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# RNA-binding ability of FUS regulates neurodegeneration, cytoplasmic mislocalization and incorporation into stress granules associated with FUS carrying ALS-linked mutations

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**Amyotrophic lateral sclerosis (ALS) is an uncommon neurodegenerative disease caused by degeneration of upper and lower motor neurons. Several genes, including SOD1, TDP-43, FUS, Ubiquilin 2, C9orf72 and Profilin 1, have been linked with the sporadic and familiar forms of ALS. FUS is a DNA/RNA-binding protein (RBP) that forms cytoplasmic inclusions in ALS and frontotemporal lobular degeneration (FTLD) patients' brains and spinal cords. However, it is unknown whether the RNA-binding ability of FUS is required for causing ALS pathogenesis. Here, we exploited a *Drosophila* model of ALS and neuronal cell lines to elucidate the role of the RNA-binding ability of FUS in regulating FUS-mediated toxicity, cytoplasmic mislocalization and incorporation into stress granules (SGs). To determine the role of the RNA-binding ability of FUS in ALS, we mutated FUS RNA-binding sites (F305L, F341L, F359L, F368L) and generated RNA-binding-incompetent FUS mutants with and without ALS-causing mutations (R518K or R521C). We found that mutating the aforementioned four phenylalanine (F) amino acids to leucines (L) (4F-L) eliminates FUS RNA binding. We observed that these RNA-binding mutations block neurodegenerative phenotypes seen in the fly brains, eyes and motor neurons compared with the expression of RNA-binding-competent FUS carrying ALS-causing mutations. Interestingly, RNA-binding-deficient FUS strongly localized to the nucleus of *Drosophila* motor neurons and mammalian neuronal cells, whereas FUS carrying ALS-linked mutations was distributed to the nucleus and cytoplasm. Importantly, we determined that incorporation of mutant FUS into the SG compartment is dependent on the RNA-binding ability of FUS. In summary, we demonstrate that the RNA-binding ability of FUS is essential for the neurodegenerative phenotype *in vivo* of mutant FUS (either through direct contact with RNA or through interactions with other RBPs).**

## INTRODUCTION

Identification of mutations in FUS and TDP-43, both RNA-binding proteins (RBPs), in familial and sporadic forms of amyotrophic lateral sclerosis (ALS) and frontotemporal lobular degeneration (FTLD) patients suggested that disruption in RNA metabolism might be a key event in ALS pathogenesis (1–6). FUS and TDP-43 are ubiquitously expressed, multifunctional proteins involved in regulating several functional aspects of RNA metabolism, such as transcription,

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pre-mRNA splicing and microRNA processing through RNA/DNA and protein–protein interactions (1,2,7–12). Interestingly, both of these ALS-causing proteins reside predominantly in the nucleus and possess similar structural organization (1,2,13,14). Cytoplasmic mislocalization of FUS in ALS and FTLD patients' neurons has been frequently observed and has been predicted to be a causative factor of neurodegeneration, suggesting that mutant FUS acquires a toxic gain-of-function in the cytoplasm (1,2).

The RNA-binding ability of FUS and TDP-43 has been suggested to be relevant to ALS pathogenesis because the physical interaction between these two proteins is RNA-dependent, and treatment with RNase blocks interaction of TDP-43 and FUS in a mammalian cell culture model system (15,16). A recent study in *Caenorhabditis elegans* demonstrated that mutating amino acid residues that mediate the RNA-binding ability of TDP-43 were sufficient to block TDP-43-mediated toxicity in the worms, suggesting that the RNA-binding ability of TDP-43 is important for ALS pathogenesis (17). Furthermore, it has been recently shown that treatment with RNase decreased complex formation between TDP-43 and Drosha in a dose-dependent manner (18). Molecular mechanisms of the RNA-binding ability of TDP-43 in regulating toxicity are not yet known.

Recent approaches combining RNA cross-linking, immunoprecipitation and high-throughput sequencing (PARCLIP/iCLIP/CLIP-seq) led to the discovery of RNA targets of FUS and TDP-43 and indicated that both proteins are involved in pre-mRNA splicing, as FUS-bound RNA target sequences were found to be preferentially localized in long intronic regions and near-splice-site acceptors (19,20). It has been demonstrated that post-transcriptional regulatory cascades controlled by RBPs (such as FUS and TDP-43) and microRNAs play critical roles in mRNA maturation and gene regulation (20–23). FUS shuttles into the cytoplasm, particularly in neurons, suggesting that FUS is also involved in regulating both mRNA transport into neurites and local protein synthesis at synapses, two processes that are essential to neurons for a rapid response to stimuli (13). Furthermore, it has been recently shown that FUS and TDP-43 incorporate into stress granules (SGs) under cellular stress conditions that negatively control mRNA translation, thus supporting the notion that FUS in the cytoplasmic compartment is in association with translation machinery (24–26). SGs are cytoplasmic aggregates that contain polyadenylated mRNAs, translation initiation factors, small ribosome subunits and RBPs (24,27). Induction of SG formation under cellular stress conditions is believed to serve as a transient protective mechanism by shifting energy expenditure toward cellular repair and recovery while blocking *de novo* translation. Incorporation of FUS and TDP-43 into the cytoplasmic SGs under oxidative stress conditions suggests that motor neurons of ALS patients may have a defect in their ability to recover from stress conditions which might be related to motor neuron degeneration in ALS patients. Despite tremendous progress in the ALS research field, the role of the RNA-binding ability of FUS in causing FUS-related neurodegeneration is not yet clear.

In the present study, we examined the contribution of the RNA-binding ability of FUS in causing neurodegenerative phenotypes in a *Drosophila* model of FUS-related proteinopathies. We mutated four conserved phenylalanines to leucines (amino acids 305, 341, 359 and 368 of human FUS) in the RNA recognition motif (RRM) domain (referred to as 4F-L herewith) and observed that, although both wild-type and mutant FUS without 4F-L mutation do bind RNA, the 4F-L mutations are sufficient to abolish RNA binding in both FUS 4F-L, FUS 4F-L R518K and FUS 4F-L R521C. We found that mutating these RNA-binding residues (4F-L) of FUS blocks its ability to cause neurodegenerative phenotypes *in vivo*. Furthermore, RNA-binding-incompetent FUS carrying ALS-causing mutations predominantly localizes to the nucleus in both a neuronal cell line and *Drosophila* motor neurons, suggesting that cytoplasmic localization of mutant FUS is dependent on its RNA-binding ability. We also observed that RNA-binding-incompetent FUS does not localize in the SGs under stress conditions. Altogether, these findings suggest that the RNA-binding ability of FUS is essential for causing ALS pathogenesis. Furthermore, identification of mechanisms regulating RNA and protein targets of FUS that result in toxicity when FUS carries ALS mutations will help in developing therapeutic interventions for this devastating disease.

## RESULTS

### RNA-binding-incompetent FUS does not bind RNA and becomes non-toxic *in vivo*

In order to determine whether mutating four conserved phenylalanines to leucines (4F-L) of FUS perturbs its RNA-binding ability, we immunoprecipitated HA-FUS UV-crosslinked to RNA in neuronal (N2a) cell lines stably expressing human FUS WT, FUS R518K, FUS 4F-L or FUS 4F-L R518K. Subsequent to radiolabeling-bound RNA, we found an RNA smear on an autoradiogram, indicating RNA binding by FUS was observed in the FUS WT and FUS R518K cells, but observed almost no RNA smear in the FUS 4F-L and FUS 4F-L R518K samples as well as none in non-transfected cells and in the pull-down with a non-specific antibody (Fig. 1A). The autoradiograph was then probed with an anti-HA antibody which showed that HA-FUS protein was pulled down in all expected lanes, demonstrating that FUS 4F-L protein levels are still maintained despite the elimination of bound RNA. Western blots of whole-cell lysates of input samples showed that HA-FUS protein is present in all samples except for the non-transfected cells (Fig. 1A, bottom panel).

To further determine the consequences of disrupting RNA binding of FUS carrying ALS-causing mutations, we expressed FUS 4F-L and FUS 4F-L R518K in yeast cells. We found that FUS 4F-L (single mutant) and FUS 4F-L R518K (double mutant) did not cause any significant toxicity when compared with FUS WT and FUS R518K expression, which did cause increased cell death in the yeast cells when expressed at equal levels (Fig. 1B and C). These findings are consistent with a previous study where ectopic expression of FUS 4F-L itself did not cause any toxicity in yeast cells (28).

