FUS regulates RAN translation through modulating the G-quadruplex structure of GGGGCC repeat RNA in C9orf72-linked ALS/FTD

Yuzo Fujino, Morio Ueyama, Taro Ishiguro, Daisaku Ozawa, Toshihiko Sugiki, Hayato Ito, Asako Murata, Akira Ishiguro, Tania F. Gendron, Kohji Mori, Eiichi Tokuda, Tomoya Taminato, Takuya Konno, Akihide Koyama, Yuya Kawabe, Toshihide Takeuchi, Yoshiaki Furukawa, Toshimichi Fujiwara, Manabu Ikeda, Toshiki Mizuno, Hideki Mochizuki, Hidehiro Mizusawa, Keiji Wada, Kinya Ishikawa, Osamu Onodera, Kazuhiko Nakatani, Hideki Taguchi, Leonard Petrucelli, and Yoshitaka Nagai

1 Department of Neurology, Kindai University Faculty of Medicine, Osaka-Sayama, Osaka 589-8511, Japan
2 Department of Neurology, Kyoto Prefectural University of Medicine, Kyoto 602-0841, Japan
3 Department of Neurotherapeutics, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan
4 Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan
5 Department of Neurology and Neurological Science, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8519, Japan
6 Laboratory of Molecular Biophysics, Institute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan
7 School of Life Science and Technology, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8503, Japan
8 Department of Regulatory Bioorganic Chemistry, The Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 565-0047, Japan
9 Research Center for Micro-nano Technology, Hosei University, Koganei, Tokyo 184-0003, Japan
10 Department of Neuroscience, Mayo Clinic, Jacksonville, FL 32224, USA
11 Department of Psychiatry, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan
12 Department of Chemistry, Keio University, Yokohama, Kanagawa 223-8522, Japan
13 Department of Neurology, Clinical Neuroscience Branch, Brain Research Institute, Niigata University, Niigata 951-8585, Japan
14 Life Science Research Institute, Kindai University, Osaka-Sayama, Osaka 589-8511, Japan
15 Department of Neurology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan
16 Cell Biology Center, Institute of Innovative Research, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8503, Japan

*Correspondence

Department of Neurology
Kindai University Faculty of Medicine
377-2 Ohnohigashi, Osaka-Sayama, Osaka 589-8511, Japan
Tel.: +81 (72) 366-0246 (ext. 3553); Fax: +81 (72) 368-4846
E-mail: yoshi.nagai@med.kindai.ac.jp
Abstract

Abnormal expansions of GGGGCC repeat sequence in the noncoding region of the C9orf72 gene is the most common cause of familial amyotrophic lateral sclerosis and frontotemporal dementia (C9-ALS/FTD). The expanded repeat sequence is translated into dipeptide repeat proteins (DPRs) by noncanonical repeat-associated non-AUG (RAN) translation. Since DPRs play central roles in the pathogenesis of C9-ALS/FTD, we here investigate the regulatory mechanisms of RAN translation, focusing on the effects of RNA-binding proteins (RBPs) targeting GGGGCC repeat RNAs. Using C9-ALS/FTD model flies, we demonstrated that the ALS/FTD-linked RBP FUS suppresses RAN translation and neurodegeneration in an RNA-binding activity-dependent manner. Moreover, we found that FUS directly binds to and modulates the G-quadruplex structure of GGGGCC repeat RNA as an RNA chaperone, resulting in the suppression of RAN translation in vitro. These results reveal a previously unrecognized regulatory mechanism of RAN translation by G-quadruplex-targeting RBPs, providing therapeutic insights for C9-ALS/FTD and other repeat expansion diseases.
**Introduction**

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are incurable neurodegenerative diseases with overlapping genetic and neuropathological features. Abnormal expansions of the GGGGCC (G\textsubscript{4}C\textsubscript{2}) repeat sequence in the noncoding region of the \textit{C9orf72} gene have been found to be the most common genetic mutation responsible for ALS/FTD (DeJesus-Hernandez et al., 2011, Gijselinck et al., 2012; Renton et al., 2011). Three major pathomechanisms are thought to be involved in the pathogenesis of \textit{C9orf72}-linked ALS/FTD (C9-ALS/FTD): first, expansion of the G\textsubscript{4}C\textsubscript{2} repeats results in decreased expression of the \textit{C9orf72} gene, leading to its haploinsufficiency (Boivin et al., 2020; DeJesus-Hernandez et al. 2011; Gijselinck et al., 2012; Shi et al., 2018; Waite et al., 2014; Zhu et al., 2020). Second, the transcribed G\textsubscript{4}C\textsubscript{2} repeat-containing RNA accumulates as RNA foci in the affected tissues, sequestering various RNA-binding proteins (RBPs) and altering their function (Conlon et al., 2016; Cooper-Knock et al., 2014; Donnelly et al., 2013; Haeusler et al., 2014; Lee et al., 2013; Mori et al., 2013a). Third, these G\textsubscript{4}C\textsubscript{2} repeat RNA is also translated into dipeptide repeat (DPR) proteins, despite the lack of an AUG initiation codon, by noncanonical repeat-associated non-AUG (RAN) translation (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013b; Mori et al., 2013c; Zu et al, 2011; Zu et al, 2013). Since RAN translation occurs in all reading frames and the expanded G\textsubscript{4}C\textsubscript{2} repeat sequence is bidirectionally transcribed, five distinct DPRs, i.e., poly(glycine-arginine) [poly(GR)], poly(glycine-alanine) [poly(GA)], poly(glycine-proline) [poly(GP)], poly(proline-arginine) [poly(PR)], and poly(proline-alanine) [poly(PA)] are produced and observed in patients’ brains (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013b; Mori et al., 2013c; Zu et al., 2013) and cerebrospinal fluid (Gendron et al., 2017; Krishnon et
DPRs have been shown to exert toxic effects in multiple C9-ALS/FTD models, such as cultured cells, flies, and mice (Choi et al., 2019; Jovičić et al., 2015; May et al. 2014; Mizielinska et al., 2014; Rudich et al., 2017; Wen et al., 2014; Zhang et al., 2016; Zhang et al., 2018). Importantly, the toxicity of DPRs was confirmed in DPR-only flies, which express DPRs translated from non-G_{4C2} repeat RNAs with alternative codons and show neurodegeneration, whereas RNA-only flies expressing G_{4C2} repeat RNAs with stop codon interruptions, which eliminate DPRs production, did not show any obvious degenerative phenotypes (Mizielinska et al., 2014). In addition, increased DPR production, but not RNA foci, was reported to correlate with G_{4C2} repeat-induced toxicity in a C9-ALS/FTD Drosophila model (Tran et al., 2015). Taken together, these studies have strongly suggested that DPRs play a central role in the pathogenesis of C9-ALS/FTD. Indeed, DPRs have been reported to disrupt various biological pathways, such as nucleocytoplasmic transport (Hutten et al., 2020; Jovičić et al., 2015; Zhang et al., 2016) and membraneless organelle dynamics (Kwon et al., 2014; Lee et al., 2016; Lin et al., 2016). Therefore, elucidating the regulatory mechanism of RAN translation is a significant challenge toward developing potential therapies for C9-ALS/FTD.

