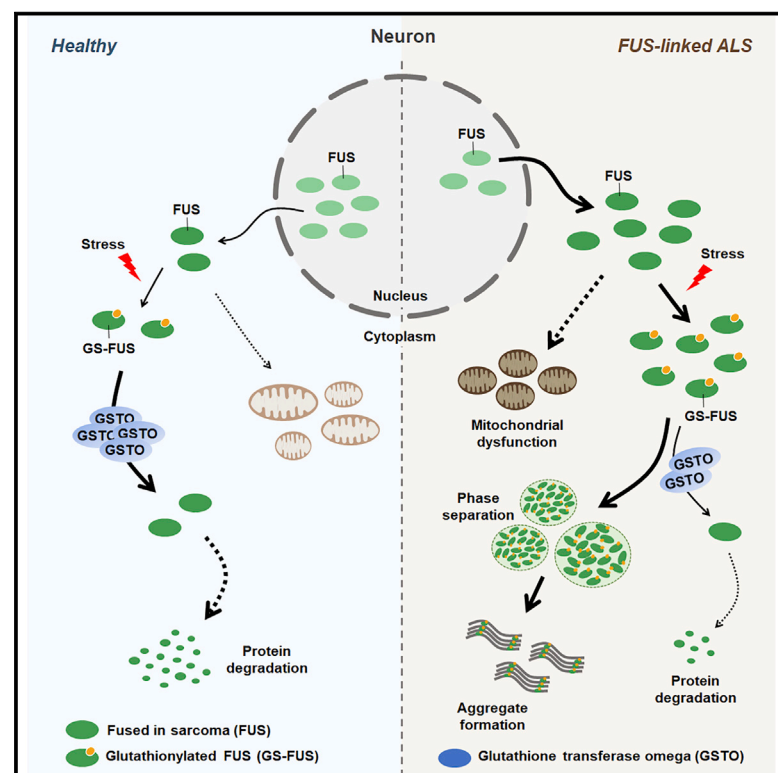


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Therapeutic modulation of GSTO activity rescues FUS-associated neurotoxicity via deglutathionylation in ALS disease models

Graphical abstract



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In brief

Fused in sarcoma (FUS) is genetically linked to amyotrophic lateral sclerosis and frontotemporal dementia. Cha and Lee et al. report that FUS glutathionylation promotes liquid-liquid phase separation and aggregate formation *in vitro*. Glutathione transferase omega activation mitigates cytoplasmic FUS aggregation by deglutathionylation *in vivo*.

Highlights

- GSTO suppresses FUS-induced neurotoxicity
- GSTO inhibits the formation of cytoplasmic FUS aggregates in neurons
- Glutathionylation decreases FUS solubility by promoting phase separation
- GSTO regulates FUS liquid-liquid phase separation by reducing glutathionylation *in vivo*



Article

Therapeutic modulation of GSTO activity rescues FUS-associated neurotoxicity via deglutathionylation in ALS disease models

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SUMMARY

Fused in sarcoma (FUS) is a DNA/RNA-binding protein that is involved in DNA repair and RNA processing. FUS is associated with neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). However, the molecular mechanisms underlying FUS-mediated neurodegeneration are largely unknown. Here, using a *Drosophila* model, we showed that the overexpression of glutathione transferase omega 2 (GstO2) reduces cytoplasmic FUS aggregates and prevents neurodegenerative phenotypes, including neurotoxicity and mitochondrial dysfunction. We found a FUS glutathionylation site at the 447th cysteine residue in the RanBP2-type ZnF domain. The glutathionylation of FUS induces FUS aggregation by promoting phase separation. GstO2 reduced cytoplasmic FUS aggregation by deglutathionylation in *Drosophila* brains. Moreover, we demonstrated that the overexpression of human GSTO1, the homolog of *Drosophila* GstO2, attenuates FUS-induced neurotoxicity and cytoplasmic FUS accumulation in mouse neuronal cells. Thus, the modulation of FUS glutathionylation might be a promising therapeutic strategy for FUS-associated neurodegenerative diseases.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder characterized by the selective death of motor neurons in the brain stem and spinal cord (Cleveland and Rothstein, 2001; Swinnen and Robberecht, 2014). Frontotemporal dementia (FTD), the second-most common form of early-onset dementia, is caused by neuronal loss in the frontal and temporal cortex and is characterized by personality and behavioral changes (Van Langenhove et al., 2012). Although most cases of ALS are sporadic, approximately 10% of cases are linked to genetic mutations in several loci, including superoxide dismutase 1 (SOD1), fused in sarcoma (FUS), TAR DNA-binding protein-43 (TDP-43), and chromosome 9 open reading frame 72 (C9ORF72) (Renton et al., 2014).

Specifically, FUS is implicated in neurodegenerative diseases, such as FTD and a rare juvenile-onset form of ALS (Lattante et al., 2013; Vance et al., 2009). FUS is a ubiquitously expressed multifunctional ribonucleoprotein (RNP) consisting of a repeated arginine/glycine-rich (RGG) domain, an RNA/DNA-recognition

motif (RRM), a zinc-finger (ZnF) motif, and a nuclear localization signal (NLS). Some ALS-associated FUS mutants promote the abnormal cytoplasmic accumulation of FUS inclusions (Dormann and Haass, 2011; Vance et al., 2013). Although it is still unclear how FUS causes neuronal toxicity, several studies have suggested that cytoplasmic FUS aggregates can induce neurodegeneration.

Some post-translational modifications (PTMs) modulate pathological protein aggregation in neurodegenerative diseases. The reported PTMs of FUS are the methylation of arginine residues by arginine methyltransferases and the phosphorylation of serine/threonine residues by a DNA protein kinase (Deng et al., 2014; Dormann et al., 2012). Hypermethylation and phosphorylation of FUS are known to affect cytoplasmic localization and aggregation, respectively (Monahan et al., 2017). However, the precise role of PTMs in FUS remains unclear.

Glutathionylation is a major redox-sensitive PTM that can alter the activity and stability of target proteins in response to several cellular stresses, including oxidative stress (Mieyal et al., 2008). Oxidative stress is a common pathological feature of most



neurodegenerative diseases, such as ALS and FTD. This type of stress can change the characteristics of certain proteins and increase the level of insoluble aggregates (Barber and Shaw, 2010; Petrov et al., 2016; Valle and Carri, 2017). Previous findings showed that oxidative stress promotes the localization of FUS to cytoplasmic stress granules, which are dynamic organelles that lack membranes (Bentmann et al., 2012; Bosco et al., 2010). Increased chronic stress and cytoplasmic accumulation of FUS most likely facilitate self-association, leading to pathological aggregation (Dormann and Haass, 2011). Therefore, PTMs of FUS may modulate pathological features under oxidative stress or other pathological conditions.

In this study, we showed that overexpression of wild-type or ALS-associated mutant FUS in *Drosophila* causes neurotoxicity and mitochondrial dysfunction similar to these manifestations in ALS pathology. These phenotypes were rescued by the overexpression of *Drosophila* glutathione transferase omega 2 (GstO2), known as a deglutathionylating enzyme. In addition, we found that glutathionylation of FUS facilitates FUS aggregation by promoting phase separation. Additional studies using a mouse neuronal cell line demonstrated that human GSTO1 (the homolog of *Drosophila* GstO2) expression reversed the FUS-induced defective phenotypes and pathology by modulating the glutathionylation status. Therefore, the modulation of FUS glutathionylation and increased GSTO1/GstO2 expression may represent a promising therapeutic strategy for FUS-associated neurodegenerative diseases.

RESULTS

GstO2 suppresses FUS-induced neurotoxicity in *Drosophila* models

A previous study that aimed to identify ALS biomarkers showed a significant reduction in glutathione transferase omega (GSTO1) levels in peripheral blood mononuclear cells and spinal cord cells from ALS patients compared with controls (Nardo et al., 2011). Furthermore, we previously reported that GstO2, which is a *Drosophila* homolog of human GSTO1, suppressed neuronal toxicity by regulating the glutathionylation of the ATP synthase β subunit in a *Drosophila* model of Parkinson's disease (PD) (Kim et al., 2012). To determine whether GstO2 expression shows changes under ALS pathogenic conditions, we performed reverse transcription-polymerase chain reaction (RT-PCR) and immunoblot analyses with extracts from the heads of FUS-, TDP-43-, and TAF15-expressing flies using pan-neuronal Gal4, *elav-Gal4*. The GstO2 mRNA level was not changed in the ALS models (Figure S1A). Interestingly, the GstO2 protein level was dramatically reduced in the FUS- and TAF15-based ALS models compared with the controls (Figure S1B). These findings are consistent with our hypothesis that GSTO1 expression and function are involved in the pathogenesis of ALS. Therefore, we used a *Drosophila* model to investigate the possible modulation of FUS-induced toxicity by GstO2 *in vivo*. We tested whether increasing GstO2 would mitigate FUS-induced neurotoxicity in *Drosophila*. To evaluate the functional relationship between GstO2 and FUS in the *Drosophila* model, we generated transgenic flies that coexpressed GstO2 and FUS driven by eye-specific Gal4 and *GMR-Gal4*. We found that FUS overexpression led to a rough eye phenotype, disrupted ommatidial organization,

and pigmentation loss. Interestingly, the coexpression of GstO2 with FUS significantly reversed the rough eye phenotype and pigmentation loss. In contrast, knocking down GstO2 enhanced the loss of eye pigment in the FUS-expressing flies. No alterations or disruptions in the eyes were observed in flies expressing GstO2 or GstO2 RNAi alone (Figure 1A).

Next, we examined the mechanisms underlying the locomotive defects caused by FUS expression using pan-neuronal Gal4, *elav-Gal4*. The flies that expressed neuronal FUS showed dramatically reduced locomotive activity compared with the control flies. Surprisingly, this decrease in locomotive activity was significantly suppressed by the coexpression of GstO2. In contrast, knocking down GstO2 caused a complete loss of locomotion in the FUS-expressing flies (Figure 1B). The overexpression or knockdown of GstO2 alone in a wild-type background had no effect on larval crawling activity (Figure 1B). These results suggested that the larval locomotive defect could be caused by morphological defects at neuromuscular junctions (NMJs). To determine the number of synaptic boutons at the NMJs, we expressed FUS under the control of *elav-Gal4*. We observed a significant decrease in the total number of boutons at the NMJs in the FUS-expressing flies compared with that in the control flies. This phenotype was also dramatically restored by the coexpression of GstO2, which had no effect when expressed alone (Figure 1C). However, knocking down GstO2 in the FUS-expressing flies had no effect on the decreased number of boutons at the NMJs (Figure 1C).

In addition, the expression of FUS in neurons resulted in a drastically shortened fly lifespan, which was rescued by the coexpression of GstO2 but was unaffected by GstO2 expressed alone (Figure 1D). In contrast, knocking down GstO2 further reduced the shortened lifespan of the FUS-expressing flies, but the lifespan remained unaffected by GstO2 RNAi alone (Figure 1E). Consistent with previous studies, the FUS-expressing flies showed substantially reduced climbing activity compared with the age-matched controls. We also found that the coexpression of GstO2 in neurons strongly suppressed the FUS-induced climbing deficit. GstO2 knockdown in the FUS-expressing flies showed a greater reduction in climbing activity than that in the single FUS-expressing flies (Figure 1F). Next, we tested whether GstO2 expression mitigated FUS-mediated motor neuron toxicity. We expressed a membrane-tethered red fluorescent protein (mCD8-RFP) using motor neuron-specific Gal4, *D42-Gal4*, to visualize motor neuronal morphology in adult fly legs and larval brains. The FUS-expressing flies showed severe eclosion defects. The coexpression of GstO2 in motor neurons improved the eclosion rate (Figure S1C). In addition, compared with the control flies, the FUS-expressing flies displayed increased axonal morphology defects in their motor neurons. GstO2 expression alleviated the axonal morphological defects in the FUS-expressing flies (Figure S1D, arrows). Moreover, GstO2 expression substantially restored the decreased RFP signal intensity and the decreased size of motor neuron cell bodies in the ventral nerve cord of larval brains (Figure S1E). These observations confirmed that GstO2 mitigates FUS-induced motor neuron toxicity *in vivo*. Taken together, these data indicate that FUS-induced neuronal toxicity can be significantly suppressed and enhanced by regulating GstO2 expression.