We recently developed a *Drosophila* model of FUS-related proteinopathies that recapitulates several key features of ALS, such as mutation-dependent toxicity, neurodegeneration and cytoplasmic mislocalization (29). In order to investigate the

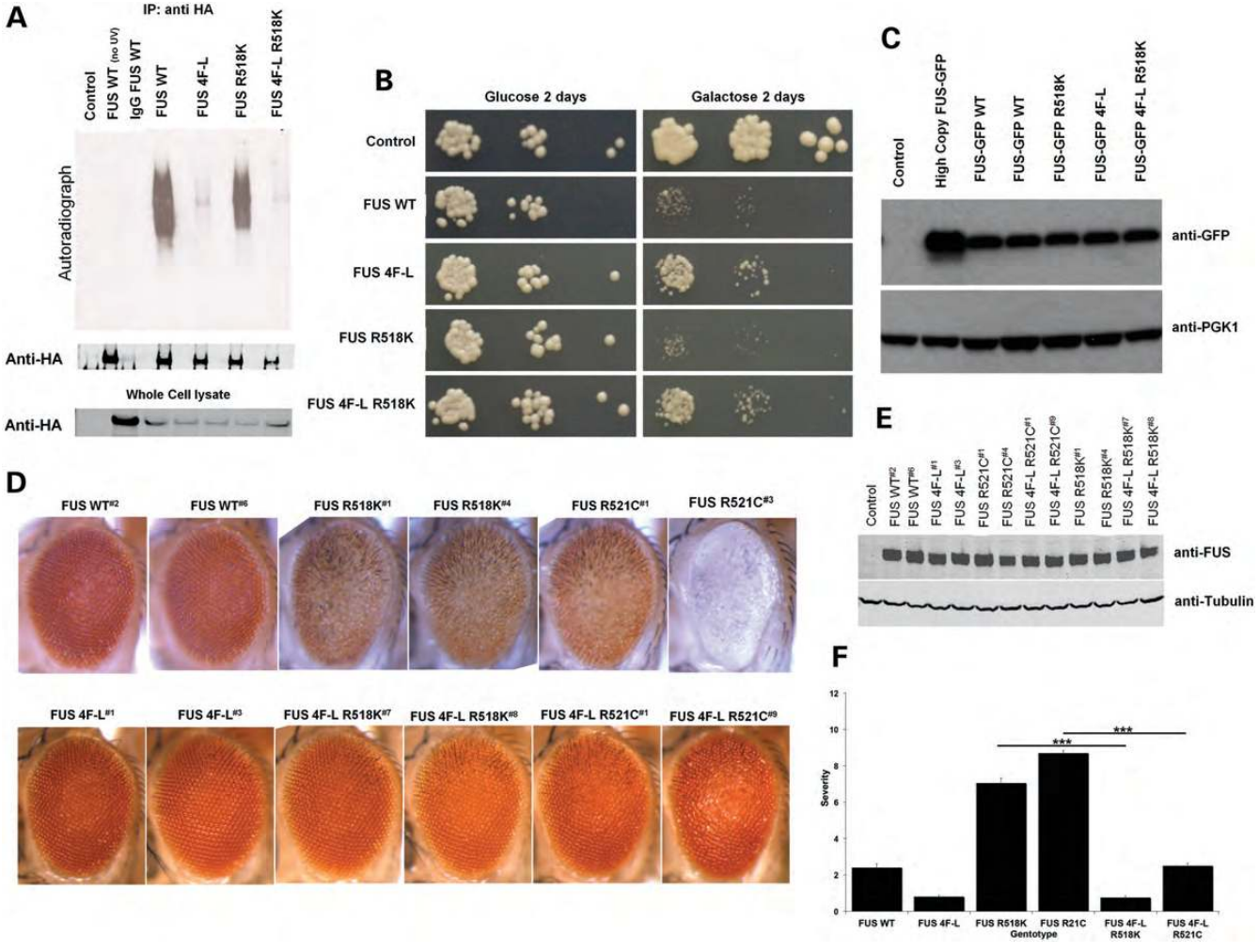


**Figure 1.** Mutating conserved RNA-binding sites 4F-L (F305L, F341L, F359L, F368L) of FUS blocks its RNA-binding ability and does not cause any obvious toxicity in yeast and *Drosophila*. (**A**) Pull-down of HA-tagged FUS constructs UV-crosslinked to RNA from neuroblastoma (N2a) cell lines stably expressing FUS WT, FUS 4F-L, FUS R518K or FUS 4F-L R518K (top panel). Subsequent to the pull-down, the RNA was radiolabeled and the complex was displayed by SDS–PAGE. An RNA smear indicating RNA binding is seen in the FUS-WT and FUS R518K lanes but is highly diminished in the RNA-binding-incompetent (FUS 4F-L and FUS 4F-L R518K) samples. Controls (first three lanes) consisted of the control of non-transfected cells (lane 1), cells transfected with FUS WT but not UV-crosslinked prior to the pull-down (lane 2) and a pull-down of UV-crosslinked FUS WT with a non-specific antibody (lane 3), all show no binding to RNA. (Middle panel) Autoradiograph probed with anti-HA antibody shows that HA-FUS protein was pulled down in all expected lanes, demonstrating that FUS 4F-L protein levels are still high despite the elimination of bound RNA. (Bottom panel) Whole-cell lysate of input shows that HA-FUS protein is present in all samples except for the non-transfected cells (first lane). Loading of whole-cell lysate input samples was done in the same order as the top two panels, but without empty wells between the samples. (**B**) Over-expression of RNA-binding-incompetent FUS alone (FUS 4F-L) or with an ALS-causing mutation (FUS 4F-L R518K) results in significantly less toxicity in yeast compared with FUS WT and FUS R518K. All proteins were expressed using the galactose-inducible *GAL1* promoter. (**C**) Western blotting indicates that FUS WT, FUS R518K and RNA-binding-incompetent FUS accumulate to similar levels in yeast following galactose-induced over-expression. (**D**) Ectopic expression of RNA-binding-incompetent FUS (4F-L constructs) does not cause any external eye degeneration in *Drosophila* (lower panel) when compared with FUS WT, FUS R518K and FUS R521C (upper panel). (**E**) Western blot of two independent transgenic fly lines expressing FUS WT, FUS 4F-L, FUS R521C, FUS 4F-L R521C, FUS R518K and FUS 4F-L R518K showed equal FUS protein levels in the fly heads. (**F**) We performed quantification of eye phenotypes using previously published criteria and found that normally toxic FUS mutations (R518K and R521C) were alleviated of their toxicity when those FUS proteins were rendered RNA-binding-incompetent (4F-L). The asterisks represent significant difference between different groups. ***P, 0.0001.

role of the RNA-binding ability of FUS on toxicity, we generated *Drosophila* lines expressing the UAS-FUS 4F-L, UAS-FUS 4F-L R518K and UAS-FUS 4F-L R521C constructs. We targeted the expression of RNA-binding-incompetent mutants to the fly eyes, using the GMR-gal4 driver. Interestingly, we observed that the expression of RNA-binding-incompetent FUS (4F-L) with or without ALS-causing mutations did not lead to any significant external eye degeneration when compared with FUS WT, FUS R518K and FUS R521C (Fig. 1C and D). We performed quantification of eye phenotypes, using previously published criteria, and found a significant difference in the external eye degenerative phenotypes of flies expressing RNA-binding-incompetent FUS when compared with mutant FUS-expressing animals (Fig. 1D). Interestingly, mutating RNA-binding residues did not alter FUS protein levels and we observed equal levels of FUS in RNA-binding-incompetent lines when compared with both WT and mutant FUS-expressing lines (Fig. 1F).

We also investigated whether deletion of the RRM (ΔRRM) domain had an effect similar to the 4F-L mutants. We generated transgenic lines expressing FUS ΔRRM, FUS ΔRRM R518K and FUS ΔRRM R521C. We targeted the expression of these FUS ΔRRM constructs to the *Drosophila* eyes, using the GMR-gal4 driver. We observed that deletion of the RRM domain of FUS strongly blocks toxicity associated with ALS-causing mutations in FUS (Supplementary Material, Fig. S1).

