans* expressing these same mutant proteins. Importantly, there are strong parallels in the genotype-to-phenotype correlations between the human disease and the phenotypes in *C. elegans* described here. These similarities strongly argue that the mutant human proteins, when expressed in *C. elegans*, are closely modelling a fundamental property of these mutations in humans.

Our data indicate that ALS-associated mutations in FUS likely cause neurodegeneration via a toxic gain-of-function effect that is likely to be a consequence of the accumulation of FUS in the cytoplasm, rather than titration of FUS from its physiological location in the nucleus. This toxic gain-of-function effect could be mediated by either of two mechanisms. First, as is widely held in the field, it is conceivable that this toxic effect could be attributable to changes in RNA metabolism induced by the mutant FUS. Secondly, it is also possible that the aggregated FUS itself is the neurotoxic moiety, as is the case for several other neurotoxic intracellular and extracellular protein aggregates (e.g. tau, alpha-synuclein and huntingtin). Under such circumstances, the mutant, aggregation-prone forms may have a novel function unrelated or only partly related to the native function. Such is the case with ALS-inducing mutations in SOD1, which do not appear to work via alterations in superoxide dismutase activity (18). The animal models described here will be of use in the necessary work that will be required to discriminate these two possibilities. Additional work will also be required to understand the functional significance of binding of mutant FUS to stress granules, which have a role in cell viability and recovery after stress (11,19,20).

**MATERIALS AND METHODS**

**Transgenic FUS worms**

Animals were maintained on nematode growth medium (NGM) plates (1.8% agar, 0.3% NaCl, 0.25% peptone, 25 mM KPO4, 1 mM MgSO4, 1 mM CaCl2 and 5 μg/ml cholesterol) at 22°C. FL human FUS/TLS cDNA (NM_004960) (WT-FUS) was mutated to contain either one of the five clinical mutations (R514G, R521G, R522G, R524S and P525L). Two C-terminal-truncated FUS constructs were generated (FUS513 and FUS501), lacking the C-terminal 13 and 25 amino acids of FUS, respectively. Each construct was injected together with *lin-15* rescuing plasmid into a host worm with temperature-sensitive *lin-15* (n765) allele, and non-Muv (multivulva) animals were selected for further analysis (21). These transgenes were integrated into the genome to generate stable transgenic lines by UV irradiation, and the lines were outcrossed more than six times with N2 WT strain. Constructs used in this study are summarized in Figure 1.

Poly (A)-binding protein (PAB-1) was used as a marker for stress granules (17). PAB-1 was cloned into the pJH897 vector with the tag replaced with mCherry at the N-terminus and was injected, solely or together with FUS constructs, along with the *lin-15* rescuing plasmid into *lin-15* worms. The N2 strain and transgenic animals expressing only GFP (GFP-only animals) were used as negative controls.

**Quantitative RT–PCR**

Transgene expression in each integrated strain was determined using quantitative real-time PCR. Total RNA was prepared from L4 stage animals with TRIzol reagent (Invitrogen) and RNeasy kit (Qiagen, USA), according to the manufacturer’s
instructions. cDNA was synthesized with AffinityScript reverse transcriptase (Stratagene, USA) and was amplified with primers: forward, 5′-TAAATTTGGTGCCCTCGGG-3′ and reverse, 5′-TCACCCAGGCCCTTGCAAAA-3′. The amplification was monitored using SYBR Green Premix Ex Taq reagent (Takara Bio, Japan) in ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, USA) with gpd-2 as an internal control.

**Immunohistochemistry**

FUS localization in neurons was assessed by fixation of the animals with 4% paraformaldehyde in MRWB buffer (80 mM KCl, 20 mM NaCl, 10 mM EGTA, 5 mM spermidine HCl, 15 mM Na PIPES and 25% methanol, pH 7.4), reduction with 1% 2-mercaptoethanol in BO_3 buffer (25 mM H_3BO_3 and 125 mM NaOH), then oxidation in 0.3% H_2O_2/BO_3 buffer and incubation with primary antibody [monoclonal anti-GFP antibody (Invitrogen, 1:200 dilution) or rabbit polyclonal anti-FUS antibody (Abcam, 1:200 dilution)] overnight at room temperature. After several washes, the animals were incubated with secondary antibody (1:400 dilution), washed again and then mounted in DAPI to stain nuclei (Molecular Probes, USA).

**Nuclear FUS quantification**

To quantify the ratio of nuclear to cytoplasmic FUS, fluorescent microscopic images of neurons in the body were used, so that signals from other nearby neurons were not counted. Using Image J, the nucleus and cytoplasm of the neuron were cropped and the pixels were counted (3-day-old animals, n = 20 animals, 10 neurons from each). Pixels that overlapped with DAPI were considered to be nuclear, and pixels that did not were considered to be cytoplasmic.

**Thrashing assay**

Animals from each transgenic line were picked at L4 stage (n = 20). Sixteen hours later, the 3-day-old young adult animals were transferred into 1 l of M9 buffer (0.6% Na_2PO_4, 0.3% KH_2PO_4, 0.5% NaCl and 0.01% NH_4Cl) on an agarose pad. Thirty seconds after the transfer, the frequency of the body bending was counted for 1 min (22). The same protocol was used to assess the motor function in 6-day-old adults.

**Heat shock treatment**

Animals were picked at L4 stage (n = 16) and kept at 22°C. Sixteen hours later, the animals were subjected to heat shock by incubation at 35°C for 30 min. After heat shock, the animals were returned to the 22°C incubator for recovery and were then assessed for functional deficits at 30 min after heat shock.

**Fluorescence microscopy**

Transgenic FUS animals with or without heat shock were anaesthetized in 20 mM sodium azide in M9 buffer, mounted on an agarose pad with cover slip and examined with a standard fluorescent microscope or confocal microscope (Nikon H600L, Japan).

**Lifespan analysis**

Animals were placed on NGM plates (n = 20) and replated daily, and the number of live animals was counted. Immobile, shrunken animals were considered to have died.

**Western blot analysis**

Animals were lysed in RIPA buffer with sonication. After removing undissolved debris by centrifugation, protein concentrations of the lysates were determined by bicinchoninic acid assay. Equal amounts of protein were subjected to NuPAGE Bis–Tris (Invitrogen); transferred onto nitrocellulose membrane (Whatman, Germany) and detected by western blotting with anti-GFP (Abcam, USA), anti-FUS (Santa Cruz Biotechnology, USA) or anti-tubulin (Abcam) antibodies. For analysis of the solubility of FUS proteins, RIPA lysates were centrifuged at 100 000g for 30 min at 4°C. The pellets were resuspended in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl, pH 8.5), sonicated and centrifuged at 100 000g for 30 min at room temperature (10).

**Statistical analysis**

For statistical analysis, the unpaired Student’s t-tests and analysis of variance were performed with SigmaPlot version 11.0.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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