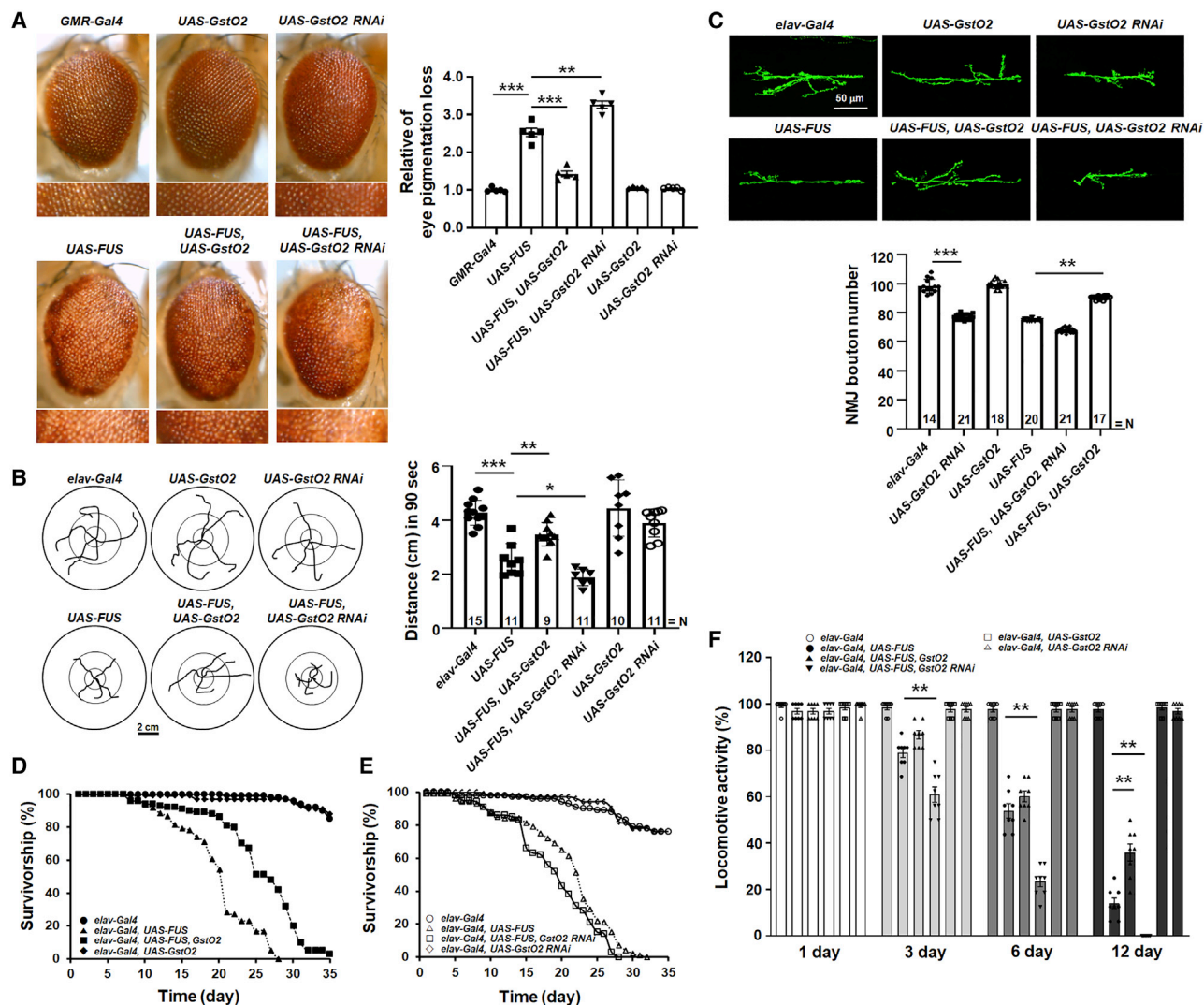


Figure 1. GstO2 modulates FUS-mediated neurodegeneration in *Drosophila* models

(A) GstO2 expression improves FUS-induced retinal toxicity in *Drosophila* eyes (magnified views). Quantification of eye pigment loss for each genotype with normalization to the value of the control flies ($n = 5$ each). Error bars represent the mean value $\pm$ SD. $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA.

(B) Locomotive traces from a 90-s trial are shown for *Drosophila* of six different genotypes ($n > 10$ each). Error bars represent the mean value $\pm$ SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA.

(C) Representative NMJ terminals of the indicated genotypes stained with anti-HRP (green) antibody. Quantification of the total number of boutons per muscle area on muscle 6/7 of abdominal segment 3 ($n > 14$ each). Scale bars, 50 μ m. Error bars represent the mean value $\pm$ SD. $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA.

(D) GstO2 expression in neurons mitigated the lifespan defects of the FUS-expressing flies.

(E) Knocking down GstO2 in neurons further reduced the shortened lifespan of the FUS-expressing flies ($n > 150$ each).

(F) Percentage of flies with locomotive activity is shown. Error bars represent the mean value $\pm$ SD. $^{**}p < 0.01$; two-way ANOVA (Tukey's multiple-comparison test).

GstO2 suppresses FUS-induced mitochondrial dynamics and oxidative phosphorylation (OXPHOS) dysfunction in *Drosophila*

Mitochondria are perturbed in ALS patients, and abnormal mitochondria are consistently observed in animal models of ALS (Canto and Gurney, 1994; Magrané et al., 2014). Although previous studies have indicated that mitochondrial dysfunction is a common characteristic in ALS, no studies have demonstrated that impairment of mitochondrial dynamics or the oxidative phosphorylation (OXPHOS) system in FUS proteinopathy is a

major contributor to ALS pathogenesis. Therefore, we investigated whether GstO2 expression suppresses enhanced mitochondrial fission in the FUS-expressing flies. The mitochondria were visualized with mitochondrion-targeted green fluorescent protein (mitoGFP). We observed that FUS overexpression using muscle-specific Gal4, *Mhc-Gal4*, in muscles led to an abnormal wing posture, which was partially restored by GstO2 coexpression (Figure S1D). Compared with the control flies, the FUS-expressing flies displayed small and fragmented mitochondria (Figure 2A). GstO2 overexpression in the control flies

did not lead to any detectable change in mitochondrial morphology. However, when GstO2 was coexpressed in the FUS-expressing flies, the mitochondrial size was dramatically restored (Figure 2A). To determine whether GstO2 also restores the morphology of mitochondria in motor neurons, we overexpressed GstO2 using motor neuron-specific Gal4, *D42-Gal4*, together with mitoGFP. The results were consistent with the previously observed rescue effect of GstO2; that is, the coexpression of GstO2 prevented mitochondrial fragmentation in the motor neurons of the FUS-expressing flies (Figure 2B). Since we previously reported that decreased Marf expression in FUS-expressing flies may induce excessive mitochondrial fission (Altanbyek et al., 2016), we assessed whether Marf expression is altered by GstO2. Interestingly, the expression of GstO2 with FUS resulted in a significant increase in Marf expression but no change in Opa1 levels (Figure 2C).

Recently, human TDP-43, another RNA/DNA-binding protein linked to ALS that shares some functional aspects with FUS, was found to induce complex I disassembly and mitochondrial dysfunction by inhibiting translation (Wang et al., 2016). However, it is unclear whether morphological changes in mitochondria under FUS overexpression affect the OXPHOS system. To determine the functional relevance of imbalanced mitochondrial dynamics in FUS-induced ALS, we indirectly measured the content of mitochondrial complexes in the electron transport chain (ETC) by analyzing the levels of several complex subunits in the FUS-expressing flies. Surprisingly, compared with the control flies, the FUS-expressing flies displayed substantially reduced levels of the complex I subunit NDUFS3 and the complex III subunit UQCRC2. However, there was little to no change in the levels of the α -subunit of complex V, ATP5A (Figure 2D). As we were unable to find antibodies that cross-react with any of the complex II and IV subunits in *Drosophila*, we could not analyze the contents of these mitochondrial complexes. As previously observed, the overexpression of GstO2 with FUS significantly restored the abundance of NDUFS3 and UQCRC2 to levels similar to those of the control (Figure 2D). To further explore complex assembly, we performed a blue native gel electrophoresis (BN-PAGE) assay using purified mitochondria from the thoracic tissues of adult flies. Consistent with the reduction in the expression of the complex I and III subunits in the FUS-expressing flies, the levels of these assembled complexes (I and III) were found to be low according to the BN-PAGE results. As in other experiments, the overexpression of GstO2 rescued FUS-induced complex I and III disassembly. However, the status of complex V assembly remained unchanged in all mutant lines (Figure 2E).

Next, to investigate whether GstO2 also restored mitochondrial activity, we used a specific mitochondrial superoxide indicator, MitoSOX, to measure the mitochondrial production of reactive oxygen species (ROS) in the FUS-expressing flies. We observed that ROS production was increased in the FUS-expressing flies and restored to normal levels by coexpressed GstO2 (Figure 2F). We found a significant decrease in the ATP level of the FUS-expressing flies compared with the control flies. By expressing GstO2 along with FUS in fly muscles, we observed a recovery in ATP levels (Figure 2G). Based on these results, we confirmed that the FUS-induced mitochondrial dy-

namic imbalance and dysfunction could be significantly prevented by GstO2.

Oxidative stress has also been suggested to be associated with the pathogenesis of various neurodegenerative diseases, including motor neuron degeneration (Lin and Beal, 2006; Niedzielska et al., 2016). We therefore examined the overall levels of protein oxidation in the FUS-expressing flies and found a 2.5-fold increase in the level of the protein carbonyl groups added through oxidative reactions. Interestingly, the accumulated protein carbonyl groups were dramatically reduced to normal levels after coexpression of GstO2 (Figure 2H), indicating that GstO2 attenuates the disruption of redox homeostasis caused by FUS expression.

GstO2 suppresses the formation of FUS aggregates in *Drosophila* neurons

Interestingly, immunoblot assays of brain extracts from the FUS-expressing adult flies showed that GstO2 overexpression reduced the total FUS protein levels (Figure 3A). However, knocking down GstO2 in the FUS-expressing flies caused a further increase in FUS expression (Figure 3B). To determine whether the expression of GstO2 suppressed the mislocalization of FUS in brain tissues, we performed subcellular fractionation assays to measure the FUS levels in both the cytoplasm and nucleus. Our results showed that with GstO2 coexpression, the levels of FUS were decreased in the cytoplasmic fraction but not in the nuclear fraction (Figure 3C). These results indicate that GstO2 can suppress FUS toxicity by decreasing FUS levels in the cytoplasm.

FUS proteins are intrinsically prone to aggregation *in vitro*, which may predispose them to form pathological inclusions (Sun et al., 2011). Furthermore, the neurotoxicity caused by FUS expression is modulated by FUS aggregation (Miguel et al., 2012). Therefore, to determine the effects of GstO2 on FUS aggregation in our transgenic flies, we separated head lysates into detergent-soluble and detergent-insoluble fractions. FUS expression resulted in the formation of the insoluble fraction. The flies coexpressing GstO2 and FUS showed significantly increased FUS levels in the soluble fraction and decreased FUS levels in the insoluble fraction. In contrast, the neuron-specific knockdown of GstO2 substantially induced a strong shift of FUS from the soluble fraction to the insoluble fraction (Figure 3D). The quantification of soluble and insoluble FUS demonstrated a significant >40% decrease in the ratio of insoluble/soluble FUS in the flies coexpressing GstO2 compared with the flies expressing only FUS. FUS expression along with GstO2 knockdown resulted in a marked increase in the ratio of insoluble/soluble FUS (Figure 3D).

In addition, to investigate the contribution of GstO1 and GstO3, which are other *Drosophila* GstOs, to FUS-induced toxicity, we overexpressed GstO1 in neurons. Transgenic flies for GstO3 are currently unavailable. Although GstO1 expression in neurons suppressed FUS-induced locomotor defects, GstO1 did not affect the total FUS protein levels or solubility (Figure S2). Notably, the knockdown of either GstO1 or GstO3 was not sufficient to enhance FUS aggregation, as shown by immunoblotting (Figure S2D). These results indicate that GstO2, in particular, has a protective function in FUS-induced ALS by regulating FUS aggregate formation in the cytoplasm.

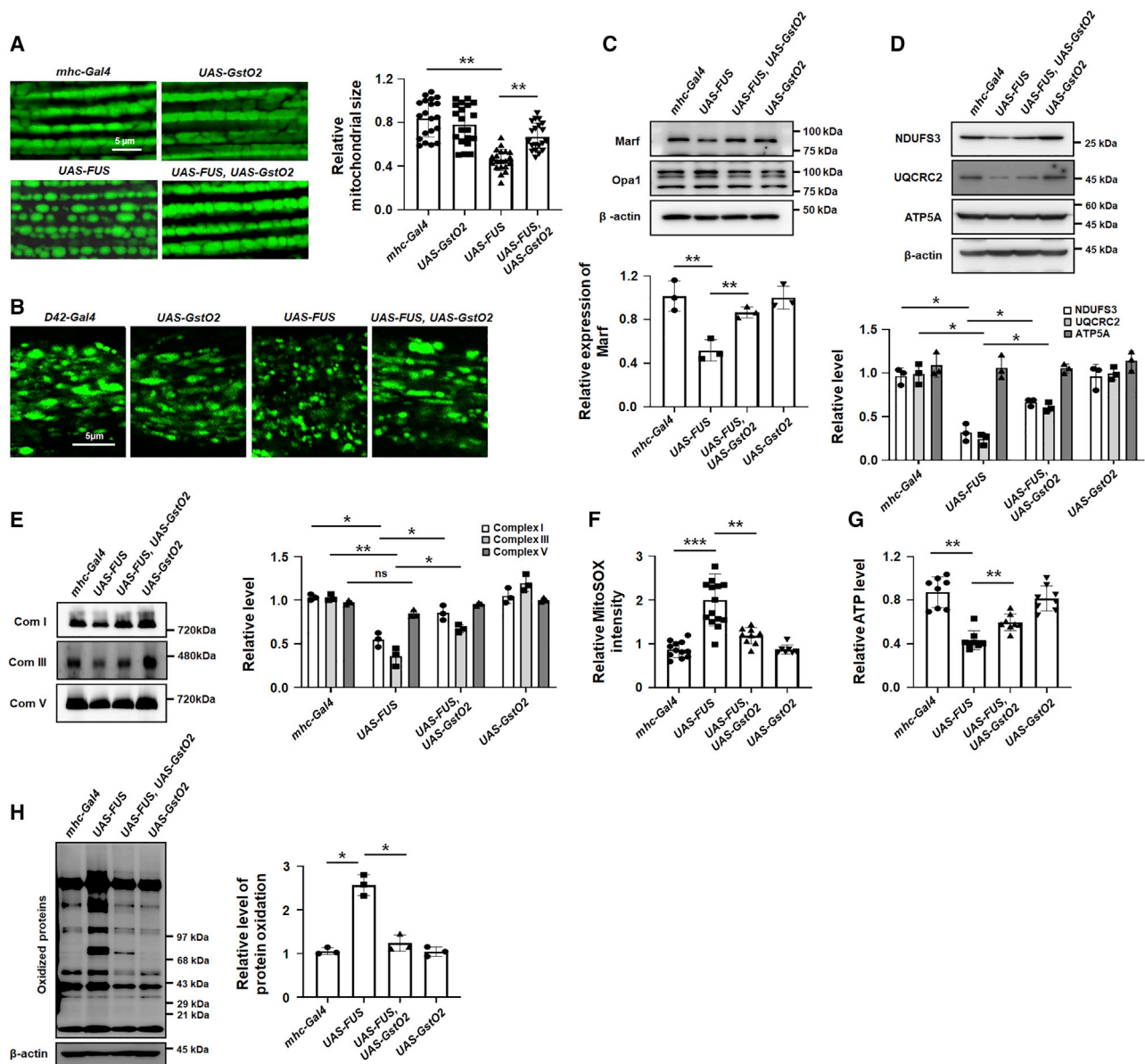


Figure 2. GstO2 reduces FUS-induced mitochondrial damage in *Drosophila*

(A) Representative images of mitochondria in the indirect flight muscles. Mitochondria were visualized by mitoGFP, and the relative mitochondrial sizes were calculated. Scale bars, 5 μ m. Error bars represent the mean $\pm$ SD. ** p < 0.01; one-way ANOVA.