### Neuronal expression of mutant FUS with 4F-L mutations (RNA-binding-incompetent) alleviates the behavioral abnormalities as well as smaller brain size caused by mutant FUS alone

Previously, we showed that motor neuron expression of mutant FUS caused eclosion defects and larval paralysis in a fly model of FUS-related ALS (29). Because ALS is a motor neuron disease, we asked whether the RNA-binding ability of FUS is required for causing an eclosion defect in animals expressing FUS under the control of the neuronal driver Elav-gal4. We found that RNA-binding-incompetent FUS-expressing lines (FUS 4F-L) eclose significantly better when compared with FUS R518K and FUS R521C. The FUS 4F-L lines demonstrated eclosion rate similar to FUS-WT-expressing animals (Fig. 2A).

In order to determine whether the expression of RNA-binding-incompetent FUS causes any larval locomotor dysfunction, we performed larval crawling assays (29–31). As expected, mutant FUS-expressing animals (UAS-FUS R518K) showed severe larval paralysis when compared with FUS WT or driver-alone controls (Fig. 2B). Interestingly, animals expressing RNA-binding-incompetent FUS (4F-L) with or without ALS-linked mutations in the motor neurons did not cause larval paralysis when compared with FUS WT or a driver-alone control, suggesting that the RNA-binding ability of FUS is important for causing larval paralysis in *Drosophila* (Fig. 2B). Importantly, we observed that mutating RNA-binding residues (4F-L) significantly blocked toxicity associated with FUS WT, and larvae expressing FUS 4F-L were able to crawl better when compared with FUS WT-expressing animals (Fig. 2B).

Normally, *Drosophila* larvae crawl in a forward direction by peristaltic movement accompanied by rhythmic and coordinated waves (32). These movements require muscle contractions passing along the body-wall segments in a posterior-to-anterior direction. To investigate consequences of the expression of FUS WT and FUS carrying ALS-causing mutations on larval body-wall contraction, we targeted the expression of transgenes to the motor neurons and performed body-wall-contraction assays. We observed a severe defect in body-wall contractions of animals expressing FUS R518K and FUS R521C when compared with FUS WT and driver-alone control (Fig. 2C). Interestingly, consistent with larval crawling assay, we found that animals expressing FUS 4F-L showed better body-wall contraction when compared with FUS WT animals, suggesting that the 4F-L mutation can significantly reduce toxicity associated with FUS WT (Fig. 2C).

To further delineate the effects of FUS expression on motor coordination, we performed larval righting assays that measure anterior–posterior and dorsal ventral motor coordination (33). Third instar larvae were turned ventral side up and the time taken for them to roll back to their dorsal side was recorded (righting assay). We found that motor neuron expression of FUS R518K and FUS R521C severely compromised their ability to right themselves (Fig. 2D). However, RNA-binding-incompetent FUS (4F-L) with or without ALS-causing mutations in FUS showed better turning when compared with FUS WT (Fig. 2D). Of note, we also observed that animals expressing FUS 4F-L were able to turn better when compared with FUS WT animals, indicating that RNA-binding-incompetent FUS alone can reduce toxicity associated with FUS WT (Fig. 2D).

Altogether, these behavioral assays strongly suggest that the RNA-binding ability of FUS is important for causing toxicity and that mutating the RNA-binding residues of FUS is enough to abolish this toxicity.

Next, we asked whether the behavioral defects in *Drosophila* larvae associated with mutant FUS can be explained by defects in gross brain morphology. We observed that the expression of mutant FUS in motor neurons (OK371-gal4 driver) caused smaller brain size due to brain atrophy in third instar larvae, suggesting that the expression of mutant FUS, but not RNA-binding-incompetent FUS, causes a defect in gross brain morphology in *Drosophila* larvae (Fig. 3). These observations are consistent with a recent study showing that the expression of a neurodegenerative disease-causing protein leads to smaller larval brains similar to the mutant FUS-expressing animals (34). It is possible that the mutant FUS expression might cause developmental defect followed by atrophy or vice versa in the early stages of development. Further investigation using conditional expression system would help in addressing the possibility if small brain sizes are due to developmental defects or atrophy.

### RNA-binding-incompetent FUS predominantly localizes to the nucleus of mammalian neuronal cells

FUS predominantly resides in the nucleus in normal cells and shuttles between the nucleus and cytoplasm (1,2,35). Abnormal cytoplasmic mislocalization of mutant FUS has been shown in human ALS patients' neurons as well as in several animal models (1,2,36). We reasoned that if the RNA-binding ability of FUS is involved in the cytoplasmic localization of mutant FUS, we would expect predominantly nuclear localization of RNA-binding-incompetent FUS. We stably expressed FUS WT, FUS R518K, FUS 4F-L and FUS 4F-L R518K (all HA-tagged) in the neuroblastoma N2a cell line. We found that the stably transfected neuronal cells have equal FUS expression as expected (Supplementary Material, Fig. S2). Next, we immunostained the stably transfected cells with anti-HA (green) and DRAQ5 (blue; nuclear staining) and examined them by confocal microscopy. As expected and previously reported, FUS WT predominantly localized in the nucleus, whereas FUS R518K was observed in both the cytoplasm and nucleus (Fig. 4A). Surprisingly, we observed predominant localization of both RNA-binding-incompetent FUS with or without ALS-causing mutations in the nucleus (Fig. 4A). We performed quantification of nuclear and cytoplasmic distribution of FUS and found significant differences