Since the discovery of RAN translation in 2011 (Zu et al., 2011), many studies to date have focused on its molecular mechanisms, that is, whether it has functional overlap with canonical AUG-dependent translation. Previous studies on C9-ALS/FTD using monocistronic reporters containing a G_{4C2} repeat sequence revealed cap-dependent translation initiation from the upstream near-cognate CUG initiation codon, requiring the cap-binding eukaryotic translation factor 4F complex (Green et al., 2017; Tabet et al., 2018). On the other hand, studies using bicistronic reporters with a G_{4C2}
repeat sequence in the second cistron also produced DPRs by RAN translation in all reading frames, suggesting cap-independent translation initiation within the G₄C₂ repeat sequence (Cheng et al., 2018; Sonobe et al., 2018). This is reminiscent of internal ribosomal entry site translation initiation, which is another type of noncanonical cap-independent translation in which specific factors are directly recruited to the highly-structured mRNA for initiation (Kwan et al., 2019). While such initiation mechanisms of RAN translation have been explored to date, specific roles of the repeat sequence on RAN translation remain enigmatic. Considering a repeat length dependency of RAN translation (Mori et al., 2013c; Zu et al., 2011; Zu et al., 2013), the repeat sequence itself would also be essential for the initiation or elongation steps of RAN translation.

Based on our previous findings of the protective role of TDP-43 on UGGAA repeat-induced toxicity in spinocerebellar ataxia type 31 (SCA31) models (Ishiguro et al., 2017), we hypothesized that RBPs specifically binding to repeat sequences of template RNA play a role in RAN translation.

Using Drosophila models of C9-ALS/FTD, we here demonstrate the regulatory roles of the ALS/FTD-linked RBP FUS on RAN translation from G₄C₂ repeat RNA, which lead to the significant modulation of neurodegeneration. We found that FUS suppresses RNA foci formation and DPR production, resulting in the suppression of repeat-induced degeneration. This suppressive effect on degeneration was abolished by mutations in the RNA-recognition motif (RRM) of FUS. In contrast, knockdown of endogenous caz, a Drosophila homologue of FUS, enhanced DPR aggregation and RNA foci formation, resulting in the enhancement of repeat-induced degeneration. Moreover, FUS was found to directly bind to G₄C₂ repeat RNA and modify its G-quadruplex structure as an RNA chaperone, resulting in the suppression of RAN
translation in vitro. In addition, other G-quadruplex-targeting RBPs also suppressed G<sub>4</sub>C<sub>2</sub> repeat-induced toxicity in our C9-ALS/FTD flies. These results strongly indicate that FUS regulates RAN translation and suppresses DPR toxicity through modulating the G-quadruplex structure of G<sub>4</sub>C<sub>2</sub> repeat RNA. Our findings shed light on the regulatory mechanisms of RAN translation by G-quadruplex-targeting RBPs, and propose novel therapeutic strategies for repeat expansion diseases by regulating RAN translation.