(B) Representative images of the mitochondria in the motor neurons of the adult fly legs. Scale bars, 5 μ m.

(C) Immunoblot analysis of Marf and Opa1 in the adult thorax extracts. β -actin was used as a loading control. Error bars represent the mean value $\pm$ SD. ** p < 0.01; one-way ANOVA.

(D) Immunoblot analysis of NDUFS3 and UQCRC2 in the adult thorax extracts. β -actin was used as a loading control. Error bars represent the mean value $\pm$ SD. * p < 0.05; one-way ANOVA.

(E) Assembly of the mitochondrial complexes assayed by BN-PAGE. Error bars represent the mean value $\pm$ SD. * p < 0.05, ** p < 0.01, not significant (ns); one-way ANOVA.

(F) Relative level of mitochondrial ROS calculated using MitoSOX indicator fluorescence in the adult thorax extracts of the flies with the indicated genotypes. Error bars represent the mean value $\pm$ SD (n > 8 each). ** p < 0.01, *** p < 0.001; one-way ANOVA.

(G) Relative ATP content in the adult thoraces normalized to total protein. Error bars represent the mean value $\pm$ SD (n = 8 each). ** p < 0.01; one-way ANOVA.

(H) OxyBlot analysis of the measurement of total protein carbonylation in the adult thoraces of the flies with the indicated genotypes. β -actin was used as a loading control. Error bars represent the mean value $\pm$ SD. * p < 0.05; one-way ANOVA.

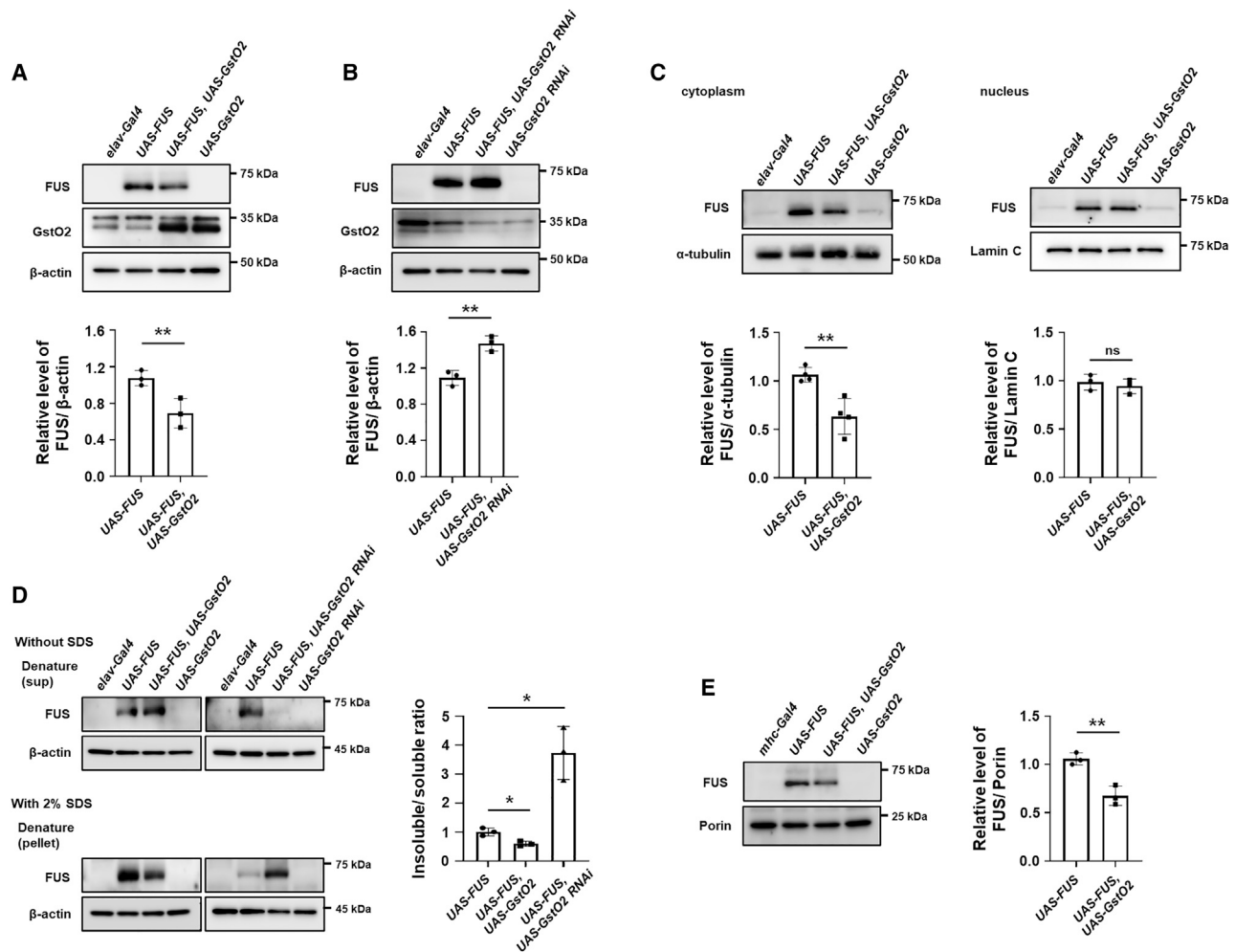


Figure 3. Gsto2 prevents FUS aggregation and promotes FUS solubility in the cytoplasm

(A and B) Immunoblot showing FUS protein levels in the adult head extracts. β -actin was used as a loading control. Error bars represent the mean value $\pm$ SD. ** $p < 0.01$; Student's t test.

(C) Immunoblot analysis of cytoplasmic and nuclear FUS protein levels in the adult head extracts. α -tubulin and lamin C were used as loading controls for the cytoplasmic and nuclear fractions, respectively. Error bars represent the mean value $\pm$ SD. ** $p < 0.01$, not significant (ns); Student's t test.

(D) Soluble (supernatant) and insoluble (pellet) fractions isolated from the adult heads of the flies with the indicated genotypes were analyzed by immunoblotting using FUS antibodies. β -actin was used as a loading control. Densitometry quantifications of insoluble to soluble FUS ratio. Error bars represent the mean value $\pm$ SD. * $p < 0.05$; one-way ANOVA.

(E) Immunoblot of the mitochondrial subcellular fraction probed for FUS. Porin was used as a loading control. Error bars represent the mean value $\pm$ SD. ** $p < 0.01$; Student's t test.

Increased FUS expression in the cytoplasm promotes the association of FUS with mitochondria and induces mitochondrial dysfunction (Deng et al., 2015). To test whether Gsto2 regulates the mitochondrial FUS level, we coexpressed Gsto2 with FUS in fly muscle tissues and then examined the FUS level in purified mitochondria by western blotting. The mitochondrial FUS level was found to be significantly decreased in the Gsto2-coexpressing flies (Figure 3E), providing further evidence that Gsto2 mediates the mitochondrial localization of FUS by reducing the level of cytoplasmic FUS.

FUS is glutathionylated at cysteine residue 447

Glutathionylation results in the formation of a mixed disulfide bond between cysteine and reduced glutathione (GSH). Human

GSTO1 was previously identified as a deglutathionylating enzyme. Therefore, we sought to identify the exact molecular mechanisms that regulate the glutathionylation process to find a way to reduce FUS-induced pathology by Gsto2. First, to determine whether FUS is glutathionylated, we performed an *in vitro* glutathionylation assay using human FUS-myc, a purified recombinant protein from human HEK293 cells. We observed that the wild-type FUS protein was glutathionylated in the presence of various concentrations of oxidized glutathione (GSSG) (Figure 4A).

For identification of putative glutathionylation sites, myc-tagged human FUS was glutathionylated *in vitro* and readily detected in Coomassie-stained gels. Peptides with a mass that differed by 307 Da, representing one GSH moiety, were detected

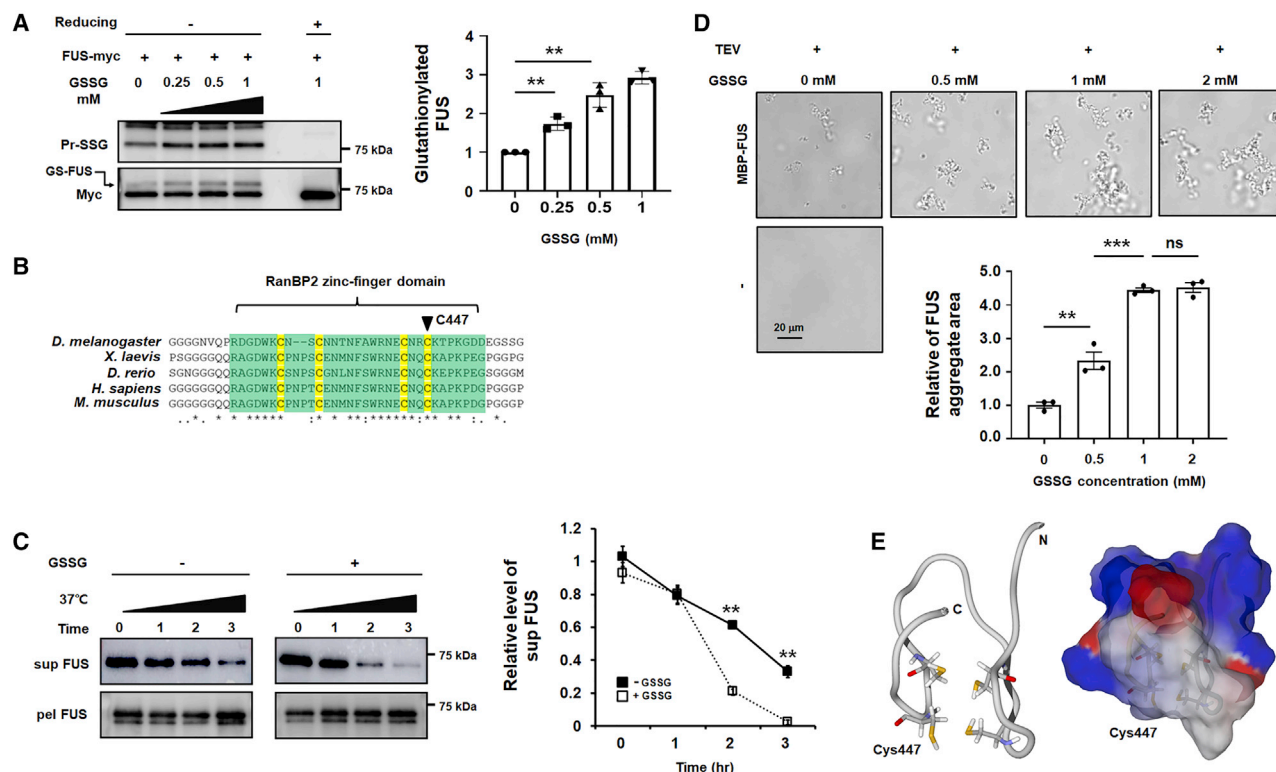


Figure 4. Glutathionylation of FUS within the ZnF motif increases the insolubility of FUS by promoting phase separation

(A) *In vitro* glutathionylation of recombinant myc-tagged FUS. Purified FUS-myc proteins were incubated with different concentrations of GSSG for 1 h at 25°C. A reducing agent-treated sample was used as a negative control. Error bars represent the mean $\pm$ SD. ** p < 0.01; one-way ANOVA.

(B) Sequence alignment of the region around Cys-447 in FUS with the FUS homologs in other organisms. The conserved RanBP2 ZnF domain is indicated with a green box. Four conserved cysteine residues are indicated with yellow boxes.

(C) The effects of glutathionylation on FUS solubility. After the induction of FUS glutathionylation, FUS in the supernatant (sup FUS) and pellet (pel FUS) fractions was analyzed by immunoblotting using an anti-FUS antibody after different incubation times (0–3 h) at 37°C. Error bars represent the mean $\pm$ SD. ** p < 0.01; one-way ANOVA.

(D) DIC images of FUS aggregates. After cleavage of the MBP tag, FUS (5 μ M) underwent LLPS and formed aggregates with or without GSSG. Scale bars, 20 μ m. Error bars represent the mean $\pm$ SD. ** p < 0.01, *** p < 0.001, not significant (ns); one-way ANOVA.

(E) The three-dimensional homology model of the human FUS ZnF domain and the relative location of the Cys-447 residue.

by MALDI-mass spectrometry (Figure S3). The RanBP2 ZnF domain of FUS has four cysteine residues (Nguyen et al., 2011) and may be sensitive to oxidative stress *in vivo*. We performed FUS sequence alignment to determine the extent of cysteine conservation among eukaryotic species and found that all four cysteine residues are well conserved from flies to humans. We identified Cys-447 as a glutathionylation site in the RanBP2-type ZnF domain (Figure 4B, arrowhead), indicating that human FUS is specifically glutathionylated at a conserved Cys-447 residue.

Glutathionylation decreases FUS solubility by promoting phase separation *in vitro*

To investigate whether the glutathionylation of Cys-447 in FUS increases FUS aggregation, we performed a solubility test with FUS. The myc-tagged human FUS proteins purified from HEK293 cells were incubated with GSSG to induce glutathionylation and then exposed to high temperature (37°C). FUS solubility was evaluated at different time points by quantifying the FUS proteins in the supernatant and pellet fractions of each sample

through western blot analysis. The FUS protein without GSSG treatment remained soluble for 2 h at 37°C. In contrast, upon induction of glutathionylation by GSSG treatment, the FUS proteins in the soluble fraction significantly decreased, from 90% to 20% after 2 h at 37°C (Figure 4C). This result showed that the glutathionylation of FUS significantly induces aggregate formation.