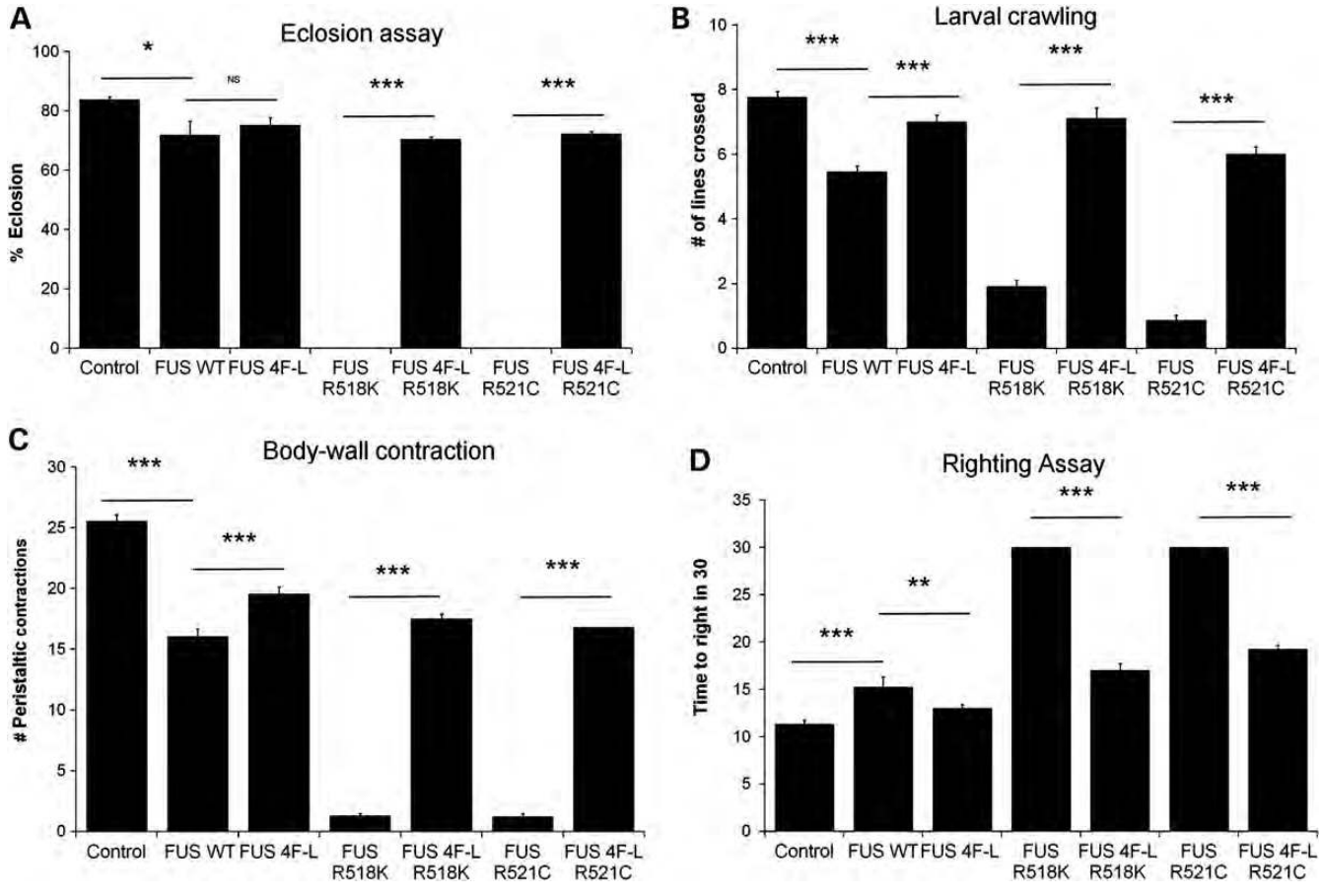


**Figure 2.** Ectopic expression of RNA-binding-incompetent FUS does not cause any behavioral abnormalities in *Drosophila*. (**A**) Eclosion assay: Motor neuron expression of either UAS-FUS R518K or UAS-FUS R521C, but not UAS-FUS WT, led to pupal lethality in *Drosophila*. The animals expressing RNA-binding-incompetent FUS (single or double mutants) in the motor neurons eclosed normally when compared with driver-alone control and WT FUS (error bars represent standard error). (**B**) Larval crawling assay: Motor neuron expression of mutant FUS R521C and R518K, but not WT, in the fly motor neurons led to larval paralysis when compared with driver alone. RNA-binding-incompetent FUS (4F-L)-expressing animals showed no defect in their crawling ability when compared with controls. (**C**) Body-wall contraction: Expression of mutant FUS led to reduced body peristaltic contractions when compared with FUS WT and driver-alone control. However, RNA-binding-incompetent FUS-expressing animals showed body peristaltic contractions similar to FUS WT and driver-alone controls. (**D**) Larval righting assay: Motor neuron expression of mutant FUS compromised the turning ability of third instar larvae when compared with FUS WT and driver-alone control. Note that FUS R521C larvae lost all their ability to right themselves. Interestingly, RNA-binding-incompetent FUS-expressing animals demonstrated normal larval turning ability similar to FUS WT and driver-alone controls. The asterisks represent significant difference between groups $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ and NS (not significant).

in the distribution pattern of RNA-binding-incompetent FUS when compared with FUS R518K (Fig. 4B). These observations suggest that cytoplasmic mislocalization of mutant FUS is mediated via the RNA-binding ability of FUS and mutating RNA-binding residues restricts FUS protein to the nucleus. However, we did not find obvious toxicity in the N2a cells transiently transfected with either FUS WT or FUS R518K in the XTT assay performed under serum starvation conditions as well as in normal serum (Supplementary Material, Fig. S3).

### RNA-binding-incompetent FUS predominantly localizes to the nucleus of *Drosophila* motor neurons

To investigate subcellular distribution of FUS in fly motor neurons, we targeted the expression of UAS FUS WT, UAS FUS R518K, UAS FUS R521C and UAS FUS 4F-L R521C to the motor neurons, using the OK371-gal4 driver. We immunostained the cells for Lamin as a nuclear marker to highlight the nuclear envelope membrane, and thus staining inside the Lamin ring is nuclear and outside the ring is cytoplasmic (29). As expected, WT FUS predominantly localized in the nucleus, whereas UAS-FUS R518K and FUS R521C were distributed in the cytoplasm and nucleus as demonstrated by the immunofluorescence of FUS (green) and Lamin (red) (Fig. 5). WT FUS can be clearly seen localized to the nucleus, whereas mutant FUS R518K and FUS R521C staining (Fig. 5C and E, arrows) is seen in the nucleus and also in the cytoplasm, outside of the Lamin ring (Fig. 5A). Interestingly, we found that RNA-binding-incompetent FUS (UAS FUS 4F-L, UAS FUS 4F-L R518K and UAS FUS 4F-L R521C) predominantly localized in the nucleus, suggesting that cytoplasmic mislocalization of mutant FUS is dependent on the RNA-binding ability of FUS, and cytoplasmic localization of mutant FUS is important for causing ALS pathogenesis.

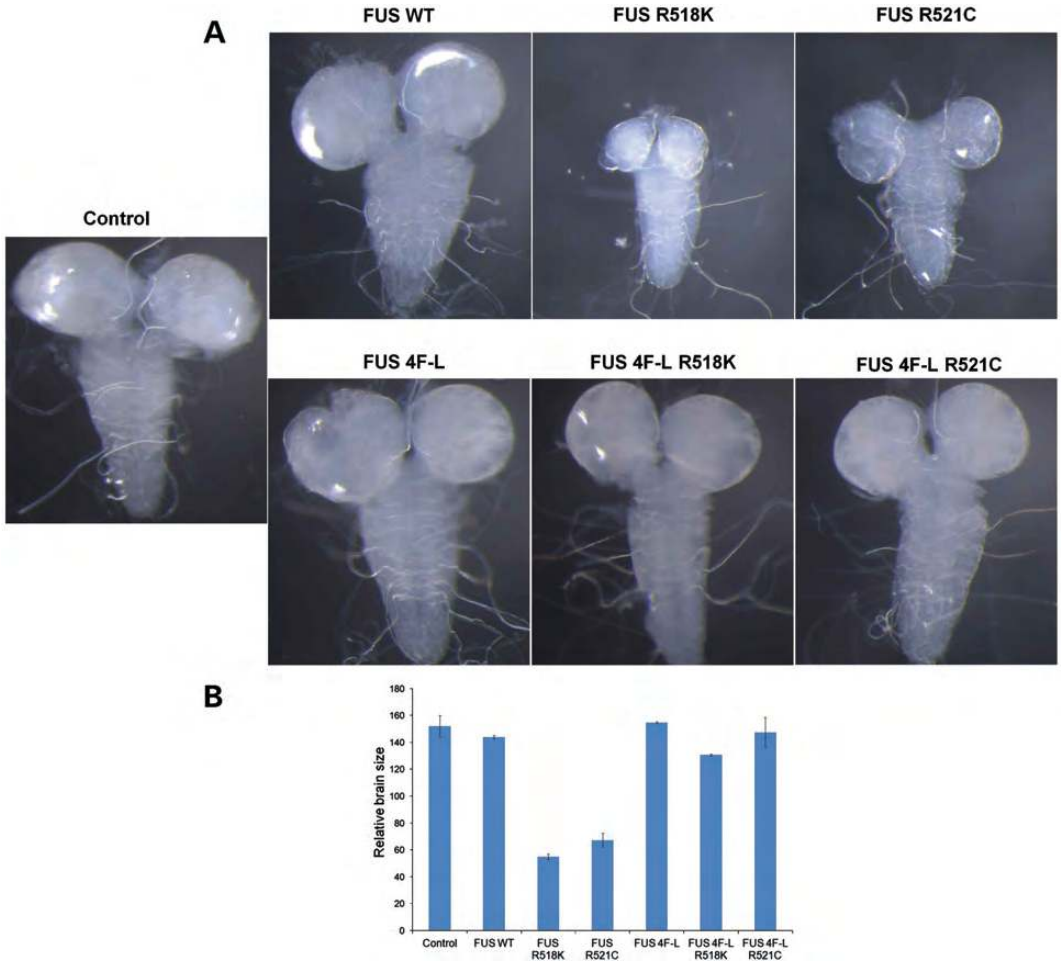


**Figure 3.** Ectopic expression of mutant FUS, but not RNA-binding-incompetent FUS, leads to brain atrophy with ubiquitin-positive pathology *in vivo*. (**A**) Ectopic expression of FUS R518K and FUS R521C in fly motor neurons leads to brain atrophy and reduced brain size. However, the expression of RNA-binding-incompetent FUS alone or with ALS-causing mutations does not cause any gross defect in the brain size. (**B**) Quantification of larval brain size.