**Results**

**Screening for RBPs that suppress G<sub>4</sub>C<sub>2</sub> repeat-induced toxicity in C9-ALS/FTD flies**

We established *Drosophila* models of C9-ALS/FTD that express pathogenic length 42 or 89 G<sub>4</sub>C<sub>2</sub> repeats [(G<sub>4</sub>C<sub>2</sub>)<sub>42</sub>, or (G<sub>4</sub>C<sub>2</sub>)<sub>89</sub> flies, respectively], and confirmed that expanded G<sub>4</sub>C<sub>2</sub> repeat sequences induce eye degeneration and motor dysfunction accompanied with the formation of RNA foci and the production of three types of DPRs (Figure 1—figure supplement 1), consistent with previous studies (Freibaum et al., 2015; Goodman et al., 2019; Mizielinska et al., 2014; Xu et al., 2013). We also established *Drosophila* expressing normal length 9 G<sub>4</sub>C<sub>2</sub> repeats as a control [(G<sub>4</sub>C<sub>2</sub>)<sub>9</sub> flies], and found that they did not show eye degeneration, motor dysfunction, RNA foci formation, or DPR aggregation (Figure 1—figure supplement 1). We selected 18 RBPs that have been reported to bind to G<sub>4</sub>C<sub>2</sub> repeat RNA (Mori et al., 2013a), as well as TDP-43, an ALS/FTD-linked RBP that does not bind to G<sub>4</sub>C<sub>2</sub> repeat RNA (Xu et al., 2013) (Figure 1—source data 1), and examined their roles in neurodegeneration in our C9-ALS/FTD fly models. We found that coexpression of *FUS, IGF2BP1*, or
hnRNP A2B1 strongly suppressed the eye degeneration in both flies expressing $(G_4C_2)_{42}$ or 89, which show decreased eye size and loss of pigmentation (Figures 1A–1D and Figure 1—source data 2). Coexpression of five RBPs, namely, *hnRNPR*, *SAFB2*, *SF3B3*, *hnRNPA1*, and *hnRNPL* also partially suppressed the eye degeneration, whereas coexpression of the other six RBPs had no effect, and two RBPs enhanced the phenotypes (Figures 1A–1D and Figure 1—source data 2). In addition, coexpression of TDP-43 had no effect on the eye degeneration in $(G_4C_2)_{42}$ flies, and resulted in lethality in $(G_4C_2)_{89}$ flies, likely due to the toxicity of TDP-43 expression itself (Figures 1A and 1D, and Figure 1—source data 2). The variation in the effects of these G4C2 repeat-binding RBPs on G4C2 repeat-induced toxicity may be due to their different binding affinities to G4C2 repeat RNA, and their different roles in RNA metabolism. We then analyzed the expression levels of G4C2 repeat RNA in flies coexpressing $(G_4C_2)_{89}$ and three RBPs that strongly suppressed eye degeneration. We found that coexpression of *IGF2BP1* or *hnRNPA2B1* significantly decreased G4C2 repeat RNA levels, whereas they were not altered upon coexpression of *FUS* (Figure 1E). Although the suppressive effects of IGF2BP1 and hnRNPA2B1 could simply be explained by the decreased levels of G4C2 repeat RNA, the molecular mechanisms by which FUS suppresses G4C2 repeat-induced toxicity remain to be clarified. The suppressive effects of FUS on G4C2 repeat-induced toxicity was confirmed using multiple FUS fly lines, showing the significant suppression of decreased eye size and loss of pigmentation in $(G_4C_2)_{42}$ or 89 flies coexpressing *FUS* (Figure 1—figure supplement 2). Therefore, we decided to further focus on FUS, which is another ALS/FTD-linked RBP, and investigated its mechanism of the suppression of G4C2 repeat-induced toxicity.
FUS suppresses G$_4$C$_2$ repeat-induced toxicity via its RNA-binding activity

We next investigated whether the suppressive effects of FUS on G$_4$C$_2$ repeat-induced toxicity are mediated by its binding to G$_4$C$_2$ repeat RNA, using flies expressing FUS with mutations in the RRM (FUS-RRMmut), which have been reported to eliminate its RNA-binding activity (Daigle et al., 2013). Western blot analysis confirmed that the FUS-RRMmut fly line expresses almost an equivalent level of the FUS proteins to the FUS fly line (Figure 2—figure supplement 1). We found that coexpression of FUS-RRMmut did not restore the eye degeneration in flies expressing (G$_4$C$_2$)$_{89}$, suggesting that the RNA-binding activity of FUS is essential for its suppressive effects on G$_4$C$_2$ repeat-induced toxicity (Figures 2A–C). We also evaluated the reduced egg-to-adult viability of (G$_4$C$_2$)$_{42}$ flies, and confirmed that this phenotype was rescued by coexpression of FUS, but not by coexpression of FUS-RRMmut (Figure 2D). Expression of G$_4$C$_2$ repeat RNA in the nervous system of flies after eclosion using the elav-GeneSwitch driver induces motor dysfunction, and coexpression of FUS significantly alleviated this motor dysfunction (Figure 2E), indicating that FUS suppresses the neuronal phenotypes of flies expressing G$_4$C$_2$ repeat RNA. It is notable that the motor dysfunction caused by the expression of FUS alone was also alleviated by coexpression of (G$_4$C$_2$)$_{42}$ (Figure 2E), indicating that the G$_4$C$_2$ repeat RNA conversely suppresses FUS toxicity. This result is consistent with our previous observations in SCA31 flies that UGGAA repeat RNA reduced the aggregation and toxicity of TDP-43 (Ishiguro et al., 2017). Moreover, recent studies demonstrated that RNA buffers the phase separation of TDP-43 and FUS, resulting in the suppression of their aggregation (Maharana et al., 2018; Mann et al., 2019). These findings hence suggest that balancing the crosstalk between repeat RNAs and RBPs neutralizes the
toxicities of each other.