Furthermore, FUS modifications were shown to influence FUS-associated diseases by increasing aggregation through liquid-liquid phase separation (LLPS), a process by which macromolecules separate from a bulk solvent to form a distinct liquid phase (Shin and Brangwynne, 2017). FUS modifications by poly (ADP-ribose) polymerase in a low-complexity domain promoted the LLPS of FUS (Altmeyer et al., 2015; Monahan et al., 2017). To investigate the relationship between glutathionylation and FUS aggregation, we assessed whether FUS could undergo LLPS *in vitro* and whether glutathionylation could regulate this process. We incubated purified recombinant maltose-binding protein (MBP)-tagged FUS with or without GSSG. After selective removal of the MBP tag by tobacco etch virus (TEV) protease,

FUS underwent phase separation and formed droplet-like aggregates. Interestingly, the addition of GSSG promoted FUS LLPS in a dose-dependent manner (Figure 4D). This finding confirms that FUS glutathionylation at the Cys-447 residue induces protein aggregation by promoting phase separation.

To further assess the relative location of Cys-447 residues in the FUS structure, we generated a three-dimensional homology model of the human FUS ZnF domain using the I-TASSER server (Roy et al., 2010). The Cys-447 residue in the ZnF domain was found to be exposed at the surface for oxidative modification (Figure 4E). These data support the high susceptibility of Cys-447 to glutathionylation and the possibility of conformational changes within the ZnF domain.

GstO2 regulates FUS phase separation by reducing glutathionylation and cytoplasmic FUS aggregation in *Drosophila* *in vivo*

The restoration of the phenotypes of the FUS-expressing flies by GstO2 may be related to FUS glutathionylation. Therefore, we evaluated whether GstO2 regulates FUS glutathionylation. We conducted *in vitro* experiments by using purified recombinant GstO2 protein and found that GstO2 decreased the level of FUS glutathionylation. As enzymatic activity is essential for GstO2 function, it was not surprising that a catalytically inactive mutant of GstO2, GstO2^{C31A}, did not decrease the level of FUS glutathionylation (Figure 5A). Additionally, we examined the effect of other *Drosophila* GstOs on FUS glutathionylation *in vitro*. Consistent with our results, recombinant GstO1 and GstO3 did not alter the level of FUS glutathionylation (Figure S3C). Based on our previous results, we hypothesized that GstO2 regulates FUS glutathionylation and aggregation in the *Drosophila* brain *in vivo*. To test this possibility, we used an immunoprecipitation method under nonreducing conditions to analyze the extracts from the heads of the FUS-expressing flies with GstO2. We observed that glutathionylated FUS immunoprecipitated with anti-GSH antibodies and was then detected by immunoblotting with total FUS antibodies. The results showed that the level of glutathionylated FUS decreased to a greater extent in the flies coexpressing FUS and GstO2 than in the flies expressing only FUS (Figure 5B). Furthermore, we administered GstO2 and GstO2 RNAi, which have opposite effects on FUS-induced neuronal toxicity and FUS glutathionylation. First, we observed that the brains of the FUS-expressing flies had cytoplasmic FUS in neuronal cells. A double immunofluorescence analysis with the brain tissues of the adult flies expressing FUS revealed that FUS was strongly colocalized with glutathionylated protein in the cytoplasm (Figure 5C, arrows). Strikingly, GstO2 coexpression suppressed the increase in cytoplasmic FUS aggregate formation and decreased FUS glutathionylation (Figure 5C). Next, we analyzed the glutathionylation-induced aggregation of cytoplasmic FUS by downregulating GstO2 with RNAi. Notably, knocking down GstO2 led to a significant increase in cytoplasmic FUS aggregates and glutathionylation (Figure 5C, arrows).

Furthermore, the findings that FUS glutathionylation induced aggregate formation by promoting phase separation and that GstO2 decreased FUS glutathionylation prompted us to examine whether GstO2 could regulate the LLPS of FUS by decreasing glutathionylation. GstO2 decreased the LLPS of FUS induced by glutathionylation in a dose-dependent manner

(Figure 5D). As a control, GstO2 without GSSG did not affect the LLPS of FUS *in vitro* under the same conditions (Figure S3D). These results strongly suggest that the GstO2-mediated deglutathionylation of FUS is necessary to prevent FUS aggregate formation in the cytoplasm of neuronal cells and to delay FUS-induced neurotoxicity.

The role of GSTO1 in regulating FUS-induced neurotoxicity is conserved in mammalian neuronal cells

Next, we sought to determine whether glutathionylation also regulates FUS-induced neurotoxicity in mammalian neuronal cells. We developed a stable N2a cell line expressing the Myc-DDK-GSTO1 fusion protein (Figure 6A). After the transfection of GFP-tagged FUS into cell lines stably expressing GSTO1, we examined the effects of GSTO1 on FUS solubility. The quantification of soluble and insoluble FUS demonstrated a significant >50% decrease in the ratio of insoluble/soluble FUS in the GSTO1 coexpression group compared with the group expressing FUS alone (Figure 6B). Furthermore, to investigate whether the expression of GSTO1 affected the mislocalization of FUS in neuronal cells, we performed subcellular fractionation assays to measure the FUS levels in both the cytoplasm and nucleus. Our results showed that GSTO1 overexpression decreased the levels of FUS in the cytoplasmic fraction but not in the nuclear fraction (Figure 6C). Consistent with previous data, these results indicate that GSTO1 acts as a suppressor of FUS toxicity by decreasing cytoplasmic FUS. We also investigated the effect of GSTO1 on FUS-induced apoptotic cell death by staining samples with anti-cleaved caspase-3 antibody. Importantly, FUS-induced neuronal cell death significantly decreased when FUS was coexpressed with GSTO1 (Figure 6D). Since cytoplasmic FUS aggregates colocalized with GSH in *Drosophila* neurons, we also assessed whether FUS colocalized with GSH in a mammalian system and determined whether the glutathionylated FUS aggregates in the cytoplasm were reduced by GSTO1 expression. The coexpression of GSTO1 with FUS was sufficient to reduce FUS aggregate formation and colocalization with GSH in the cytoplasm (Figure 6E, arrows). Taken together, these results suggest that the GSTO1-mediated deglutathionylation of FUS may attenuate the neurotoxicity induced by FUS in mammalian neuronal cells.

Deglutathionylation of FUS alleviates FUS-induced toxicity in neuronal cells

To test whether the cysteine glutathionylation of FUS is required for the cellular properties of FUS and its toxicity, we generated a GFP-tagged FUS construct and substituted Cys-447 in the ZnF domain with alanine. Then, we expressed cysteine mutant or wild-type FUS in N2a cells and examined the solubility of the different FUS proteins. Interestingly, the single cysteine mutant FUS^{C447A} exhibited an insoluble/soluble ratio approximately similar to that of FUS^{WT}. Therefore, we generated a double cysteine mutant form of FUS, FUS^{C444A/C447A}. The ratio of insoluble to soluble FUS decreased >20% in the FUS^{C444A/C447A}-GFP-expressing cells compared with the FUS^{WT}-GFP-expressing cells (Figure S4A). Since our mass spectrometric analysis after an *in vitro* glutathionylation assay demonstrated that glutathionylation occurs at a single site, Cys-447, in FUS (Figures 4B and S3B), these mutagenesis

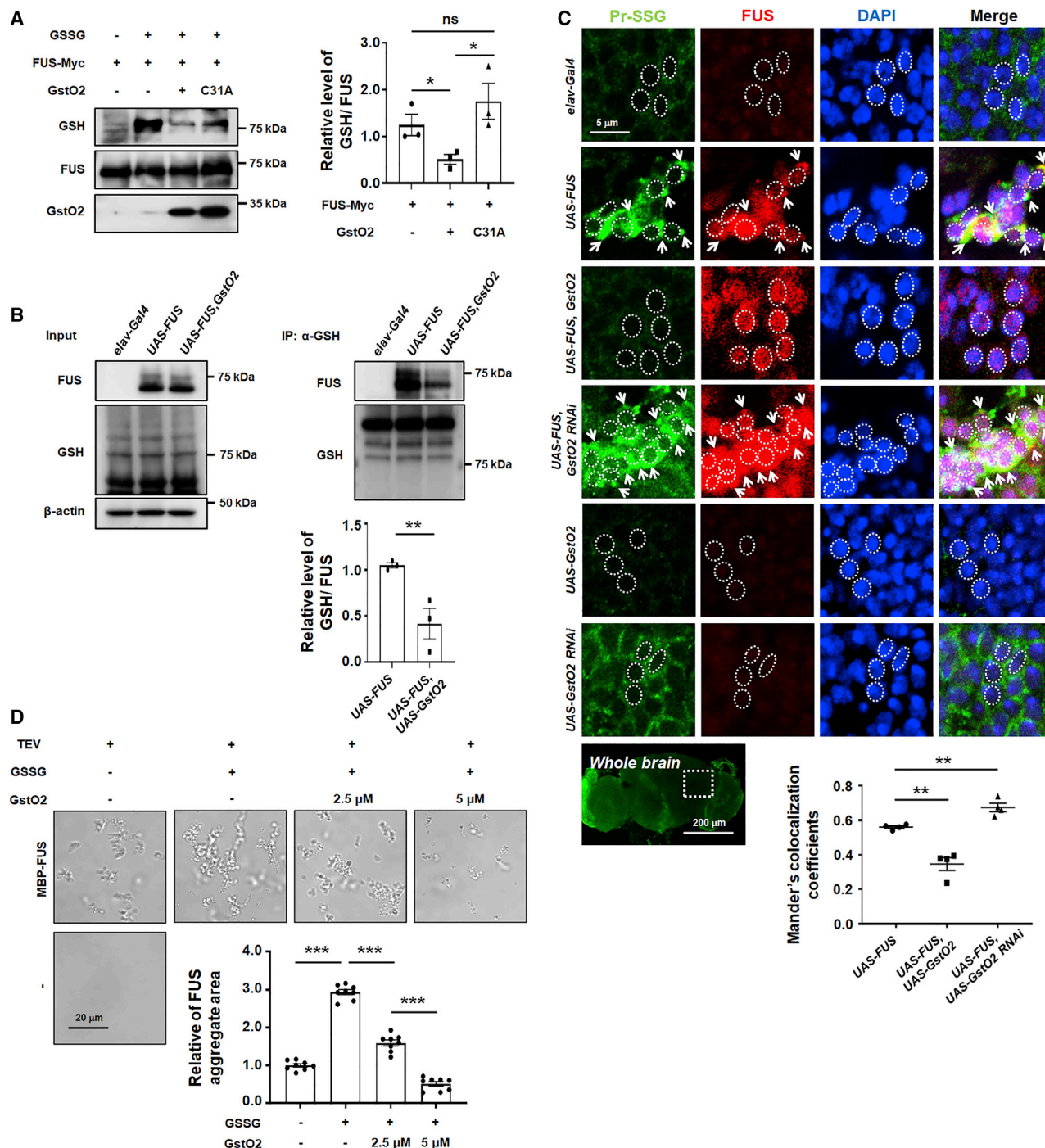


Figure 5. GstO2 deglutathionylates FUS in the *Drosophila* neurons

(A) *In vitro* glutathionylation of recombinant myc-tagged FUS. Purified FUS-myc proteins were incubated with GSSG. Error bars represent the mean $\pm$ SD. *p < 0.01, not significant (ns); one-way ANOVA.

(B) Immunoprecipitation of glutathionylated FUS from the head extracts of the FUS-expressing flies with GstO2. β -actin was used as a loading control. Error bars represent the mean $\pm$ SD. **p < 0.01; Student's t test.

(C) Immunostaining of FUS (red) and glutathionylated protein (green) in the whole brains of flies with the indicated genotypes. The nuclei (dotted circles) were stained with DAPI (blue). Scale bars, 5 and 200 μ m. The bar graph shows Mander's overlap coefficient between Pr-SGG and FUS (n = 4 brains). Error bars represent the mean value $\pm$ SD. **p < 0.01; one-way ANOVA.

(D) DIC images of FUS aggregates. After cleavage of the MBP tag, FUS (5 μ M) underwent LLPS and formed aggregates with or without GSSG. Scale bars, 20 μ m. Error bars represent the mean $\pm$ SD. ***p < 0.001; one-way ANOVA.

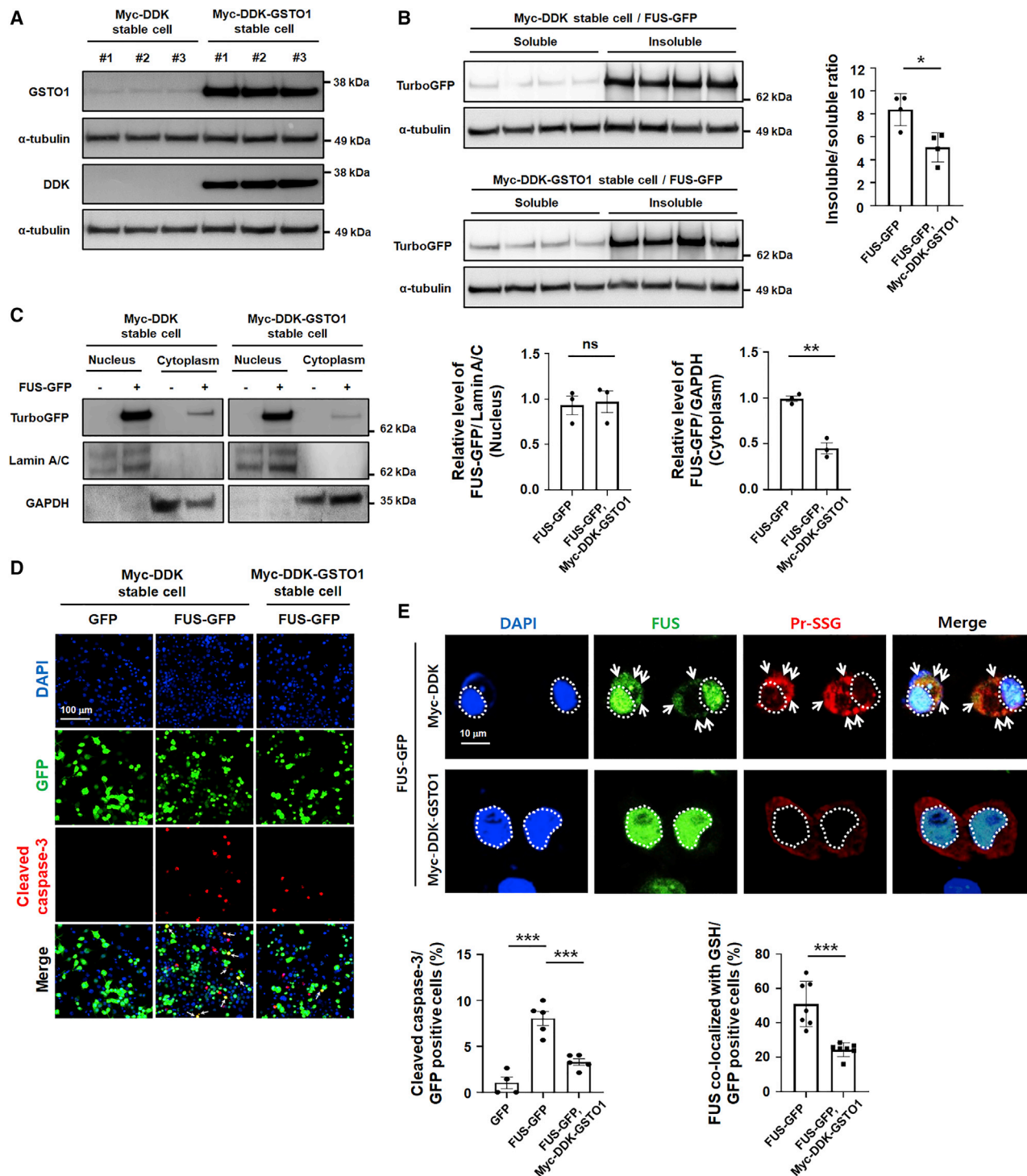


Figure 6. GSTO1 attenuates the formation of FUS aggregates and mitigates FUS-induced neuronal toxicity in Neuro2a cells

(A) Immunoblot analysis of GSTO1 and DDK levels in the control and N2a cell lines that stably overexpressed Myc-DDK-GSTO1.