### RNA-binding-incompetent FUS does not incorporate into the cytoplasmic SGs

It has been previously demonstrated that FUS carrying ALS-causing mutations incorporates into the cytoplasmic SGs under stress conditions and is important for ALS pathogenesis (24). We asked whether this incorporation of mutant FUS is dependent on the RNA-binding ability of FUS. To address this issue, we stressed neuronal N2a cell lines stably expressing FUS WT, FUS R518K, FUS 4F-L and FUS 4F-L R518K (all HA-tagged), with sodium arsenite for 1 h as described previously (24). We immunostained the cells with anti-HA (green, FUS), anti-TIAR (red, SG marker) and DRAQ5 (blue, nuclear marker) and examined them by confocal microscopy. We observed that FUS R518K, but not WT FUS, incorporates into the cytoplasmic SGs under stressed condition (Fig. 6). Importantly, RNA-binding-incompetent FUS (FUS 4F-L and FUS 4F-L R518K) did not incorporate into the cytoplasmic SGs under stress conditions. We performed analogues experiment under unstressed condition and we did not find any evidence of colocalization of FUS with SGs (Supplementary Material, Fig. S4). We performed quantification of cells showing localization of FUS in the cytoplasmic SGs and found a significant difference in the incorporation of FUS R518K and RNA-binding-incompetent FUS R518K into the cytoplasmic SGs (Supplementary Material, Fig. S5).

## DISCUSSION

FUS and TDP-43 are two key RBPs implicated in ALS and several other neurodegenerative diseases (1–3). Recent studies demonstrated that FUS and TDP-43 interact with a large number of RNA targets, suggesting that these proteins' interactions with RNA may have important roles in pathways involved in neurodegenerative diseases (19,20). Interestingly, disease-causing mutations in FUS do not disrupt the RNA-binding ability of FUS, suggesting the possibility that mutant FUS might interact with a different set of mRNAs or with the same set of RNAs with different affinity when compared with WT FUS (1). Massive numbers of RNAs bind to both WT and mutant forms of FUS, suggesting that FUS is part of a large RNA–protein network and thus a slight disruption in this network due to disease-associated mutations might

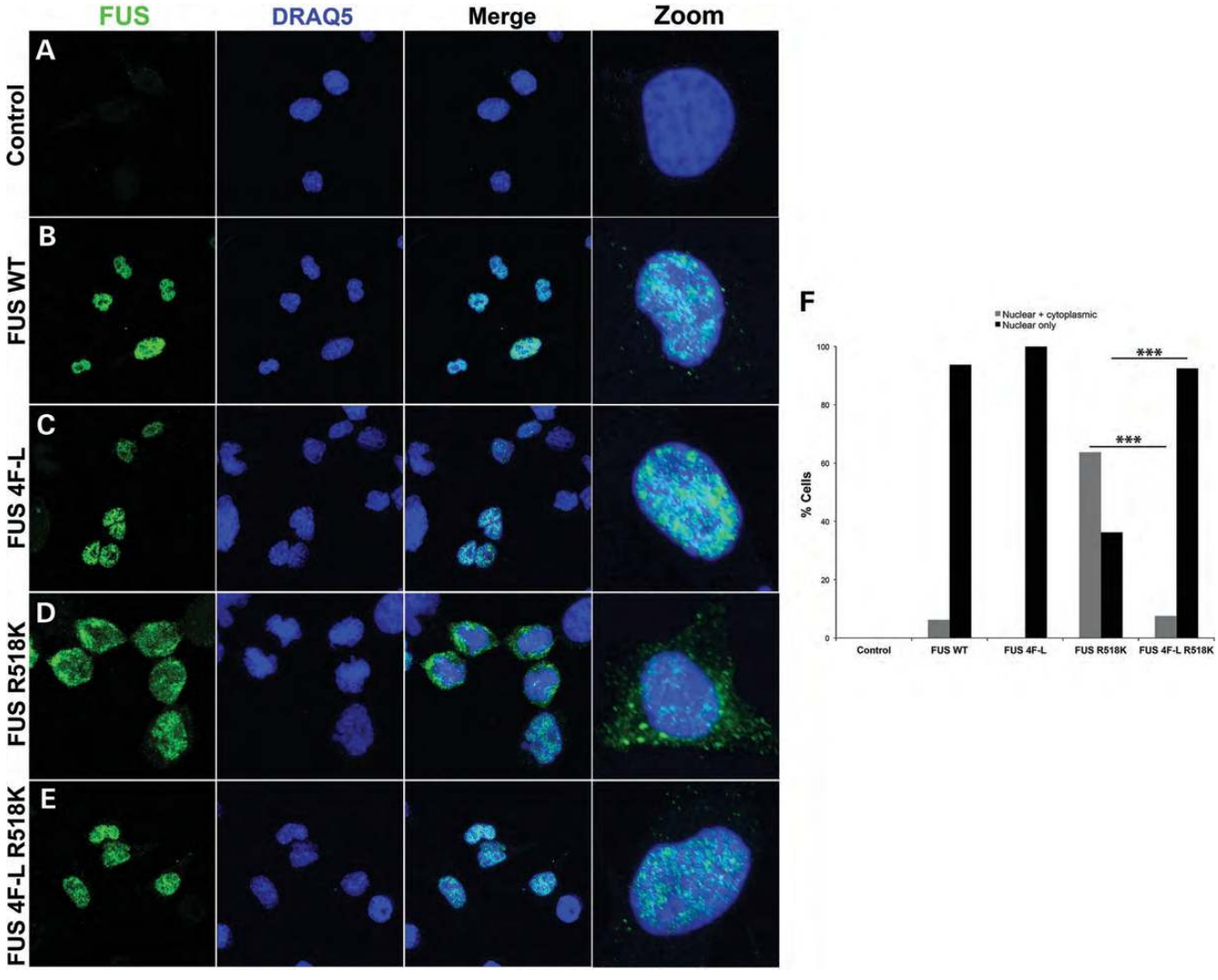


**Figure 4.** FUS carrying the ALS-causing mutation R518K alone distributes itself to the nucleus and cytoplasm in mammalian neuronal cells but RNA-binding-incompetent FUS (4F-L alone and 4F-L R518K) localizes in the nucleus similar to WT FUS. (**A**) FUS WT (anti-HA: green) predominantly localizes to the nucleus (DRAQ5: blue), (**B**) whereas FUS R518K is distributed to the nucleus and cytoplasm (**D**). Interestingly, RNA-binding-incompetent FUS 4F-L (**C**) itself or with an ALS-associated mutation FUS 4F-L R518K (**E**) localized exclusively to the nucleus in the neuronal (N2a) cells. (**F**) Quantification of cytoplasmic and nuclear distribution of FUS. The asterisks represent significant difference between different groups. ***P, 0.0001.

be enough to cause the motor neuron degeneration observed in ALS (19,20).

We found that mutating conserved phenylalanine residues to leucine (4F-L) in the RRM of FUS impairs the RNA-binding abilities of both FUS 4F-L and FUS 4F-L R518K, suggesting that these residues are critical for mediating RNA–protein interactions without destabilizing FUS protein expression. Previously, it has been shown that the expression of FUS 4F-L does not cause any obvious toxicity in yeast cells (28). We observed that the ectopic expression of FUS 4F-L with ALS-causing mutations (R518K or R521C) does not cause toxicity in yeast cells (Fig. 1A), suggesting that RNA-binding-incompetent FUS with ALS-causing mutations becomes non-toxic in yeast cells similar to FUS 4F-L. We also investigated whether the RNA-binding-incompetent FUS with or without ALS-causing mutations becomes non-toxic in a whole animal model system. To address this, we utilized a *Drosophila* model of FUS-related proteinopathies that recapitulates several key features of ALS, such as mutation-dependent toxicity and locomotor dysfunctions when expressed in the fly eyes and motor neurons, respectively. We found that targeted expression of RNA-binding-incompetent FUS in the *Drosophila* eyes does not lead to any significant external eye degeneration, suggesting that the RNA-binding ability of FUS is important for causing neurodegeneration *in vivo*.