FUS suppresses RNA foci formation and RAN translation from G₄C₂ repeat RNA

We next analyzed the effects of FUS expression on RNA foci and DPR production in flies expressing G₄C₂ repeat RNA. We performed RNA fluorescence in situ hybridization (FISH) of the salivary glands of fly larvae expressing (G₄C₂)₈⁹, and found that coexpression of FUS significantly decreased the number of nuclei containing RNA foci in (G₄C₂)₈⁹ flies, whereas it was not altered by coexpression of FUS-RRMmut (Figures 3A and 3B). We confirmed that the expression levels of G₄C₂ repeat RNA in (G₄C₂)₈⁹ flies were not altered by coexpression of FUS or FUS-RRMmut (Figure 3C). These results were in good agreement with our previous study on SCA31 showing the suppressive effects of FUS and other RBPs on RNA foci formation of UGGAA repeat RNA as RNA chaperones (Ishiguro et al., 2017), raising the possibility that FUS has RNA-chaperoning activity also for G₄C₂ repeat RNA. Immunohistochemistry of the eye imaginal discs of fly larvae expressing (G₄C₂)₈⁹ revealed that coexpression of FUS significantly decreased the number of DPR aggregates in (G₄C₂)₈⁹ flies, whereas coexpression of FUS-RRMmut did not (Figures 3D and 3E). Quantitative analyses of poly(GP) by immunoassay also demonstrated that poly(GP) levels were greatly decreased in (G₄C₂)₈⁹ flies upon coexpression of FUS, but not FUS-RRMmut (Fig 3F), indicating that FUS suppresses RAN translation from the G₄C₂ repeat RNA to reduce DPR production. Considering that the 5′ upstream sequence of the G₄C₂ repeat in the C9orf72 gene is reported to affect RAN translation activity (Green et al., 2017; Tabet et al., 2018), we used flies expressing the G₄C₂ repeat sequence with the upstream intronic sequence of the C9orf72 gene, namely, LDS-(G₄C₂)₄⁴GR-GFP (Goodman et al., 2019).
Since this construct has a 3′-green fluorescent protein (GFP) tag in the GR reading frame downstream of the G₄C₂ repeat sequence, the GR-GFP fusion protein is produced by RAN translation. We confirmed that coexpression of FUS significantly decreased the expression level of GR-GFP, whereas coexpression of FUS-RRMmut had no effect (Figures 3G–3I).

We further excluded the possibility that FUS directly interacts with DPRs, rather than with G₄C₂ repeat RNA, to decrease DPR levels and exert its suppressive effects. Using DPR-only flies expressing DPRs translated from non-G₄C₂ RNAs with alternative codons (Mizielinska et al., 2014), we confirmed that FUS did not suppress the eye degeneration in DPR-only flies expressing poly(GR), but rather enhanced their phenotypes, likely due to the additive effects of FUS toxicity (Figure 3—figure supplement 1). Together with the finding that FUS decreases not only DPR expression but also RNA foci formation (Figures 3A and 3B), these results collectively indicate that FUS indeed interacts with G₄C₂ repeat RNA and regulates RAN translation from G₄C₂ repeat RNA in Drosophila models of C9-ALS/FTD.

Reduction of endogenous caz expression enhances G₄C₂ repeat-induced toxicity, RNA foci formation, and DPR aggregation

To elucidate the physiological role of FUS on RAN translation, we also investigated the role of endogenous caz, a Drosophila homologue of FUS, on G₄C₂ repeat-induced toxicity in flies expressing G₄C₂ repeat RNAs. Coexpression of caz as well as FUS suppressed eye degeneration in flies expressing (G₄C₂)₄₂ or ₈₉ (Figure 4—figure supplement 1). These data suggest that caz is a functional homologue of FUS. In contrast, knockdown of caz by RNA interference or its hemizygous deletion modestly
but significantly enhanced the eye degeneration in ($G_4C_2$)$_{89}$ flies (Figures 4A–4D), indicating that reduced $caz$ expression enhances $G_4C_2$ repeat-induced toxicity. We next analyzed the effects of $caz$ knockdown on RNA foci formation and DPR production in flies expressing ($G_4C_2$)$_{89}$. FISH analysis of the salivary glands revealed that knockdown of $caz$ significantly increased the number of nuclei containing RNA foci in ($G_4C_2$)$_{89}$ flies (Figures 4E and 4F). We also confirmed that the expression levels of $G_4C_2$ repeat RNA in ($G_4C_2$)$_{89}$ flies were not altered by the knockdown of $caz$ (Figure 4G). Immunohistochemical analysis showed that knockdown of $caz$ significantly increased the number of DPR aggregates in ($G_4C_2$)$_{89}$ flies (Figures 4H and 4I). These results indicate that the reduction of $caz$ expression enhances RNA foci formation and DPR aggregation, compatible with the results of $FUS$ coexpression in flies expressing ($G_4C_2$)$_{89}$ (Figure 3), and that $FUS$ functions as an endogenous regulator of RAN translation.

**FUS directly binds to and modulates the G-quadruplex structure of $G_4C_2$ repeat RNA, resulting in the suppression of RAN translation in vitro**