(B) Cells that stably expressed GSTO1 were transiently transfected with the FUS-expression vector. Error bars represent the mean value $\pm$ SD. * $p < 0.05$; Student's t test.

(legend continued on next page)

experiments suggest that either Cys-444 or Cys-447, but not both, are the predominant target of glutathionylation. Moreover, this result suggests that if glutathionylation of FUS is blocked at the Cys-447 residue, the site of glutathionylation is shifted to a nearby cysteine residue, Cys-444. Next, subcellular fractionation was performed to further investigate whether the deglutathionylated mutations of FUS can prevent the mislocalization of FUS in neuronal cells. Although there was no significant effect on the amounts of both types of FUS in the nucleus, the FUS^{C444A/C447A} mutant was less detectable in the cytoplasm than FUS^{WT} (Figure S4B). In immunohistochemical analysis, cytoplasmic mislocalization of FUS was lower in the FUS^{C444A/C447A}-expressing cells than in the FUS^{WT}-expressing cells (Figure S4C), which is consistent with subcellular fractionation data in N2a cells transiently transfected with FUS^{WT} or FUS^{C444A/C447A}. Next, we investigated the effect of the deglutathionylated mutation FUS^{C444A/C447A} on neurotoxicity by detecting apoptotic cell death with an anti-cleaved caspase-3 antibody. Importantly, the cleaved caspase-3 signals were significantly decreased in the N2a cells expressing FUS^{C444A/C447A} compared with the FUS^{WT}-expressing cells (Figure S4D). Collectively, these findings suggest that deglutathionylation of FUS may attenuate the cytoplasmic aggregation of FUS and the neurotoxicity induced by the formation of cytoplasmic FUS aggregates.

Potential therapeutic effects of GSTO1/GstO2 activators on FUS-associated neurodegenerative diseases

Based on the observation that *Drosophila* GstO2 expression is downregulated during disease progression in the extracts from the heads of the FUS-induced model, we focused on activating or inducing GSTO1 as a neuroprotective effector. In addition, some factors that can increase GSTO1-specific expression have recently been found. Transient hypoxia was found to increase mitochondrial ROS levels in and extend the lifespan of *Caenorhabditis elegans* by upregulating GSTO-1 expression (Schieber and Chandel, 2014). Carboplatin treatment of breast cancer cells induced hypoxia-inducible factor-dependent expression of GSTO1 (Lu et al., 2017). Hypoxic exposure did not change GstO2 protein expression in *Drosophila* (Figure S5A). Therefore, to assess whether pharmacological restoration of GstO2 expression rescues the FUS-induced toxicity observed in the nervous system of the FUS-expressing flies, we treated the FUS-expressing flies with carboplatin. *Drosophila* larvae expressing FUS were raised on food supplemented with carboplatin throughout the larval stages. Carboplatin treatment (2 μ g/mL) led to a dramatic increase in the GstO2 expression in the head extracts of the FUS-expressing flies (Figure S5B). We found that FUS-induced larval movement dysfunction was significantly

suppressed in a dose-dependent manner by carboplatin treatment (Figure 7A). Immunoblot assays of the brain extracts from the FUS-expressing adult flies showed that carboplatin treatment slightly reduced FUS levels (Figure 7B). Next, we determined the effects of carboplatin on FUS solubility. The quantification of soluble and insoluble FUS revealed a significant >50% decrease in the ratio of insoluble/soluble FUS in the FUS-expressing flies treated with carboplatin compared with the untreated flies (Figure 7C). Furthermore, carboplatin treatment suppressed FUS-induced apoptotic cell death and NMJ defects in fly neurons (Figures 7D and S5C). Indeed, to determine whether the protective effect of carboplatin treatment against FUS-induced neurotoxicity is mediated by GstO2 expression, we examined the effects of carboplatin in the FUS-expressing flies with GstO2 RNAi. Knocking down GstO2 in the FUS-expressing flies treated with carboplatin had no significant effects on FUS-induced toxicity, including locomotor defects and apoptotic cell death (Figure 7). We also found that knockdown of GstO2 in the FUS-expressing flies treated with carboplatin did not affect the reduction in FUS protein levels or insolubility (Figures 7B and 7C). Taken together, our results suggest that modulation of GSTO1/GstO2 activity presents a potential therapeutic strategy for FUS-associated neurodegenerative diseases, including ALS and FTD.

Glutathionylated FUS aggregates are linked to ALS

We previously observed cytoplasmic FUS aggregates in the motor neurons of a patient with ALS carrying the FUS p.G504Wfs*12 or FUS R495* mutation (Lim et al., 2016). The above *in vitro* and *in vivo* studies showed that FUS is glutathionylated and that glutathionylation of FUS facilitates the formation of FUS aggregates in the cytoplasm of neuronal cells. Therefore, to investigate whether cytoplasmic FUS is glutathionylated in motor neurons in FUS-ALS cases, we conducted immunohistochemical analyses for FUS and glutathionylated protein in postmortem brain tissue obtained from a patient with sporadic ALS (sALS) carrying the FUS p.G504Wfs*12 mutation and compared these results with those of the brain tissues of non-neurological controls. As expected, consistent with the results in fly and mammalian neuronal cells, glutathionylated protein colocalized with FUS in the cytoplasm of neurons in the brain tissue of the patient with ALS-FUS (Figure S6A). Thus, these results from human brain tissue reveal that glutathionylated FUS is associated with pathologic FUS aggregates in the ALS-FUS brain and support glutathionylation-induced FUS aggregation as a pathological mechanism in FUS-linked ALS.

Based on the observation that *Drosophila* GstO2 expression is downregulated during disease progression in the extracts from the heads of the FUS-induced model, we examined the expression level of GSTO1 in the patient with ALS-FUS. The expression

(C) Immunoblot analysis of FUS protein levels in cytoplasmic and nuclear fractions of the cells expressing DDK or DDK-GSTO1. The amount of cytoplasmic and nuclear FUS normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lamin A/C, respectively. Error bars represent the mean value $\pm$ SD. **p < 0.01, not significant (ns); Student's t test.

(D) Cells that stably expressed GSTO1 were transiently transfected with the FUS-expression vector. N2a cells were stained for cleaved caspase-3 (red) and DAPI (blue). Arrows indicate the colocalization of cleaved caspase-3 with GFP-positive cells. Scale bars, 100 μ m. Error bars represent the mean value $\pm$ SEM. ***p < 0.001; one-way ANOVA.

(E) N2a cells were stained for GSH (red) and DAPI (blue). Arrows indicate glutathionylated FUS aggregates in the cytoplasm. Scale bars, 10 μ m. Data are presented as the mean value $\pm$ SD. ***p < 0.001; Student's t test.

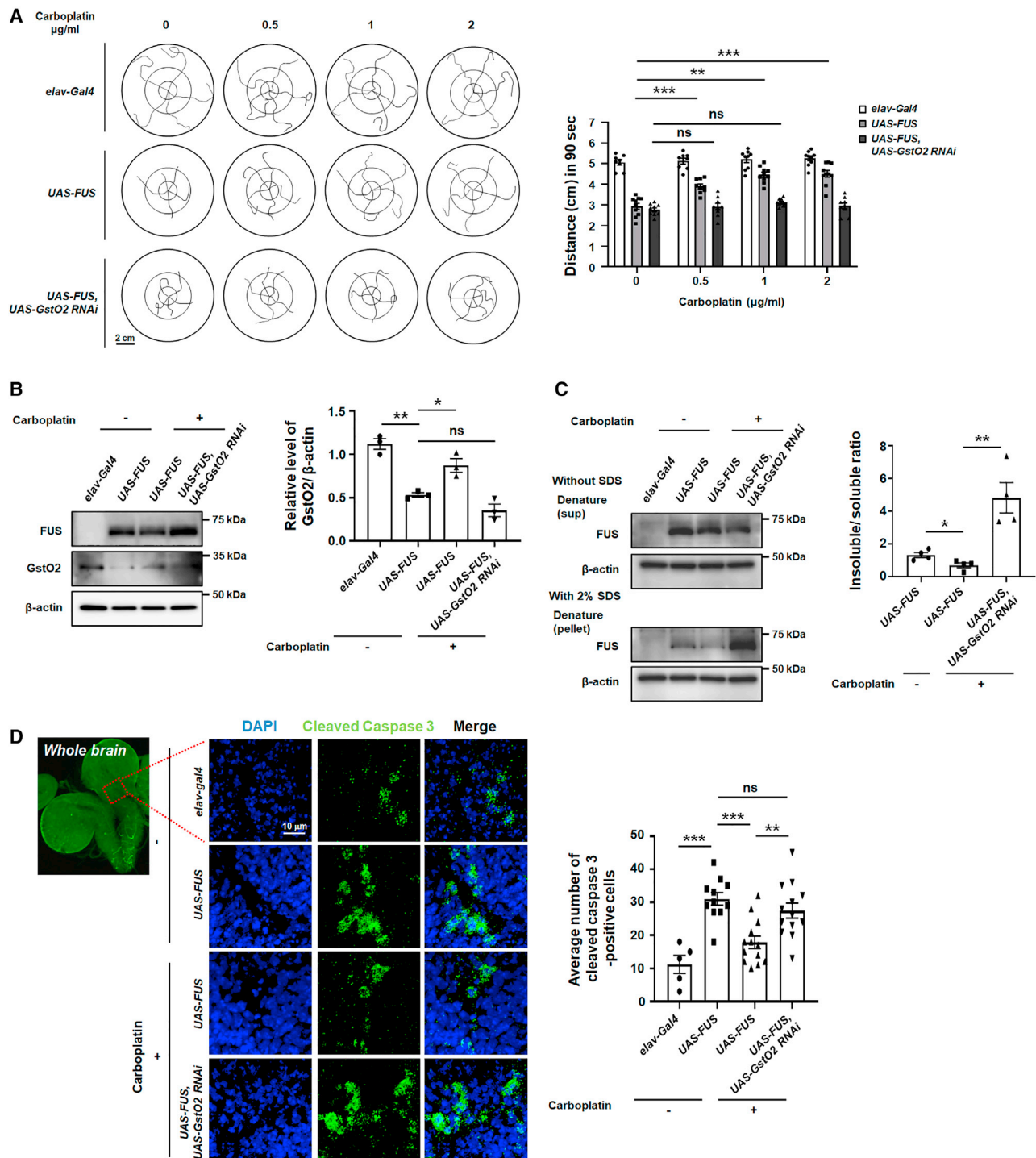


Figure 7. Carboplatin treatment rescues FUS-induced toxicity by increasing GstO2 expression in *Drosophila* models

(A) Larvae expressing FUS were treated with different concentrations of carboplatin. The movement of the 3rd-instar larvae was monitored for 90 s, and the average distance of the larval trails was calculated ($n > 10$ each). Error bars represent the mean value $\pm$ SD. ** $p < 0.01$, *** $p < 0.001$, not significant (ns); one-way ANOVA.

(B) Immunoblot showing FUS and GstO2 protein levels in the brain extracts of larvae. β -actin was used as a loading control. Error bars represent the mean value $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, not significant (ns); one-way ANOVA.

(C) Soluble (supernatant) and insoluble (pellet) fractions isolated from the larval brains of the flies with the indicated genotypes were analyzed by immunoblot using FUS antibodies. β -actin was used as a loading control. Error bars represent the mean value $\pm$ SD. * $p < 0.05$, ** $p < 0.01$; one-way ANOVA.

(D) Representative confocal images showing cleaved caspase-3 (green) in the brains of the FUS-expressing larvae. The nuclei were stained with DAPI (blue). Scale bars, 20 μm . Error bars represent the mean value $\pm$ SD ($n > 16$). ** $p < 0.01$, *** $p < 0.001$, not significant (ns); one-way ANOVA.

of GSTO1 was measured in fibroblasts of the patients carrying the *FUS* p.G504Wfs*12 and *FUS* R495* mutations by using immunoblot analysis. Human GSTO1 protein, but not mRNA, was significantly reduced in the fibroblasts of the patients with ALS-FUS compared with control fibroblasts obtained from healthy individuals (Figures S6B and S6C). These results were consistent with those observed in the *FUS*-expressing flies and indicate that *FUS* glutathionylation caused by the disruption of GSTO1 expression may be implicated in *FUS*-linked ALS pathogenesis.