We next asked whether mutating the RNA-binding ability of FUS can also block locomotor dysfunctions associated with motor neuron expression of mutant FUS. Previously, we demonstrated that motor neuron expression of mutant FUS leads to an eclosion rate defect and larval paralysis in *Drosophila* (29). We observed that the RNA-binding-incompetent FUS with or without ALS-causing mutations did not cause an eclosion rate defect or larval paralysis

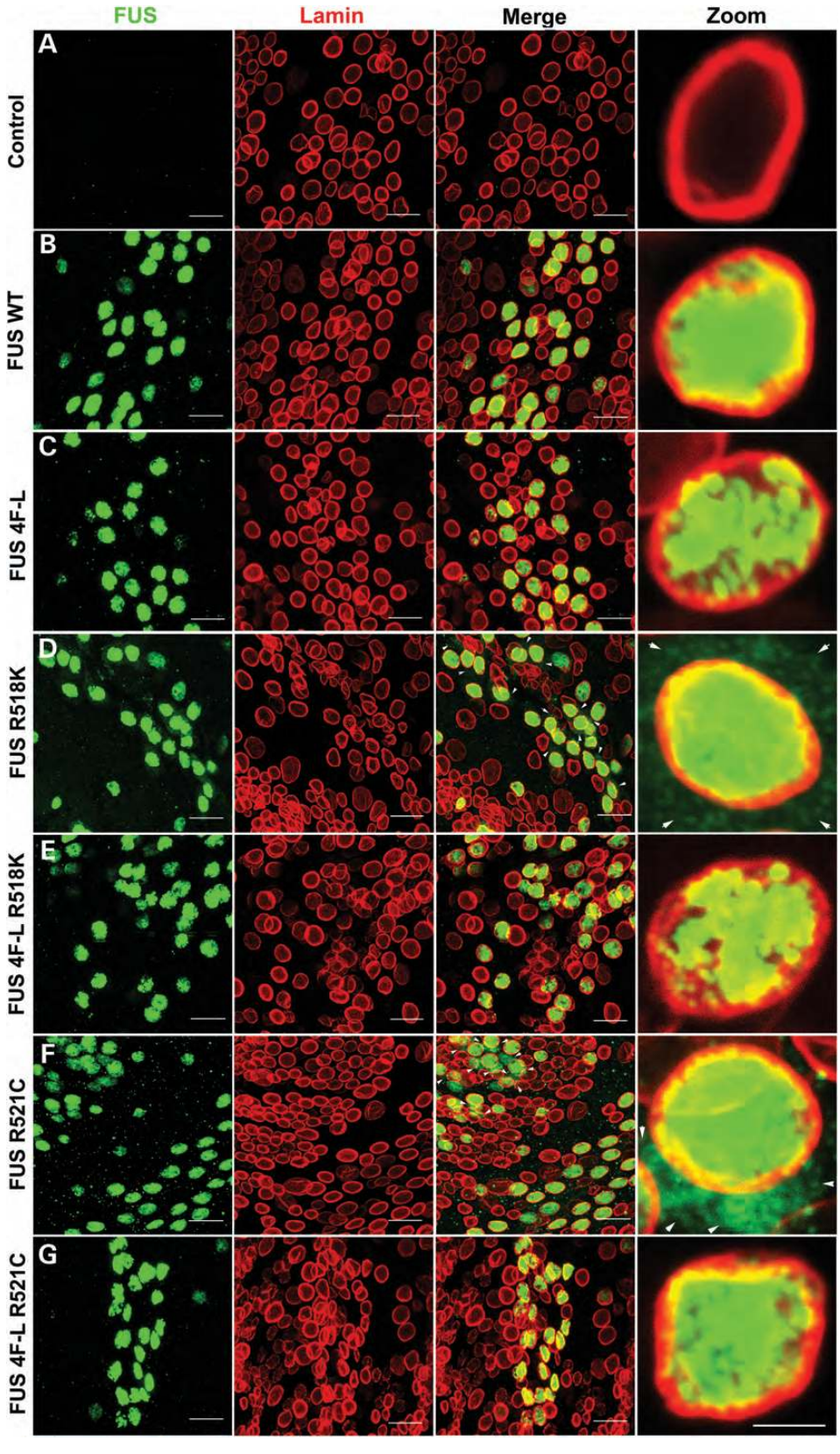


**Figure 5.** Cytoplasmic mislocalization of FUS carrying ALS-causing mutations (R518K and R521C) in *Drosophila* motor neurons is reversed by making FUS RNA-binding-incompetent. (**A**) FUS WT (anti-HA: green) predominantly localized in the nucleus (anti-Lamin: red) of the *Drosophila* motor neurons (**B**), whereas FUS R518K or FUS R521C distributed itself to both the nucleus and cytoplasm (**D** and **F**). Interestingly, RNA-binding-incompetent FUS itself (**C**) or with ALS-associated mutations were predominantly nuclear (**E** and **G**).

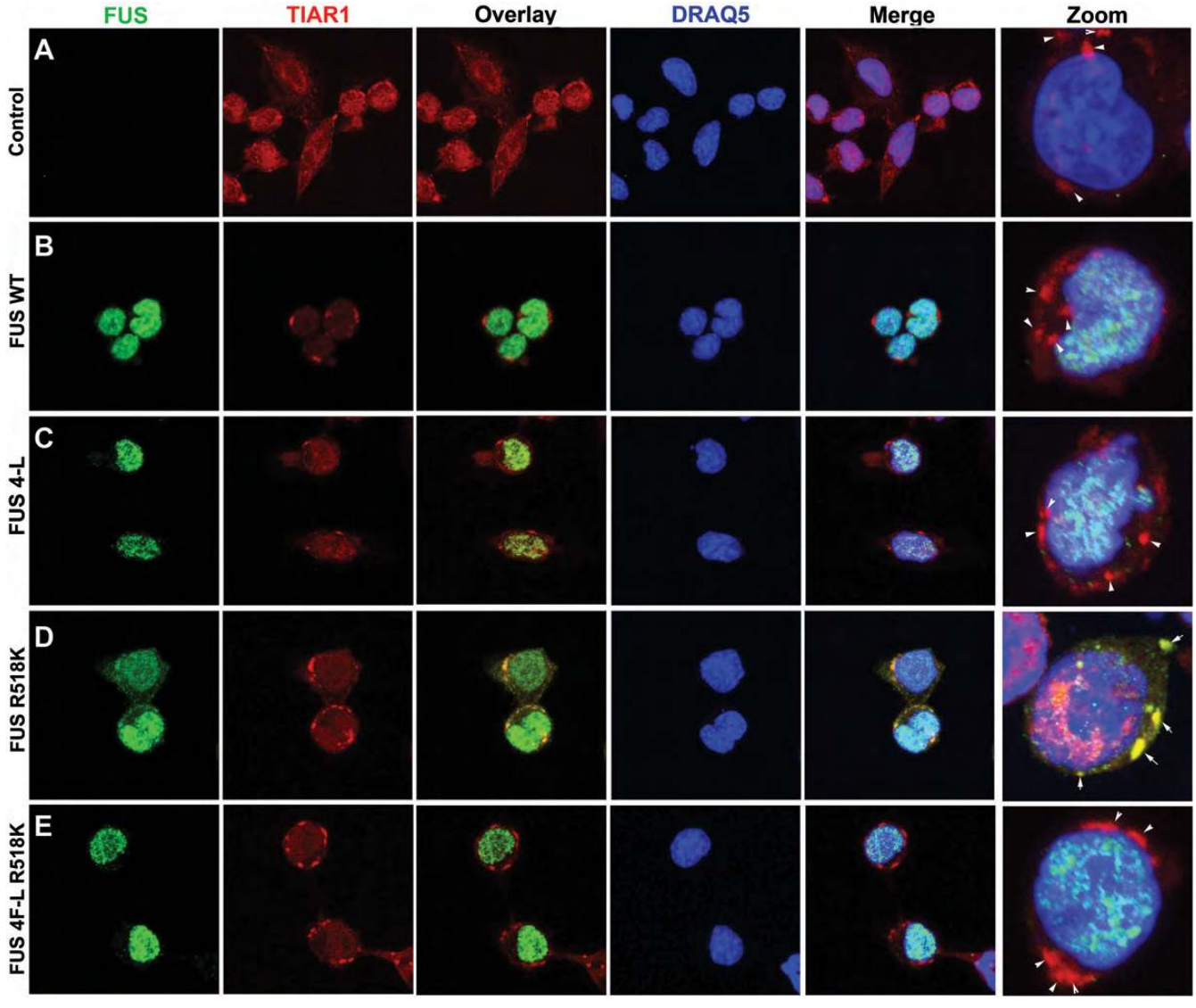


**Figure 6.** Incorporation of mutant FUS into SGs is dependent on its RNA-binding ability. Each HA-FUS stable N2a cell line was treated with 0.5 mM sodium arsenite for 1 h, stained with the nuclear dye DRAQ5 (blue), anti-HA (green) and anti-TIAR (red). (**A**) SGs were induced in untransfected N2a cells. (**B**) Anti-HA staining reveals HA-FUS is primarily nuclear and does not incorporate into SGs. (**C**) RNA-binding-incompetent mutations do not cause HA-FUS incorporation into SGs. (**D**) HA-FUS R518K colocalizes with the SG marker TIAR. (**E**) RNA-binding-incompetent FUS carrying the ALS-causing mutation FUS R518K was excluded from TIAR SGs.

when compared with FUS WT-, FUS R518K- or FUS R521C-expressing animals. Moreover, mutating the RNA-binding ability of FUS also strongly suppressed decreased body-wall contractions and larval turning phenotypes associated with the expression of FUS carrying ALS-causing mutations. These results further support our hypothesis that disrupting the RNA-binding property of FUS is enough to make FUS carrying ALS mutations non-toxic.