We next confirmed the direct interaction of FUS with $G_4C_2$ repeat RNA by the filter binding assay. We found that His-tagged FUS binds to the ($G_4C_2$)$_4$ RNA in a dose-dependent manner, but not to the control (AAAAAA)$_4$ RNA (Figure 5A), and His-tagged FUS-RRMmut had almost no binding affinity to the ($G_4C_2$)$_4$ RNA, consistent with a previous study (Mori et al., 2013a). We also confirmed the interaction of FUS with the $G_4C_2$ repeat RNA in our C9-ALS/FTD flies by showing the colocalization of FUS with the RNA foci (Figure 5—figure supplement 1), consistent with a recent study using C9-ALS/FTD patient fibroblasts (Bajc Česnik et al., 2019). Since $G_4C_2$ repeat...
RNA was reported to form both G-quadruplex and hairpin structures (Fratta et al., 2012; Hauesler et al., 2014; Reddy et al., 2013; Su et al., 2014), we next characterized the interactions of FUS with G₄C₂ repeat RNA having different structures. G₄C₂ repeat RNA is known to form G-quadruplex structures in the presence of K⁺, whereas they form hairpin structures in the presence of Na⁺ (Su et al., 2014). Surface plasmon resonance (SPR) analyses demonstrated that FUS preferentially binds to (G₄C₂)₄ RNA with the G-quadruplex structure in KCl buffer (Table 1, dissociation constant (K_D) = 1.5×10⁻⁸ M), and weakly to (G₄C₂)₄ RNA with the hairpin structure in NaCl buffer (Table 1, K_D = 1.3×10⁻⁷ μM). We also confirmed that FUS has poor binding affinity to (G₄C₂)₄ RNA in LiCl buffer (Table 1, K_D = 1.4×10⁻⁵ μM), which destabilizes the G-quadruplex structure (Herdy et al., 1992), and was an almost similar level to its binding affinity to the negative control (A₄C₂)₄ RNA (not shown). These results suggest the preferential binding of FUS to G₄C₂ repeat RNA with the G-quadruplex structure, which is consistent with a previous report showing preferential binding of FUS to G-quadruplex structured Sc1 and DNMT RNAs (Ozdilek et al., 2017). Considering that higher-order structures, including G-quadruplex and hairpin structures, are reported to be involved in RAN translation (Mori et al., 2021; Simone et al., 2018; Wang et al., 2019; Zu et al., 2011), we next investigated the effects of FUS on the structure of G₄C₂ repeat RNA. The circular dichroism (CD) spectrum of (G₄C₂)₄ RNA in KCl buffer was found to exhibit a positive peak at approximately 260 nm and a negative peak at 240 nm (Figure 5B, black line), consistent with previous reports (Fratta et al., 2012; Hauesler et al., 2014; Reddy et al., 2013; Su et al., 2014). Interestingly, upon the addition of FUS, these two peaks were notably shifted to longer wavelengths with substantial CD spectrum changes, indicating a significant structural alteration in (G₄C₂)₄ RNA (Figure
5B, red line). We confirmed that the CD spectrum of FUS alone in the wavelength range of 240 to 300 nm was almost negligible (Figure 5—figure supplement 2A, green line), indicating that this change in CD spectrum is attributed to structural changes in the (G₄C₂)₄ RNA. We also observed CD spectrum changes to some extent in the (G₄C₂)₄ RNA upon the addition of FUS in NaCl buffer, but not in LiCl buffer, confirming an interaction between FUS and hairpin-structured (G₄C₂)₄ RNA as well (Figures 5C and 5D). We further analyzed the interaction between FUS and G₄C₂ repeat RNA by imino proton nuclear magnetic resonance (NMR). In KCl buffer, the NMR signals of the imino proton for the G-quadruplex structure of (G₄C₂)₄ RNA were detected in the region around 10 to 12 ppm (Figure 5—figure supplement 2D), consistent with previous studies (Fratta et al., 2012; Su et al., 2014). Upon the addition of FUS, the NMR intensities of (G₄C₂)₄ RNA were decreased in an FUS concentration-dependent manner (Figure 5—figure supplement 2D), further supporting the possibility that FUS interacts with and modulates the G-quadruplex structure of (G₄C₂)₄ RNA. Collectively, these results indicate that FUS directly binds to G₄C₂ repeat RNA, preferentially to its G-quadruplex form, and modulates its higher-order structures. These structural alterations of G₄C₂ repeat RNAs by FUS did not require ATP or interactions with other proteins, suggesting its role as an RNA chaperone for G₄C₂ repeat RNA (Rajkowitsch et al., 2007).

To further clarify the direct link between the binding of FUS to G₄C₂ repeat RNA and its effects on RAN translation, we employed a cell-free in vitro translation assay using rabbit reticulocyte lysate. We designed a reporter construct containing the 80 G₄C₂ repeat sequence with the 5’ upstream intronic sequence of the C9orf72 gene and the Myc tag sequence in the GA reading frame at the 3’ downstream (Figure 5E).
This upstream sequence contained multiple stop codons in each reading frame and lacked AUG initiation codons. We confirmed by Western blotting that this reporter system indeed produces GA-Myc by RAN translation, consistent with previous studies (Green et al., 2017; Tabet et al., 2018). Notably, the addition of FUS to this system decreased the expression level of GA-Myc in a dose-dependent manner, whereas the addition of the control bovine serum albumin (BSA) did not (Figure 5F). Taken together, these results indicate that FUS suppresses RAN translation from G₄C₂ repeat RNA in vitro as an RNA chaperone.

Identification of G-quadruplex-targeting RBPs that suppress G₄C₂ repeat-induced toxicity in C9-ALS/FTD flies

Considering that FUS suppresses G₄C₂ repeat-induced toxicity as an RNA chaperone through its preferential binding to the G-quadruplex structure of G₄C₂ repeat RNA (Figure 5 and Table 1), we hypothesized that other G-quadruplex-targeting RBPs might have similar suppressive effects on G₄C₂ repeat-induced toxicity. To investigate this possibility, we selected 6 representative G-quadruplex-targeting RBPs, all of which are known to bind to G₄C₂ RNA as well (Cooper-Knock et al., 2014; Haeusler et al., 2014; Mori et al., 2013a; Xu et al., 2013) (Figure 6—source data 1). Intriguingly, coexpression of EWSR1, DDX3X, DDX5, or DDX17 significantly suppressed eye degeneration in (G₄C₂)₈₉ flies without altering G₄C₂ RNA expression (Figure 6). Their effects on G₄C₂ repeat-induced toxicity and repeat RNA expression were consistent with those of FUS. In support of our results, DDX3X was previously reported to suppress RAN translation and G₄C₂ repeat-induced toxicity in cell culture in a helicase-activity-dependent manner (Cheng et al., 2019). On the other hand, coexpression of DHX9 or
DHX36 suppressed eye degeneration by reducing G₄C₂ repeat RNA levels (Figure 6). Since G-quadruplex-targeting RBPs have diverse biological functions, including transcription, RNA processing, translation, and RNA stabilization (Dumas et al., 2021), these different effects among G-quadruplex-targeting RBPs on G₄C₂ repeat RNA expression might be attributed to their different roles in RNA metabolism. Thus, some G-quadruplex-targeting RBPs regulate G₄C₂ repeat-induced toxicity by binding to and possibly by modulating the G-quadruplex structure of G₄C₂ repeat RNA.
Discussion