DISCUSSION

The molecular regulators and cellular pathogenic events that contribute to *FUS*-linked ALS, FTD, and other proteinopathies are still poorly defined. In this study, we focused on Gsto2 as a potential regulator of *FUS* cytoplasmic mislocalization and accumulation in a *Drosophila* model system. Interestingly, the Gsto2 protein levels were significantly decreased in the heads of the *FUS*-expressing flies. The restoration of Gsto2 expression suppressed various abnormal phenotypes, including mitochondrial dysfunction and axonal defects in motor neurons, in the *FUS*-expressing flies. Mitochondria are involved in the pathogenesis of various neurodegenerative diseases, including ALS (Kawamata and Manfredi, 2017). Recent studies have indicated that TDP-43 accumulation can cause OXPHOS defects (Wang et al., 2016) and that the overexpression of *FUS* can cause mitochondrial dysfunction, including mitochondrial dynamic imbalance in *Drosophila* (Altanbyek et al., 2016). In the current study, we found that Gsto2 restored *FUS*-induced mitochondrial fragmentation and complex I/III disassembly, increased ROS production, and decreased ATP levels by decreasing *FUS* levels in mitochondria (Figure 2). Furthermore, we identified *FUS* glutathionylation as a PTM regulating the protein properties of *FUS*, including cytoplasmic inclusion formation, which provided strong evidence for the modulation of *FUS*-induced neurotoxicity by glutathionylation using *Drosophila* and mouse neuronal cell models. Given the critical importance of cytoplasmic *FUS* aggregation in the regulation of *FUS* stability and in disease pathology, we speculate that increased glutathionylation in the cytoplasm could be responsible for aggregate formation and neurotoxicity in *FUS*-linked ALS. To our knowledge, the glutathionylation of *FUS* decreases *FUS* solubility by promoting phase separation (Figure 4). We showed that the induction of *FUS* glutathionylation affects cytoplasmic accumulation in the *Drosophila* brain (Figure 5).

Neuroprotective mechanisms that inhibit the formation of toxic protein aggregation and degrade aggregated proteins in the cytoplasm may be promising therapeutic targets for ALS (Neumann et al., 2006; Sharma et al., 2016; Wils et al., 2010). We speculate that the deglutathionylation of *FUS* may reduce cytoplasmic *FUS* aggregates and maintain soluble *FUS* in the cytoplasm, which are critical conditions for toxic protein degradation and neuronal cell survival. One potential candidate to target is human GSTO1, which was previously identified as a deglutathionylating enzyme. A previous proteomic analysis based on a two-dimensional gel electrophoresis approach to detect ALS biomarkers in peripheral blood mononuclear cells and spinal cord cells from sALS patients identified GSTO1 as a major target pro-

tein (Nardo et al., 2011). Furthermore, we previously reported that Gsto2, which is a *Drosophila* homolog of human GSTO1, suppressed neuronal toxicity by regulating glutathionylation in a *Drosophila* PD model (Kim et al., 2012). The regulation of *FUS* glutathionylation status in the cytoplasm by Gsto2 may reduce the level of *FUS* translocated to mitochondria and abolish the toxicity induced by *FUS* in neurons. These findings prompted us to explore the role of protein glutathionylation/deglutathionylation by Gsto2 in the regulation of *FUS* neurotoxicity. We found that *Drosophila* Gsto2 is also involved in the regulation of *FUS* glutathionylation status and in the suppression of *FUS*-induced neurotoxic phenotypes, including those associated with locomotive defects and mitochondrial dysfunction. Thus, we demonstrated that Gsto2 in *Drosophila* and GSTO1 in N2a cells are physiological modulators of *FUS* glutathionylation *in vivo*. These studies indicate that increased *FUS* glutathionylation may act in a pathogenic mechanism to induce aggregate formation and neurodegeneration.

Glutathionylation is a reversible PTM characterized by the conjugation of GSH to a target protein and is emerging as a crucial pathogenic mechanism in neurodegenerative diseases that are characterized by the aggregation of mislocalized proteins (Hong et al., 2015; McAlary et al., 2013). However, the precise molecular mechanism and the importance of glutathionylation in pathological protein aggregates remain unclear. Our *in vitro* experiments provide strong evidence that the Cys-447 residue of *FUS* in the ZnF domain is glutathionylated when it is exposed to oxidative stress (Figure 4A). Therefore, upon an increase in oxidative stress in the cytoplasm, various proteins that cause ALS may be reversibly modified by ROS. TDP-43 undergoes cysteine oxidation in response to oxidative stress and leads to aggregation (Cohen et al., 2012; Kim et al., 2014). Indeed, *FUS* expression dramatically increased oxidative stress in *Drosophila* (Figure 2H). Notably, we detected glutathionylated *FUS* pathology in the brains of ALS-FUS patients, suggesting that *FUS* glutathionylation is a pathological feature associated with *FUS* aggregates in the ALS-FUS brain and supporting glutathionylation-induced *FUS* aggregation as a pathological mechanism in *FUS*-linked ALS (Figure S6). The increased glutathionylation of *FUS* led to aggregate formation by decreasing *FUS* solubility (Figure 4D). GSH conjugation adds a negative charge to the modified *FUS*, which likely causes a conformational change within the ZnF domain of the protein. This modification appears to induce *FUS* aggregation and change *FUS* function with respect to RNA processing. *FUS* modifications were reported to influence *FUS*-associated diseases by increasing aggregation through phase separation. We found that *FUS* glutathionylation induces protein aggregation by promoting phase separation (Figure 4E). Additionally, we found that the deglutathionylated form of *FUS*, which eliminates the glutathionylation site, is less toxic than wild-type *FUS* in neuronal cells (Figure S4). Thus, our results showing *FUS* glutathionylation-induced aggregate formation *in vitro* and *in vivo* indicate that *FUS* glutathionylation at Cys-447 in the ZnF domain may influence protein properties through the modulation of its LLPS and RNA-binding properties.

Interestingly, our investigations revealed that *Drosophila* Gsto2 and human GSTO1 were sufficient to reduce the level of cytoplasmic *FUS* and enhance the solubility of *FUS* by

modulating glutathionylation status (Figures 5 and 6). Furthermore, GstO2 suppressed the neurotoxicity induced by FUS^{P525L}, the ALS-associated mutant form of FUS (Figures S7A–S7C). Moreover, we observed that the GstO2 protein level was decreased in the TAF15-expressing flies and that GstO2 suppressed TDP-43- and TAF15-induced rough eye phenotype in *Drosophila* (Figures S7D–S7G). Further studies are required to investigate whether glutathionylation-induced aggregate formation is limited only to the FUS protein or whether this PTM is also implicated in TDP-43- or TAF15-induced neurodegeneration. These results potentially expand the therapeutic relevance of the FUS modification that is modulated by glutathione transferase omega.

Our results demonstrate that reversible FUS glutathionylation plays a critical role in regulating FUS aggregate formation. Finally, the cause of FUS-induced neurotoxicity may be explained by the lack of sufficient GstO2 levels and activity levels *in vivo*. Therefore, our findings suggest that FUS deglutathionylation or GSTO1 (GstO2 in *Drosophila*) activation can be pharmacologically targeted in FUS-linked neurodegenerative diseases, including ALS and FTD (Figures 7 and S7H). We propose that the modulation of FUS biochemical properties by its glutathionylation/deglutathionylation represents a general mechanism by which to regulate cytoplasmic protein aggregation in neurons.

Limitations of the study

Our study showed that reversible FUS glutathionylation plays a critical role in regulating FUS aggregate formation through phase separation in neurons, and it provided strong evidence for the modulation of FUS-induced neurotoxicity by glutathionylation using *Drosophila* and mouse neuronal cell models. Furthermore, the GSTO-mediated deglutathionylation of FUS is necessary to prevent FUS aggregate formation by modulating phase separation in the cytoplasm of neuronal cells and to delay FUS-induced neurotoxicity. Thus, future work should aim to complement our findings, particularly *in vivo* mouse models. It will be important to assess whether the roles of GSTO enzymes in regulating FUS aggregate formation are conserved as implied by our findings. Moreover, the functional relevance and physiological importance of GSTO enzymes at the onset of neurodegenerative diseases including ALS and FTD are not fully understood. This would require carrying out detailed analyses in animal models on whether the knockout of GSTO leads to age-dependent neurodegenerative disease.

STAR★METHODS

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Supplemental information can be found online at <https://doi.org/10.1016/j.devcel.2022.02.022>.

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AUTHOR CONTRIBUTIONS

K.K. designed the study. S.J.C., Seongsoo Lee, H.-J.C., Y.J.H., M.N., S.M.L., Y.-M.J., M.J., and K.K. performed molecular biology experiments. S.J.C., Shinrye Lee, M.N., S.H.K., H.-J.K., and K.K. analyzed the data. K.K. wrote the manuscript. All authors commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti- β -Actin	Cell Signaling Technology	Cat #4967; RRID: AB_330288
Mouse anti-ATP5A	Abcam	Cat #ab14748; RRID: AB_301447
Rabbit anti-Cleaved caspase-3	Cell Signaling Technology	Cat #9661; RRID: AB_2341188
Mouse anti-DDK	Origene	Cat #TA50011-100; RRID: AB_2622345
Rabbit anti-FUS	Bethyl laboratories	Cat #A300-292A; RRID: AB_263408
Rabbit anti-FUS	Bethyl laboratories	Cat #A300-302A; RRID: AB_309445
Rabbit anti-FUS	Abnova	Cat #PAB20448; RRID: AB_10964225
Mouse anti-GAPDH	Santa Cruz Biotechnology	Cat #sc-47724; RRID: AB_627678
Mouse anti-turboGFP	Origene	Cat #TA150041; RRID: AB_2622256
Mouse anti-Glutathione	Virogen	Cat #101-A; N/A
Mouse anti-Glutathione	Abcam	Cat #ab19534; RRID: AB_880243
Mouse anti-Glutathione	Thermo Fisher Scientific	Cat #MA1-7620; RRID: AB_1074679
Rabbit anti- <i>Drosophila</i> GstO1	(Kim et al., 2012)	N/A
Rabbit anti- <i>Drosophila</i> GstO2	(Kim et al., 2012)	N/A
Mouse anti-human GSTO1	Abnova	Cat #H00009446-B01P; RRID: AB_1673788
Mouse anti-human GSTO1	Abcam	Cat #ab88604; RRID: AB_2041452
Mouse anti- <i>Drosophila</i> Lamin C	DSHB	Cat #LC28.26; RRID: AB_528339
Mouse anti-Marf	Gift from Dr. Alexander J. Whitworth	N/A
Mouse anti-Myc	Cell Signaling Technology	Cat #2276; RRID: AB_331783
Mouse anti-NDUFS3	Abcam	Cat #ab14711; RRID: AB_301429
Rabbit anti-Mitofusin 2	Sigma Aldrich	Cat #M6319; RRID: AB_477221
Rabbit anti-TAF15	GeneTax	Cat #GTX77901; RRID: AB_424849
Rabbit anti-TARDBP (TDP-43)	Proteintech Group	Cat #10782-2-AP; RRID: AB_615042
Rabbit anti- α -Tubulin	Cell Signaling Technology	Cat #9099; RRID: AB_10695471
Mouse anti-UQCRC2	Abcam	Cat #ab14745; RRID: AB_2213640
Goat anti-rabbit HRP	Millipore	Cat #AP307P; RRID: AB_11212848
Goat anti-mouse HRP	Millipore	Cat #AP308P; RRID: AB_11215796
TrueBlot Goat anti-rabbit HRP	Rockland	Cat #18-8816-31; RRID: AB_469528
TrueBlot Goat anti-mouse HRP	Rockland	Cat #18-8817-30; RRID: AB_10126987
Goat anti-rabbit IgG Cy3	Invitrogen	Cat #A10520; RRID: AB_2534029
Goat anti-rabbit IgG Alexa 488	Invitrogen	Cat #A11034; RRID: AB_2576217
Goat anti-mouse IgG Cy3	Invitrogen	Cat #A10521; RRID: AB_1500665
Goat anti-mouse IgG Alexa 488	Invitrogen	Cat #A11001; RRID: AB_2534069
Bacterial and virus strains		
DH5 α Competent Cells	Thermo Fisher Scientific	Cat #18265017
BL21(DE3) Chemically Competent E. coli	Enzynomics	Cat #CP110
Chemicals, recombinant protein		
Ampicillin	Bioshop	Cat #AMP201.5
4',6-diamidino-2-phenylindole (DAPI)	Sigma Aldrich	Cat #D9564
Dulbecco Modified Eagle Medium (DMEM)	Gibco	Cat #11995073
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	Cat #D8418
Fetal Bovine Serum (FBS)	Gibco	Cat #16000-044
FUS	Origene	Cat #TP301808
G418	Gibco	Cat #10131027

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Gold Antifade Reagent	Invitrogen	Cat #S36936
IP Lysis Buffer	Thermo Fisher Scientific	Cat #87788
Isopropyl-D-thiogalactopyranoside	Biosesang	Cat #IF1006-001
Kanamycin	Gibco	Cat #11815-024
L-Glutathione Oxidized	Sigma Aldrich	Cat #G4376
L-Glutathione Reduced	Sigma Aldrich	Cat #G6529
2-Mercaptoethanol	Sigma Aldrich	Cat #M6250
MitoSox-Red	Invitrogen	Cat #M36008
Non-Essential Amino Acids Solution	Gibco	Cat #11140050
NuPAGE LDS Sample Buffer	Invitrogen	Cat #NP007
NuPAGE Sample Reducing Agent	Invitrogen	Cat #NP0009
Penicillin-streptomycin	Gibco	Cat #15140-122
Protease and Phosphatase Inhibitor Cocktail	Thermo Fisher Scientific	Cat #78440
Protein G Sepharose 4 Fast Flow	Amersham	Cat #71-7083-00 AI
RIPA buffer	Sigma Aldrich	Cat #R0278
Tetramethylrhodamine (TMRM)	Molecular Probe	Cat #T668
TEV Protease	New England BioLabs	Cat #P8112S
Trizol reagent	Thermo Fisher Scientific	Cat #15596026