Because cytoplasmic mislocalization of FUS carrying an ALS-causing mutation is a pathological hallmark of ALS in human patients as well as in several animal models, we wished to determine the subcellular distribution of RNA-binding-incompetent FUS in a mammalian neuronal cell culture model system. We observed that similar to WT FUS, RNA-binding-incompetent FUS with or without ALS-causing mutations predominantly resides in the nucleus, suggesting that cytoplasmic mislocalization of FUS associated with disease-causing mutations is dependent on the RNA-binding ability of FUS. We further investigated this question in flies expressing RNA-binding-incompetent FUS in the motor neurons. We found that RNA-binding-incompetent FUS predominantly localizes in the nucleus, further supporting our hypothesis that cytoplasmic localization of mutant FUS is mediated through the RNA-binding ability of FUS. It is possible that regulation of subcellular distribution of FUS by its RNA-binding ability might be important to ALS pathogenesis and that cytoplasmic mislocalization of mutant FUS might be mediated by RNA binding. Along this line, the FUS protein could form an FUS–RNA complex before being transported to the cytoplasm, and mutating 4F-L residues might disrupt

this complex formation, which in turn leads to predominant nuclear localization of RNA-binding-incompetent FUS. Alternatively, FUS carrying ALS-causing mutations might be transported to the cytoplasm via other RBPs, and, along these lines, the 4F-L mutations could disrupt a complex of FUS with other RBPs, thereby blocking transport to the cytoplasm. Additionally, mutant FUS might be involved in cytoplasmic sequestering of protein or RNAs critical for maintaining nuclear functions, and this non-specific transport could lead to motor neuron degenerative pathologies observed in ALS patients.

Cytoplasmic SGs are transient structures that arise upon exposure to cellular stress such as infection, heat, oxidative insult and hypoxia. SGs are considered as a protective response against adverse cellular conditions, storing mRNAs important for maintaining cellular homeostasis. The SG, which is a non-membranous structure, sequesters mRNAs encoding for housekeeping proteins responsible for triage, degradation and translational re-initiation (37). On the one hand, SGs selectively down-regulate the translation of housekeeping mRNAs to conserve energy to cope with the stress-mediated damage, and on the other hand, SGs up-regulate synthesis of proteins such as DNA-repair proteins, chaperone proteins and various transcriptional factors. In addition to mRNAs, SGs also contain several RBPs, such as TDP-43, FUS, poly(A)-binding protein 1 and T-cell intracellular antigen 1. Previous studies have demonstrated that marker proteins that label SGs colocalize with the pathological FUS inclusions in postmortem brains of ALS and FTLD (26,38).

Molecular mechanisms underlying incorporation of FUS with ALS-associated mutations into SGs still remain an enigma. We observed that RNA-binding-incompetent FUS with ALS-causing mutations does not colocalize with SGs in the neuronal cells under stress conditions, suggesting that incorporation of mutant FUS into SGs is RNA-dependent. It is possible that transportation of mutant FUS is either directly or indirectly regulated by the RNA-binding ability of FUS or through other RBPs and that mutating the RNA-binding residues of FUS might impair the ability of FUS to localize into SGs under cellular stress conditions. A recent study supports our observations that the RNA-binding abilities of FUS and TDP-43 play important roles in SG formation (26). Moreover, the study from Bentmann *et al*. (39) also suggests that FUS domains that do not bind artificial RNA (UG-rich RNA) in *in vitro* assays also contribute to SG assembly. The experiments performed by Bentmann *et al.* (39) rely on the deletion of different functional domains of FUS which might disrupt the three-dimensional architecture of FUS protein when compared with our study, where we mutated the conserved phenylalanine residues to leucines (4F-L); hence, it is difficult to conclude that non-RNA-binding domains of FUS also contribute to SG formation. Moreover, it is possible that differences between our study and Bentmann *et al.* (39) are due to the use of different cell lines for performing the assays.

Our data demonstrate the role of the RNA-binding ability of FUS in ALS pathogenesis and support the notion that incorporation of mutant FUS into the cytoplasmic SGs is important for FUS-mediated ALS pathogenesis. Our study cannot rule out the possibility of any structural changes in the FUS protein due to mutations in the RNA-binding residues. However, further investigations would help in determining whether 4F-L mutation also causes structural changes in the FUS protein structure, which in turn blocks the toxicity. On the basis of our results, we expect that the RNA-binding ability of mutant FUS would be a logical target for a potential drug for treating ALS patients.

## MATERIALS AND METHODS

### Antibodies, plasmids and cell culture

The human FUS cDNA plasmid (pDONR-FUS-RRM-4F-L) containing four phenylalanine-to-leucine mutations (amino acids 305, 341, 359 and 368 of human FUS) in the RRM domain of FUS was a generous gift of Dr Aaron Gitler (28). Analogous mutation of four phenylalanines to leucines in the closely related TDP-43 protein has been shown to abolish RNA binding (40). The 713 bp fragment spanning the 4F-L mutations was isolated following double digestion with restriction enzymes *Bsu*36I and *Ppu*I (New England Biolabs, MA, USA) and inserted into similarly prepared pUAST-based vectors (*Drosophila* expression vectors pUAST-FUS-WT, pUAST-FUS-R518K and pUAST-FUS-R521C). Subcloning was done using standard methods, and isolated clones were verified as correct by analytical restriction digests and DNA sequencing. These pUAST constructs (pUAST FUS 4F-L, pUAST FUS 4F-L R518K and pUAST FUS 4F-L R521C) were sent to BestGene (Chino Hills, CA, USA) to generate transgenic flies.

To transfer each of the three FUS constructs into the pCIneo eukaryotic expression vector, the pUAST plasmids were digested with *Xho*I and *Xba*I. The isolated fragments were subcloned into the similarly prepared pCIneo vectors to generate pCIneo FUS 4F-L and pCIneo FUS-4F-L R518K. Isolated clones were verified by analytical restriction digests. The aforementioned two 4F-L clones and also pCIneo FUS-WT and pCIneo FUS-R518K were then transfected into the mouse neuro-N2a cell line, using TurboFect (Fermentas, MD, USA), following the manufacturer's suggested protocol. Positive clones were selected and maintained by including 500 μg G418/ml in the culture medium. The medium was advanced DMEM supplemented to contain 10% FBS and 1× GlutaMax (all from Life Technologies, Grand Island, NY, USA).

### XTT assay to measure cell toxicity

To measure differences in cell proliferation of Neuro2a cells, a standard curve was constructed to determine the number of cells within the range of detection for the assay. Standards were performed in triplicate. For the assay, 50 000 cells were seeded in triplicate in a 96-well tissue culture plate and were allowed 24 h to adhere. Fresh DMEM media containing 5% FBS was added and assayed using ATCC XTT Cell Proliferation Assay Kit (Cat. # 30-1011k) for 3 h using the manufacturer's instructions. Data from three trials containing three replicates of each sample were compiled.

To measure cell viability by XTT assay, cells were serum-starved in DMEM media without FBS and treated with aphidicolin (400 ng/ml, Calbiochem) for 48 h. Two independent trials containing triplicates of each sample were averaged

together. Data were analyzed using ANOVA with *post hoc* analysis using Tukey's at an alpha level of 0.05.