In this study, we revealed a novel regulatory mechanism of RAN translation from expanded G\(_4\)C\(_2\) repeat RNA by the ALS/FTD-linked RBP FUS, which suppresses DPR production and neurodegeneration in C9-ALS/FTD Drosophila models (Figures 1–4). FUS directly binds to G\(_4\)C\(_2\) repeat RNA and modulates its G-quadruplex structure, as evident by CD and NMR analyses (Figure 5), suggesting its functional role as an RNA chaperone. This is reminiscent of our recent study on SCA31, in which we demonstrated a novel role of the ALS/FTD-linked RBPs TDP-43, FUS, and hnRNPA2B1 as RNA chaperones binding to UGGAA repeat RNA and altering its structure, resulting in the suppression of its neurotoxicity through reducing RNA foci formation and repeat polypeptide translation (Ishiguro et al., 2017). Considering the similarities of the effects of FUS on G\(_4\)C\(_2\) repeat RNA and UGGAA repeat RNA, we conclude that FUS functions as an RNA chaperone also for G\(_4\)C\(_2\) repeat RNA to regulate its RAN translation.

The suppressive effects of RBPs in several noncoding repeat expansion diseases by the amelioration of their sequestration into RNA foci have been reported. For example, in myotonic dystrophy type 1, MBNL1 was shown to be sequestered into CUG repeat RNA foci, and overexpression of MBNL1 in a mouse model was found to compensate its functional loss, resulting in the reversal of myotonia (Kanadia et al., 2006). Similarly, previous studies reported the suppressive effects of other RBPs on neurodegeneration, such as hnRNPA2B1 in fragile X ataxia/tremor syndrome (Sofora et al., 2007), and Pur-\(\alpha\) and Zfp106 in C9-ALS/FTD (Xu et al., 2013; Celona et al., 2017). The suppressive effects of these RBPs have been thought to result from the supplementation against their loss-of-function due to their sequestration into RNA foci,
although their effects on gain-of-toxic disease pathomechanisms, that is, RAN translation and repeat RNA expression, remain to be elucidated. In contrast, in this study we demonstrated that FUS suppresses neurodegeneration in C9-ALS/FTD by directly targeting G\textsubscript{4}C\textsubscript{2} repeat RNA and inhibiting RAN translation as an RNA chaperone. Similar suppressive effects of RBPs by targeting UGGAA repeat RNA in SCA31 as RNA chaperones have also been reported (Ishiguro et al., 2017). In addition, we also showed that the expression of IGF2BP1, hnRNPA2B1, DHX9, and DHX36 decreased G\textsubscript{4}C\textsubscript{2} repeat RNA expression and suppressed eye degeneration in our C9-ALS/FTD Drosophila model (Figures 1 and 6), likely via the reduction of DPR levels. In myotonic dystrophy type 2 models, MBNL1 was also reported to retain CCUG repeat RNA in the nucleus, resulting in the suppression of RAN translation (Zu et al., 2017), implying various mechanisms of the effects of RBPs depending on the combination of RBPs and repeat RNA. Nevertheless, our findings highlighted the previously unrecognized roles of RBPs directly interacting with repeat RNA and modulating gain-of-toxic pathomechanisms, including RAN translation in noncoding repeat expansion diseases.

Several studies have indicated the importance of higher-order structures of repeat RNA in RAN translation. In SCA8 models, hairpin-forming CAG repeat RNA was shown to be RAN-translated to produce polyglutamine proteins, but switching the CAG repeats to non-hairpin-forming CAA repeats abolished the RAN translation (Zu et al., 2011). In C9-ALS/FTD, G\textsubscript{4}C\textsubscript{2} repeat RNA has been reported to form both hairpin and G-quadruplex structures (Fratta et al., 2012; Hauesler et al., 2014; Reddy et al., 2013; Su et al., 2014). Although the effect of each structure on RAN translation remains largely unknown, small molecules binding to the hairpin structure or the G-quadruplex
structure were both reported to inhibit RAN translation from the G₄C₂ repeat RNA, resulting in reduced DPR levels (Wang et al., 2019, Mori et al., 2021). These findings are in accordance with our results showing that FUS modifies the G-quadruplex structure as well as the hairpin structure of G₄C₂ repeat RNA as an RNA chaperone and reduces DPR production. We further found that G-quadruplex-targeting RNA helicases, including DDX3X, DDX5, and DDX17, which are known to bind to G₄C₂ repeat RNA (Cooper-Knock et al., 2014; Haeusler et al., 2014; Mori et al., 2013a; Xu et al., 2013), also alleviate G₄C₂ repeat-induced toxicity without altering the expression levels of G₄C₂ repeat RNA in our Drosophila models. These results suggest that not only ATP-independent RNA chaperones, but also ATP-dependent RNA helicases may regulate RAN translation through modifying the higher-order structures of template repeat RNA. Consistently, a previous study also reported that DDX3X inhibits RAN translation from G₄C₂ repeat RNA in a helicase activity-dependent manner (Cheng et al., 2019). Knockdown of another RNA helicase, DHX36, has been reported to both promote (Cheng et al., 2019) and inhibit (Liu et al., 2021; Tseng et al., 2021) RAN translation, possibly due to the different effects on repeat RNA structures depending on the experimental conditions. Unfortunately, most of these studies reporting the effects of RBPs on RAN translation have limitations of the detailed structural analyses of repeat RNA. In this study, focusing on FUS, we performed a series of molecular structural analyses, in vitro translation assays, and in vivo genetic analyses to clarify the structure-function relationship of G₄C₂ repeat RNA, and provide compelling evidence for the modifying effects of FUS on repeat RNA structures leading to the suppression of RAN translation and repeat-induced toxicity in vivo.