Critical commercial assays

Amicon Ultra-0.5 Centrifugal Filter Unit	Millipore	Cat #UFC501096
BCA protein assay kit	PIERCE	Cat #23227
ECL Prime Western Blotting Detection Reagent	Amersham	Cat #RPN2236
ENLITEN ATP Assay System	Promega	Cat #FF2000
EZ-Western Lumi Femto	DoGenBio	Cat #DG-WF100
Mitochondria Isolation Kit	Thermo Fisher Scientific	Cat #89801
Native PAGE Sample Prep Kit	Invitrogen	Cat #BN2008
NE-PER Nuclear and Cytoplasmic Extraction Reagents	Thermo Fisher Scientific	Cat #78833
OxyBlot Protein Oxidation Detection Kit	Millipore	Cat #S7150
Slide-A-Lyzer Dialysis Cassettes	Thermo Fisher Scientific	Cat #66383

Deposited data

Raw immunoblot images	This paper	Mendeley Data: https://doi.org/10.17632/hrynsbggv.1
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Experimental models: Cell lines

Mouse: Neuroblastoma (N2a) cells	ATCC	Cat #CCL-131
Human: Skin fibroblasts from human ALS patients	(Lim et al., 2016)	N/A

Experimental models: Organisms/strains

<i>Drosophila</i> : UAS-FUS	Gift from Dr. Nancy M. Bonini	N/A
<i>Drosophila</i> : UAS-FUS ^{P525L}	Gift from Dr. Nancy M. Bonini	N/A
<i>Drosophila</i> : UAS-TDP-43	(Kim et al., 2014)	N/A
<i>Drosophila</i> : UAS-TAF15	Gift from Dr. Nancy M. Bonini	N/A
<i>Drosophila</i> : UAS-GstO2	(Kim et al., 2012)	N/A
<i>Drosophila</i> : UAS-LacZ	Bloomington Drosophila Stock Center	BDSC: 1776; FlyBase: FBti0002128
<i>Drosophila</i> : UAS-mitoGFP	Bloomington Drosophila Stock Center	BDSC: 8443; FlyBase: FBst0008443
<i>Drosophila</i> : UAS-mCD8-RFP	Bloomington Drosophila Stock Center	BDSC: 27398; FlyBase: FBst0027398
<i>Drosophila</i> : elav ^{3A} -Gal4	Bloomington Drosophila Stock Center	BDSC: 8760; FlyBase: FBst0008760
<i>Drosophila</i> : GMR-Gal4	Bloomington Drosophila Stock Center	BDSC: 84247; FlyBase: FBst00084247
<i>Drosophila</i> : mhc-Gal4	Bloomington Drosophila Stock Center	BDSC: 55133; FlyBase: FBst00055133
<i>Drosophila</i> : D42-Gal4	Bloomington Drosophila Stock Center	BDSC: 8816; FlyBase: FBst0008816
<i>Drosophila</i> : UAS-GstO1 RNAi	Bloomington Drosophila Stock Center	BDSC: 34727; FlyBase: FBgn0035907

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Drosophila</i> : UAS-GstO1 RNAi	Vienna <i>Drosophila</i> RNAi Center	VDRC: v26711; FlyBase: FBgn0035907
<i>Drosophila</i> : UAS-GstO2 RNAi	Vienna <i>Drosophila</i> RNAi Center	VDRC: v109255; FlyBase: FBgn0035906
<i>Drosophila</i> : UAS-GstO3 RNAi	Vienna <i>Drosophila</i> RNAi Center	VDRC: v105274; FlyBase: FBgn0035904
Recombinant DNA		
Plasmid: pCMV6-AC-GFP	This paper	N/A
Plasmid: pCMV6-AC-FUS ^{WT} -GFP	This paper	N/A
Plasmid: pCMV6-AC-FUS ^{C444A/C447A} -GFP	This paper	N/A
Plasmid: pTHMT-MBP-FUS _{FL} -WT	Addgene	Cat #98651
Plasmid: pCMV6-Myc-DDK	This paper	N/A
Plasmid: pCMV6-Myc-DDK-GSTO1	This paper	N/A
Plasmid: pET15b- <i>Drosophila</i> GstO1	(Kim et al., 2012)	N/A
Plasmid: pET15b- <i>Drosophila</i> GstO2	(Kim et al., 2012)	N/A
Plasmid: pET15b- <i>Drosophila</i> GstO2 ^{C31A}	(Kim et al., 2012)	N/A
Plasmid: pET15b- <i>Drosophila</i> GstO3	(Kim et al., 2012)	N/A
Software and algorithms		
ImageJ 1.47	NIH	http://imagej.nih.gov/ij/download.html
I-TASSER Server	(Roy et al., 2010)	http://zhanglab.ccmb.med.umich.edu/I-TASSER/
Molegro Molecular Viewer 2.5.0	Molegro Aps	http://molegro-molecular-viewer.software.informer.com/2.5/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kiyoun Kim (kiyoung2@sch.ac.kr).

Materials availability

Any materials generated in this study will be made available by the lead contact, Kiyoun Kim (kiyoung2@sch.ac.kr).

Data and code availability

- Original immunoblot images have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the [key resources table](#).
- This paper did not generate original code requiring public database deposition.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila melanogaster

All fly stocks were fed a standard diet (5% cornmeal, 3% yeast, 10% sucrose, 1% agar, 0.3% propionic acid, and 0.3% methyl 4-hydroxybenzoate) and housed under normal temperature (25°C) and normal humidity conditions (60%). Genetic crosses between flies were performed according to the standard procedure, and all progeny were cultured at 25°C.

Cell line

Mouse neuroblastoma cells, Neuro2a, were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 10% fetal bovine serum (FBS) (Gibco) and penicillin-streptomycin (50 mg/ml) (Gibco) in solution at 37°C in 5% CO₂/95% air. The culture medium was initially changed every 5 days and then changed every 3 days.

Patient cells

Human skin fibroblasts were obtained from two healthy controls (Control-1, 35-year-old female; Control-2, 57-year-old female) and two patients carrying FUS mutations (p.R495* and p.G504Wfs*12) as previously described (Lim et al., 2016). Fibroblasts were

cultured and maintained in DMEM (Gibco) with nonessential amino acids (Gibco), sodium bicarbonate (Sigma-Aldrich), 1% (vol/vol) penicillin/streptomycin/fungizone (Cellgro), and 20% heat-inactivated FBS (Gibco). The use of human skin fibroblasts has been approved by the Institutional Review Boards of Hanyang University.

Human tissue samples

The use of human *post-mortem* brain sections has been approved by the Institutional Review Boards of Hanyang University, and informed consent was obtained from all participants.

METHOD DETAILS

Semiquantitative RT-PCR

Total RNA was isolated from the heads of adult male flies using TRIzol reagent (Sigma). The RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega), and PCR was carried out for 25 cycles. We used a previously reported *GstO2* primer. The following primer pairs were used: *rp49*-For (GCT TCA AGA TGA CCA TCC GCC C), *rp49*-Rev (GGT GCG CTT GTT CGA TCC GTA AC); *human GSTO1*-For (GAA CGG CTG GAA GCA ATG AAG), *human GSTO1*-Rev (TGC CAT CCA CAG TTT CAG TTT). Each mRNA level was normalized to the levels of *rp49* and *GAPDH* (PPH00150F, Qiagen).

Transgenic flies

The UAS-FUS and UAS-FUS^{P525L} lines were a gift from Nancy M. Bonini (University of Pennsylvania). The UAS-GstO1 and UAS-GstO2 lines were previously described (Kim et al., 2012). The UAS-GstO1 RNAi (BL34727, v26711), UAS-GstO2 RNAi (v109255), UAS-GstO3 RNAi (v105274), and UAS-LacZ (BL1776) lines were obtained from the Bloomington Drosophila Stock Center and the Vienna Drosophila RNAi Center. UAS-LacZ was included as a control transgene. The UAS-mitoGFP line was a gift from H. J. Bellen (Baylor College of Medicine). Pan-neuronal driver *elav*^{3A}-Gal4, eye-specific driver *GMR*-Gal4, muscle-specific driver *mhc*-Gal4, and motor neuron-specific driver *D42*-Gal4 lines were also obtained from the Bloomington Drosophila Stock Center. In accordance with the genetic background, we used *W*¹¹¹⁸ flies as controls.

External eye microscopy

For adult fly eye images, the heads of the male flies were glued to a glass slide, and images were captured by a digital camera. Five-day-old male flies were used for the experiments. Representative digital eye images were taken using a Leica MZ10 F stereomicroscope and Leica DFC450 camera system. Adobe Photoshop 7.0 imaging software was used to create the final images (grayscale images). To analyze pigment loss in a quantitative manner, a comparable area of eyes for each genotype was selected. The threshold of grayscale images was adjusted to determine the area of depigmentation in the eyes. Quantification of white regions in the selected area of eyes was analyzed using the ImageJ software. Five eyes were analyzed in each experiment.

Locomotive activity and lifespan assays

For the larval crawling assay, 3rd-instar larvae were washed with PBS to remove any remaining food medium. The larvae were dried on clean filter paper and placed on a 2% grape juice-agar petri plate. The larvae of each genetic line were allowed to crawl for 90 seconds. To quantify the crawling behavior of the larvae, we drew lines to track the larvae and measured the distance using ImageJ software. The results from at least 10 larvae were averaged for each transgenic line.

For the adult climbing assay, 10 male flies of each age group were anesthetized with CO₂ and placed in a column vial (a total of 15 vials per genotype were used in this assay, and at least 150 flies per genotype were used). The flies were transferred to an empty vial and incubated for 1 h at room temperature to acclimatize to the environment. The vial was then tapped, and the flies dropped to the bottom. We counted the number of flies that could climb to the top of the vial within 10 seconds. The experiment was independently repeated four times for each transgenic line at 5 min intervals. All climbing experiments were performed at 25°C.

For the lifespan assay, 20 male flies were grouped per vial (a total of 8–9 vials per genotype were used in this assay) and maintained at 25°C. On each of the subsequent days, the flies in all groups were transferred to fresh vials. The number of dead flies was recorded. The lifespan of more 150 flies was recorded.

Immunohistochemistry

For analysis of the NMJs in *Drosophila*, 3rd-instar larvae were dissected in PBS and then fixed in 4% formaldehyde in PBS for 15 min. The samples were washed three times in PBS containing 0.1% Triton X-100 for 10 min per wash and blocked with 5% BSA in PBST. Then, the samples were incubated with primary antibody for 12 h at 4°C. FITC-conjugated anti-HRP (Jackson ImmunoResearch Laboratories) was used at 1:150. Larval preparations were mounted in *SlowFade*TM Gold antifade reagent (Invitrogen). All images were collected from a Leica TCS SP5 AOBS confocal microscope.

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and then washed three times in PBS containing 0.3% Triton X-100 (PBST) for 10 min per wash. The cells were incubated with blocking buffer (PBST containing 5% normal goat serum) for 1 h at 25°C and then with primary antibodies diluted in blocking buffer for 12 h at 4°C. The following antibodies were used for immunohistochemistry as indicated: mouse anti-GSH (1:100; ViroGen Corp.), rabbit anti-FUS (1:100; Bethyl Laboratories), rabbit anti-FUS (1:1000; Abnova), mouse anti-GFP (1:200; Roche), and rabbit anti-cleaved caspase-3 (1:500; Cell Signaling). After

incubation with primary antibodies, the cells were washed three times in PBST for 10 min per wash and then incubated with secondary antibodies diluted to 1:2000 in PBST for 1 h at 25°C. The following secondary antibodies were used for the immunohistochemistry assays: Alexa 594-conjugated goat anti-rabbit IgG, Alexa 488-conjugated goat anti-rabbit IgG, Alexa 594-conjugated goat anti-mouse IgM, and Alexa 488-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). The samples were mounted and observed with a Leica TCS SP8 confocal microscope.

Motor neuron morphology

For imaging of motor axons in the leg, 10-day-old adult male flies were dissected in PBS. The front legs were fixed with 4% formaldehyde in fixative buffer (100 mM PIPES, 1 mM EGTA, 1% Triton X-100, and 2 mM MgSO₄, pH 6.9) for 25 min. The samples were washed in PBST and mounted in *SlowFade*TM Gold antifade reagent (Invitrogen). All images were collected using a Carl Zeiss confocal microscope (LSM710) at the Soonchunhyang Biomedical Research Core-Facility of Korea Basic Science Institute (KBSI).

For imaging of motor neuron cell bodies in the brain, 3rd-instar larvae were dissected in PBS on Sylgard plates and then fixed in 4% formaldehyde in PBS for 25 min. After a rinse with PBST, the samples were mounted onto coverslips using *SlowFade*TM Gold antifade reagent (Invitrogen). Images were collected using a Carl Zeiss confocal microscope (LSM710). For cell size determination, the outline of each cell body was extracted, and its area was measured using ImageJ software.

Mitochondrial imaging and morphology

For imaging of the mitochondria in thorax muscle tissues, 7-day-old adult male flies were dissected in PBS and fixed with 3% formaldehyde in fixative buffer (100 mM PIPES, 1 mM EGTA, 1% Triton X-100, and 2 mM MgSO₄, pH 6.9) for 25 min. After a rinse with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, and 0.5 mg/ml BSA, pH 6.8), the samples were mounted onto coverslips using *SlowFade*TM Gold antifade reagent (Invitrogen). Images were collected using a Carl Zeiss confocal microscope (LSM710).

For imaging of the mitochondria in the motor neurons of the leg, 7-day-old adult male flies were dissected in PBS. The front legs were fixed with 4% formaldehyde in fixative buffer (100 mM PIPES, 1 mM EGTA, 1% Triton X-100, and 2 mM MgSO₄, pH 6.9) for 25 min. The samples were washed in PBST and mounted in *SlowFade*TM Gold antifade reagent (Invitrogen). All images were collected using a Carl Zeiss confocal microscope (LSM710).