### RNA-binding assay

The protocol used was adapted from the HITS-CLIP protocol used in Chi *et al*., 2009 (http://ago.rockefeller.edu/Ago_HITS_CLIP_Protocol_June_2009.pdf). Briefly, the cells were suspended in PXL lysis buffer and homogenized followed by the addition of a 1:2500 dilution of RNase I (Ambion, AM2295) and a 1:250 dilution of Turbo DNase. The samples were then incubated for 3 min at 37°C and then transferred to ice. The cell lysate was spun at 4°C at 22 500*g* for 30 min and the supernatant was carefully collected. The supernatant was incubated with Protein G Dynabeads that had been pre-bound with monoclonal anti-HA antibody HA-7, and then rotated for 4 h at 4°C. On the beads, the protein-bound RNA was treated with calf intestinal alkaline phosphate and then ligated with a radiolabeled 3′RNA linker. After stringent washing, the samples were run on a NuPage 4–12% Bis–Tris gel and transferred to a nitrocellulose membrane for autoradiography exposure to detect the bound RNA and subsequently probed with anti-HA to detect protein levels, using the Li-COR Odyssey system.

### Yeast expression and immunoblotting

FUS-GFP fusion proteins were expressed in *Saccharomyces cerevisiae* strain W303 as previously described (41). Yeast cells were grown at 30°C in standard synthetic media containing either 2% glucose or 2% galactose (for the induction of protein expression). FUS proteins were expressed using the *GAL1* promoter from plasmids derived from pFPS298 (*CEN*, *URA3*). The plasmids are designated as follows: pDK346 (FUS-GFP), pDK400 (FUS 4F-L-GFP), pDK405 (FUS R518K-GFP), pDK518K 406 (FUS 4F-L R518K-GFP). The high-copy plasmid DK248 (*2μ, URA3*, FUS-GFP) was used as an additional control during immunoblotting.

Protein expression levels were confirmed by western blotting, using cultures grown for 6 h in 2% galactose, harvested and washed once with water. Cell pellets were re-suspended in lysis buffer [50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 3 mM EDTA, 5% glycerol, 5 mM DTT and Complete protease inhibitor mixture (Roche, Indianapolis, IN, USA)]. Cells were physically disrupted by vigorous agitation with acid-washed glass beads for 3 min at 4°C. Cellular debris was removed by low-speed centrifugation (10 min at 1500*g*). BCA reagent (Pierce, Rockford, IL, USA) was used to normalize protein concentrations. Yeast lysates were subjected to SDS/PAGE (Any kD gels, Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Immunoblotting was performed according to standard protocols. The following antibodies were used: mouse monoclonal α-GFP (Roche), mouse monoclonal α-PGK1 and secondary AP-conjugated α-mouse (Invitrogen).

### *Drosophila* culture, light microscopy and quantification

The FUS WT, FUS R518K and FUS R521C transgenic flies and GMR-gal4 and OK371-gal4 drivers were described previously (29). Eye phenotypes of 1-day-old flies were analyzed with a Leica M205C stereomicroscope and photographed with a Leica DFC420 digital camera. For each genotype and condition, more than 25 flies were evaluated and quantification of eye phenotypes was done as described previously (29).

### Larval brain size determination

All isolated fly brains were isolated and photographed with a dissecting scope (Leica M205C), using the same distance and magnification. All images were analyzed using NIH ImageJ under identical conditions. A line was drawn around the perimeter of each brain. The relative area was determined. For each brain, this was done in triplicate, and the average was used as the relative area of the brain. This was done for multiple brains of each genotype. This cumulative data were then analyzed.

### *Drosophila* behavioral assays

*Eclosion assays*
Eclosion assays were carried out using the methodology published previously (29).

*Crawling assay*
We performed the larval crawling assay, using a previously published method (29,30). Before starting the assays, vials containing larvae were kept at room temperature for 20 min. After removing third instar larvae from food vials, they were washed in PBS to remove any residual food and were allowed to crawl on a 1% agar plate for 1 min as described previously (29).

*Righting assay*
For the righting assay, third instar wandering larvae were placed on agar plates and allowed to acclimatize for 5 min at room temperature. The larvae were then placed ventral side up and the time taken to return back to a crawling position/dorsal side upward was recorded. Twenty to 25 larvae were used per genotype and time taken was recorded in seconds.

*Contraction assay*
For the contraction assay, larvae were placed on agar plates and allowed to acclimatize for 5 min. The number of full-body peristaltic contractions in 30 s was recorded (31). Note that FUS R521C larvae lost all their ability to right themselves. Interestingly, RNA-binding-incompetent FUS-expressing animals demonstrated normal larval turning ability similar to FUS WT and driver-alone controls. The asterisks represent significant difference between groups $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ and NS (not significant).

### Statistical analysis

For all behavioral assays, data were analyzed using one-way ANOVA. Tukey's *post hoc* analysis was then applied with multiple comparisons between each group under an alpha level of 0.05. Differences were considered statistically significant when $P < 0.05$; however, we do report when $P < 0.001$ and $P < 0.0001$.

### Immunohistochemistry

*Subcellular localization*

Wandering third instar larvae were dissected and fixed in 4% formaldehyde for 15–25 min. Antibodies used included anti-FUS (Bethyl Labs, cat. # A300–302A, 1:1000), anti-Lamin DmO (ADL67.10, at 1:100, developed by Paul Fisher and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biology, University of Iowa, Iowa City, IA, USA). For secondary staining, anti-rabbit Alexa Fluor 488 (Invitrogen, cat. # A-11008, 1:500) and anti-mouse Alexa Fluor 546 (Invitrogen, cat. # A-11003, 1:500) were used. After staining, larval cuticles with intact CNS were mounted in ProLong Gold (Invitrogen, cat. # P36930). Images were captured with a Leica SP2-TCS confocal microscope. All motor neurons imaged were located in the dorsal midline of the ventral ganglion.

Stable Neuro2a cell lines were taken off of G418 selection for 5 days prior to assay. In total, 100 000 cells were allowed to adhere to cover slips pre-coated with 0.01% poly-L-lysine (Sigma, cat. #P4832). The medium was changed to contain DRAQ5 [Abcam, cat. # ab108410 (5 mM), 1:1000] and incubated at 37°C for 15 min. The medium was removed and cells were washed with PBS and fixed with 4% formaldehyde for 15 min. Antibodies used included anti-HA [Y11] (Santa Cruz, cat. # sc-805, 1:200) and anti-rabbit Alexa Fluor 488 (Invitrogen, cat. # A-11008, 1:500). Cover slips were mounted in ProLong Gold (Invitrogen, cat. # P36930).

For the subcellular localization analysis, only cells positive for FUS were considered. For the quantification of subcellular localization, cytoplasmic staining was counted as present when pixel intensity was at least 10 times the background fluorescence. Pixel intensity was determined using ImageJ (http://rsbweb.nih.gov/ij/).

### SG induction

Stable Neuro2a cell lines were taken off of G418 selection for 5 days prior to the assay. In total, 100 000 cells were allowed to adhere to cover slips coated with 0.01% poly-L-lysine (Sigma, cat. #P4832). The medium was changed to contain 0.5 mM sodium arsenite (Ricca Chemical Co., cat # 7142-16) as described previously (24). The medium was then changed to contain DRAQ5 [Abcam cat., # ab108410 (5 mM), 1:1000] and incubated at 37°C for 15 min. Cells were washed with sterile PBS, pH 7.0, and fixed with 4% formaldehyde for 15 min. Antibodies used included anti-HA [Y11] (Santa Cruz, cat. # sc-805, 1:200), anti-TIAR (BD Transduction Labs, cat # 610352, 1:250), anti-rabbit Alexa Fluor 488 (Invitrogen, cat. # A-11008, 1:500) and anti-mouse Alexa Fluor 568 (Invitrogen, cat. # A-11004). Cover slips were mounted in ProLong Gold.

For quantifying FUS incorporation into SGs, only cells which both stained positive for FUS and contained cytoplasmic SGs were considered in the analysis (60 cells). Cytoplasmic staining was counted as present when pixel intensity was at least 10 times the background fluorescence. Pixel intensity was determined using ImageJ (http://rsbweb.nih.gov/ij/). Incorporation of FUS into SGs was considered positive when FUS directly colocalized with the SG marker TIAR.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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*Conflict of Interest statement*. None declared.

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