FUS has an RRM domain for RNA binding and a low complexity (LC) domain.
involved in protein interactions, and exerts multifaceted functions, such as RNA transcription, RNA splicing, RNA transport, and formation of membraneless organelles, such as stress granules and nuclear paraspeckles via liquid-liquid phase separation (Lagier-Tourenne et al., 2010). Recent studies reported that arginine-rich DPRs, such as poly(GR) and poly(PR), interact with LC domain-containing RBPs, including FUS, and alter their liquid-liquid phase separation, resulting in the disruption of the dynamics and functions of membraneless organelles (Kwon et al., 2014; Lee et al., 2016; Lin et al., 2016). These findings raise the possibility that FUS may exert its suppressive effects by directly interacting with DPRs. However, we showed that FUS does not suppress eye degeneration in DPR-only flies (Figure 3—figure supplement 1) indicating that a direct interaction between FUS and DPRs is unlikely to be the mechanism of the suppression of DPR toxicity in our C9-ALS/FTD flies. This result supports our conclusion that FUS suppresses G4C2 repeat-induced toxicity through direct binding to G4C2 repeat RNA.

In summary, we here provided evidence that FUS modulates the structure of G4C2 repeat RNA as an RNA chaperone, and regulates RAN translation, resulting in the suppression of neurodegeneration in C9-ALS/FTD fly models. Recent advances in genome sequencing technology unveiled that such expansions of repeat sequences cause more than 50 monogenic human diseases (Malik et al., 2021), and are also associated with psychiatric diseases such as autism (Mitra et al., 2021; Trost et al., 2020). Thus, our findings contribute not only towards the elucidation of repeat-associated pathogenic mechanisms underlying a wider range of neuropsychiatric diseases than previously thought, but also towards the development of potential therapies for these diseases.
Materials and Methods

Flies

All fly stocks were cultured and crossed at 23 °C or 25 °C in standard cornmeal-yeast-glucose medium. Male adult flies were used for the climbing assay and GeneSwitch experiments. Three to 5-day-old female adult flies were used for the evaluation of eye phenotype using a stereoscopic microscope model SZX10 (Olympus). Female third instar larvae were used for quantitative real-time polymerase chain reaction (PCR), RNA FISH and immunohistochemistry experiments. The transgenic fly line bearing the GMR-Gal4 transgene has been described previously (Yamaguchi et al., 1999). The transgenic fly lines bearing elav-Gal4 (#8765), elav-GeneSwitch (#43642), UAS-EGFP (#6874), UAS-DsRed (#6282), UAS-GFP-IR (inverted repeat) (#9330), UAS-(GR)36 (#58692), UAS-(GA)36 (#58693), UAS-(GR)100 (#58696), UAS-(GA)100 (#58697), and UAS-EWSRI (#79592) were obtained from Bloomington Drosophila Stock Center. The transgenic fly line bearing UAS-caz-IR (#100291) was obtained from Vienna Drosophila Resource Center. The fly line with the caz null allele (caz\(^2\)), UAS-LDS-(G\(_4\)C\(_2\))\(_{44}\)GR-GFP, and UAS-caz (UAS-FLAG-caz) and UAS-FUS-4 (UAS-FLAG-FUS) were kind gifts from Dr. Erik Storkebaum (Frickenhaus et al., 2015), Dr. Nancy Bonini (Goodman et al., 2019), and Dr. Brian McCabe (Wang et al., 2011), respectively. Other transgenic fly lines were generated in this study. Full genotypes of the fly lines used in all figures and their cultured temperatures are described in Supplementary file 1.

Generation of constructs and transgenic flies

Artificially synthesized (G\(_4\)C\(_2\))\(_{50}\) sequences flanked at the 5′ end with an EagI recognition site and at the 3′ end with a PspOMI recognition site were subcloned into T-
vector pMD20 (Takara Bio). To generate a longer repeat size, the pMD20-(G₄C₂)₅₀ vector was digested with *Eag*I and *PspOM*I, followed by ligation into the pMD20-(G₄C₂)₅₀ vector linearized by digestion with *Eag*I. This vector was digested with *EcoRI* and *Hind*III, and subcloned into the pcDNA™3.1/myc-His(-)A vector (Thermo Fisher Scientific). We accidentally obtained the pcDNA™3.1/myc-His(-)A-(G₄C₂)₉ vector at this step. The pcDNA™3.1/myc-His(-)A-(G₄C₂)ₙ vector was digested with *EcoRI* and *Xba*I, and subcloned into the *Drosophila* pUAST vector. These constructs have no start codon sequence (ATG) upstream of the G₄C₂ repeat sequence (Figure 1—figure supplement 1A). These pUAST-(G₄C₂)ₙ vectors were amplified with a recombinase-mutated SURE®2 *Escherichia coli* strain (Agilent Technologies) at 28 °C for 72 hours to prevent repeat length contraction. The number of G₄C₂ repeats in the pUAST-(G₄C₂)₉ or ₅₀ vectors was determined by sequencing. To determine the number of G₄C₂ repeats in the pUAST-(G₄C₂)₈₉ vector, transposable element insertional mutagenesis using EZ-Tn5™ <KAN-2> Insertion Kit (Epicentre) and sequencing were performed.