Immunoblot analysis

Protein extracts for western blot analysis were prepared in LDS sample buffer (Invitrogen) by homogenizing ten 14-day-old male fly heads. The total protein extracts were separated using a 4–12% gradient SDS-PAGE gel and transferred to PVDF membranes (Millipore). The membranes were blocked with Tris-buffered saline (TBS) containing 4% nonfat dry milk or 4% bovine serum albumin (BSA) for 1 h and incubated with primary antibodies for 12 h at 4°C. The following primary antibodies were used: rabbit anti-*Drosophila* Marf (1:1000; a gift from Leo Pallanck, University of Washington), mouse anti-Opa1 (1:1000; Enzo Life Sciences), mouse anti-NDUFS3 (1:5000; Abcam), mouse anti-UQCRC2 (1:1000; Abcam), mouse anti-ATP5A (1:10000; Abcam), rabbit anti-FUS (1:1000; Bethyl Laboratories), rabbit anti-*Drosophila* GstO1 (1:1000), rabbit anti-*Drosophila* GstO2 (1:1000), rabbit anti-lamin C (Developmental Studies Hybridoma Bank, DSHB), rabbit anti- α -tubulin (1:2000; Sigma), rabbit anti- β -actin (1:4000; Cell Signaling), and mouse anti-GSH (1:1000; ViroGen Corp). The blots were washed in TBS containing 0.1% Tween-20 (TBST) and incubated with secondary antibodies. The primary antibodies were detected with the following horseradish peroxidase (HRP)-conjugated secondary antibodies: goat anti-rabbit IgG HRP conjugate and goat anti-mouse IgG HRP conjugate (1:2000; Millipore). Detection was carried out using an ECL-Plus kit (Amersham).

Protein extracts from the cells were homogenized in RIPA buffer (Cell Signaling) with a protease and phosphatase inhibitor cocktail (Roche) and lysis buffer (50 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl, pH 7.4 and protease inhibitors). The protein extracts were mixed with LDS sample buffer (Invitrogen) with a reducing agent. The protein sample was separated on a 4–12% Bis-Tris gel (Novex) and transferred to a PVDF membrane (Novex). The membrane was used for western blot analyses using the following primary antibodies: mouse anti-GSTO1 (1:1000; Abnova), mouse anti-GSTO1 (1:500; Abcam), rabbit anti-DDK (1:1000; OriGene), rabbit anti-TurboGFP (1:2000; OriGene), rabbit anti- α -tubulin (1:2000; Sigma), and mouse anti-GAPDH (1:1000, Santa Cruz Biotechnology).

Mitochondrial isolation assay

For mitochondrial isolation, the thoraces of 30 male flies were lysed in a reagent included in a mitochondrial isolation kit (Pierce) according to the manufacturer's protocol and fractionated by sequential centrifugation. The total protein extracts in the subcellular fractions were mixed with SDS loading buffer, boiled, and subjected to SDS-PAGE for western blot analysis.

BN-PAGE

Mitochondria were isolated from 14-day-old male fly thoraces using a mitochondrial isolation kit (Pierce) following the manufacturer's protocol. The purified mitochondrial extracts were resuspended in 60 μ l of 1 $\times$ native PAGE sample buffer (Invitrogen) containing 2% N-dodecyl- β -D-maltoside (DDM), 1% digitonin, and protease inhibitor (Halt). The sample was incubated on ice for 15 min and centrifuged at 12000 $\times g$ for 30 min. We measured the mitochondrial protein concentration in the supernatant. We mixed the supernatant (20 μ g) with 0.5% G-250 sample additive and performed blue native electrophoresis using a 3–12% native PAGE Bis-Tris gel (Invitrogen) at 4°C. An anode running buffer was used outside the gel, and a cathode running buffer was used inside the gel. After PAGE,

western blot analyses were performed with the following antibodies: mouse anti-NDUFS3 (1:5000; Abcam), mouse anti-UQCRC2 (1:1000; Abcam), and mouse anti-ATP5A (1:10000; Abcam).

Measurement of mitochondrial superoxide

Mitochondrial ROS production in the flies was measured using the mitochondrial oxygen free radical indicator MitoSOX Red (Invitrogen) according to a protocol. The muscle tissues from 11-day-old fly thoraces were dissected in cold PBS and incubated with 5 μ M MitoSOX Red in DMSO at 25°C for 20 min. After three washes in cold PBS, the muscle tissue samples were quickly mounted in *SlowFade*TM Gold antifade reagent (Invitrogen) and observed within 15 min with a Carl Zeiss confocal microscope (LSM710). Quantification of the fluorescence intensity was performed using ImageJ software.

ATP assay

A 28-day-old fly thorax was homogenized in 100 μ l of extraction buffer (6 M guanidine-HCl, 100 mM Tris, and 4 mM EDTA, pH 7.8) to inhibit ATPase. The extracts were immediately frozen in liquid nitrogen and then boiled for 5 min to denature endogenous ATP synthase. The samples were centrifuged at 20000 $\times g$ for 15 min, and the supernatant was transferred to a new tube. The supernatant was diluted (1:100) with extraction buffer. An appropriate amount of the sample was placed in each well of a 96-well plate and mixed with the luminescent solution included in the ENLITEN ATP assay kit (Promega). We measured luminescence at 10-second intervals using a GloMax microplate reader (Promega). The supernatant was diluted (1:2) with extraction buffer to measure the protein concentration using a BCA protein assay kit (Pierce). We calculated the ATP level relative to the standard by dividing the value by the total protein concentration.

Protein oxidation assay

For detection of protein oxidation, an OxyBlot protein oxidation detection kit (Millipore) was used according to the manufacturer's protocol. Eight thoraces from 28-day-old flies were briefly homogenized in lysis buffer containing 2% β -mercaptoethanol with 6% SDS. The homogenates were centrifuged for 30 min at 20000 $\times g$, and the pellet debris was discarded. The denatured protein was then derivatized with 2,4-dinitrophenylhydrazine (DNPH) at 25°C and neutralized with neutralization solution. The DNPH-labeled proteins were electrophoresed by SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were probed with anti-DNP antibody (1:150; Millipore), and the signal was detected using goat anti-rabbit IgG HRP conjugate secondary antibody (1:300; Millipore). Detection was carried out using an ECL-Plus kit (Amersham). The band density was measured using ImageJ software.

Nuclear/cytoplasmic fractionation assay

For subcellular fractionation, the heads of 20 male flies were lysed in the reagent included in a nuclear extraction kit (Active Motif) according to the manufacturer's protocol. The protein extracts in the different subcellular fractions were mixed with SDS loading buffer, boiled, and subjected to SDS-PAGE for western blot analysis.

Protein solubility assay

Total proteins were fractionated according to their solubility by making some modifications to a protocol previously described. The heads of twenty 7-day-old or 14-day-old flies were homogenized in ice-cold lysis buffer without SDS (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, and 10% glycerol, pH 7.5). The homogenates were centrifuged at 100,000 $\times g$ for 30 min at 4°C. The supernatant from this step was collected as a soluble fraction. The remaining pellets were washed two times with ice-cold lysis buffer containing protease inhibitors and re-centrifuged. The insoluble proteins were further resuspended, extracted with 50 μ l of 2 $\times$ SDS loading buffer with 2% SDS, sonicated, and centrifuged at 100,000 $\times g$ for 30 min at room temperature. Supernatants were boiled for 10 min at 95°C. The supernatant from this step was collected as an insoluble fraction. Samples were then processed for western blot.

In vitro glutathionylation assay

The recombinant full-length protein of human FUS (0.51 μ g) with a myc tag, purified from HEK293 cells (OriGene), was incubated at 25°C in 50 mM Tris-HCl (pH 7.5) in the presence of various concentrations of GSSG. After 1 h, the samples were placed on ice, and 3 $\times$ nonreducing LDS sample buffer (Invitrogen) was added to the mixtures. The samples were separated by 12% SDS-PAGE, transferred, and probed with mouse anti-GSH (1:1,000; ViroGen Corp.) and mouse anti-myc (1:1,000; Millipore) antibodies.

Homology modeling

The three-dimensional structure of the FUS ZnF domain [32 amino acid residues (422-RAGDWKCPNPTCENMNFNSWRNECNQCK APKPD-453)] was predicted by the I-TASSER server for protein structure and function prediction accessed through Molegro Molecular Viewer 2.5.0 software (Molegro ApS, Aarhus C, Denmark).

Preparation of recombinant protein and purification

The pET15b-GstO1, pET15b-GstO2, pET15b-GstO2^{C31A}, pET15b-GstO3, and pTHMT-MBP-FUS (Addgene) plasmids were previously described (Burke et al., 2015; Kim et al., 2012). All cloned DNA constructs were transformed into competent *Escherichia*

coli BL21 (DE3) cells. The recombinant GstO proteins were expressed with an N-terminal 6× His tag from the pET15b and pTHMT plasmids. Transformed cells were grown to an optical density (OD_{600nm}) of 0.4–0.5 in 100 ml of Luria-Bertani (LB) medium containing 50 µg/ml ampicillin or 50 µg/ml kanamycin and then induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG). After 4 h of incubation at 37°C, the cells were harvested by centrifugation and suspended in 8 ml of ice-cold binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9) (Novagen). Next, the cells were homogenized using a sonicator, and the supernatant was collected by centrifugation at 20,000 ×g for 30 min at 4°C, followed by purification with His-Bind Resin according to the standard manufacturer's protocol (Novagen). Purified proteins were dialyzed against storage buffer (50 mM Tris-HCl, 50 mM NaCl, and 5% glycerol, pH 7.5). All proteins were concentrated via centrifugal filtration (Millipore) and frozen with liquid nitrogen.

LLPS assay

MBP-FUS full-length protein was diluted to a final concentration of 5 µM into 50 mM Tris-HCl, 50 mM NaCl, and 5% glycerol, pH 7.5. Droplets of FUS were formed by incubating 10 units/µl TEV protease (New England BioLabs) for 20 min at 30°C. Then, 20 µl of sample was dropped on a glass coverslip and droplet formation was evaluated by imaging with differential interference contrast (DIC) on a Leica DMi8 microscope and DFC7000T camera system. The aggregate area was calculated using ImageJ software.

In vitro protein aggregation assay

After in vitro glutathionylation, the samples were incubated on a heat block at 37°C. At different time points after incubation at 37°C, the samples were divided into supernatant and pellet fractions by centrifugation at 20000 ×g for 30 min at 4°C. The pellet was washed five times in 50 mM Tris-HCl (pH 7.5) for 10 min per wash and then dissolved in LDS sample buffer (2% SDS) (Invitrogen) with a reducing agent. The FUS in both fractions was detected by western blot analysis with rabbit anti-FUS antibody (1:1000; Bethyl Laboratories).

Immunoprecipitation assay

The heads of 7-day-old male flies were homogenized in lysis buffer with 100 µM diamide (Sigma-Aldrich) (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40, 10% glycerol, pH 7.5 and protease inhibitor cocktail). The extracts were centrifuged and then incubated with an anti-GSH antibody (3 µg; ViroGen Corp.) for 4 h at 4°C. After incubation, protein A/G Sepharose (GE Healthcare) was added to lysis buffer and incubated for 12 h at 4°C. Beads were washed five times with lysis buffer and boiled in SDS sample buffer (Invitrogen). Samples were separated by 12% SDS-PAGE, transferred, and probed with rabbit anti-FUS (1:1000; Bethyl Laboratories) antibody. The signal was detected using TrueBlot secondary antibodies: goat anti-rabbit IgG HRP (1:1000; Rockland) and goat anti-mouse IgG HRP (1:1000; Rockland).

Whole-brain immunostaining

The brains of 7-day-old male flies and 3rd-instar larvae were fixed with 4% formaldehyde in fixative buffer (100 mM PIPES, 1 mM EGTA, 1% Triton X-100, and 2 mM MgSO₄, pH 6.9) for 25 min and blocked in wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, and 0.5 mg/ml BSA, pH 6.8) with 10 mg/ml BSA for 1 h at 25°C. Then, the brains were incubated with primary antibodies diluted in blocking buffer for 12 h at 4°C. The following antibodies were used: rabbit anti-FUS (1:100; Bethyl Laboratories), mouse anti-FUS (1:50; Santa Cruz Biotechnology), mouse anti-GSH (1:100; ViroGen Corp.), rabbit anti-GstO2 (1:20), and rabbit anti-cleaved caspase-3 (1:500; Cell Signaling). The samples were then incubated with Alexa 488-conjugated secondary antibodies (1:200; Invitrogen), Cy3-conjugated secondary antibodies (1:200; Jackson ImmunoResearch Laboratories), and DAPI (1:500; Sigma-Aldrich). The brains were washed three times in washing buffer for 10 min per wash and mounted in *SlowFade*TM Gold antifade reagent (Invitrogen). All images were obtained on a Carl Zeiss confocal microscope (LSM710).

Hypoxic exposure

Flies were placed into an airtight chamber at 25°C supplied with 5% oxygen/95% nitrogen.

Stable cell line generation

N2a cells in 24-well plates were transfected with 1 µg of human GSTO1 cDNA using Lipofectamine 3000 reagent (Invitrogen). An empty pCMV6-Myc-DDK vector was used as a control. Stable transfectants were selected in the presence of G418 (600 µg/ml) 2 days after transfection. The overexpression of GSTO1 protein in the stable transfectants was confirmed by immunoblot analysis.

Transfection

N2a cells were plated in six-well plates and transfected with 4 µg of pCMV6-FUS labeled with GFP. The pCMV6-FUS-GFP-tagged human wild-type and mutant FUS vectors were added to each well with Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's manual. An empty pCMV6-AC-GFP plasmid was used as a negative control.

Drug feeding assay

Standard fly food was mixed with various concentrations of carboplatin (LKT Laboratories). The same amount of distilled water was used as a vehicle control for carboplatin. Parent flies were crossed on food supplemented with carboplatin. The 3rd-instar larvae were selected and subjected to several experiments.

QUANTIFICATION AND STATISTICAL ANALYSIS

Experimental analyses and data collection were performed in a blinded fashion. All datasets were organized and analyzed in Microsoft Excel using two-tailed Student's *t* tests. The differences in the mean values of multiple datasets were analyzed using one-way ANOVA with Tukey's multiple-comparison test. The number of flies and replicates used are reported in the figure legend. The statistical test used for determining the significance of the comparisons (*p* values) is specified in the appropriate figure legends. Significance was set at $p < 0.05$. The data from multiple experiments were quantified and are expressed as the mean $\pm$ SD and SEM.