To generate pUAST-*FUS* or pUAST-*TDP-43* vectors, the Gateway® Vector Conversion System (Thermo Fisher Scientific) was used. The human *FUS* or human *TARDBP* cDNA was subcloned into the pENTR™/D-TOPO® vector (Thermo Fisher Scientific). To generate the Gateway destination vector pUAST-DEST, we inserted the Gateway cassette A sequence (Thermo Fisher Scientific) into the pUAST vector. The pUAST-*FUS* or pUAST-*TDP-43* vectors were generated using Gateway recombination reactions (Thermo Fisher Scientific). The FUS RRM mutant construct (pUAST-*FUS-RRMmut*), in which leucine residues at positions 305, 341, 359, and 368 in the FUS protein were substituted to phenylalanine, was generated by PCR and the In-Fusion® Cloning system (Takara Bio). To generate the other pUASTattB-*RBP* vectors, each
cDNA encoding the RBP shown in Figure 1—source data 1 and Figure 6—source data 1 was subcloned into the pUASTattB vector (VectorBuilder). To establish transgenic flies harboring $UAS-(G_4C_2)_n$, $UAS-FUS$, $UAS-FUS-RRMmut$, and $UAS-TDP-43$, the pUAST-$(G_4C_2)_n$, pUAST-$FUS$, pUAST-$FUS$ line 2, pUAST-$FUS-RRMmut$, and pUAST-$TDP-43$ vectors, respectively, were injected into fly embryos of the $w^{1118}$ strain. To establish transgenic flies harboring the other $UAS-RBP$ constructs including $UAS-FUS$ line 3, pUASTattB-$RBP$ vectors were injected into fly embryos of the attP40 strain. These transgenic flies were established using standard methods at BestGene Inc.

The number of repeats in $UAS-(G_4C_2)_9$ or $42$ transgenic flies were determined by genomic PCR using the forward (5′-AACCAGCAACCAAGTAAATCAAC-3′), and reverse (5′-TGTTGAGAGTCAGCAGTAGCC-3′) primers, which amplifies a part of the $UAS-(G_4C_2)_n$ sequence, including G4C2 repeat sequence, followed by sequencing using the forward (5′-GCCAAGAAGTAATTATGA-3′) and/or reverse (5′-TCCAATTATGTCACACC-3′) primers.

**Quantitative real-time PCR**

Total RNA was extracted from female third instar larvae of each genotype using TRIzol® reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. First-strand cDNA was synthesized using QuantiTect Reverse Transcription Kit (QIAGEN). Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara Bio) and the Mx3000P Real-time quantitative PCR system (Agilent Technologies) or the CFX96™ Real-Time PCR Detection System (Bio-Rad). For G4C2 repeat RNA quantification of flies expressing $(G_4C_2)_n$ in Figure 1—figure supplement 1C, the forward (5′-ATGAATGGGAGCAGTGGTGG-3′) and reverse (5′-
TGTTGAGAGTCAGCAGTAGCC-3′) primers were used. For G₄C₂ repeat RNA quantification of flies expressing (G₄C₂)₈⁹(H) and FUS, FUS-RRMmut, other RNA-binding proteins, or caz-IR in Figures 1E, 3C, 4G, and 6D, the forward (5′-CCCACCACATAGACTAGATCC-3′) and reverse (5′-TGTAGGTAGTTTGTCCAATTATGTCA-3′) primers were used. Both of the above-mentioned primer pairs recognize sequences downstream of the G₄C₂ repeats. For gal4 mRNA quantification, the forward (5′-TTGAAATCGCGTCGAAGGA-3′) and reverse (5′-GGCTCCAATGGCTAATATGCA-3′) primers were used (Li et al., 2008). Data were analyzed using the standard curve method. The amounts of G₄C₂ repeat transcripts were normalized to those of gal4 transcripts in the same sample. At least three independent biological replicates per genotype were analyzed. Data were normalized by setting the values of the samples from flies expressing one copy of EGFP (Figure 4D), those expressing two (G₄C₂)₈⁹(H) and EGFP (Figures 1E, 3C, and 6D), or both (G₄C₂)₈⁹(H) and GFP-IR (Figure 4G) as 100.

Imaging and quantification of fly eyes

Light microscopic images of the eyes of 3 to 5-day-old female flies were taken using a stereoscopic microscope model SZX10 (Olympus) with a CCD camera DP21 (Olympus). Images shown are representative eye phenotypes of the fly crosses. Crosses were performed three times to validate the specific phenotypes. Eye size and pigmentation were quantified as previously reported (Saitoh et al., 2015). Five or 10 eyes per genotype were analyzed. Data were normalized by setting the values of samples from flies expressing one copy of EGFP (Figure 4D), those expressing two
copies of *EGFP* (Figures 1B, 1C, 1D, 2B, 2C, 6B, 6C, Figure 1—figure supplements 2B-2D, and Figure 4—figure supplements 1B-1D), or those expressing both *EGFP* and *GFP-IR* (Figure 4B), as 100.

**Egg-to-adult viability of flies**

Mated female flies were placed on grape juice agar with yeast paste for 24 hours. Eggs were collected from the surface of the grape juice agar, and the number of eggs were counted and placed on new standard fly food. After eclosion, the number of adult flies was counted. Egg-to-adult viability was calculated by dividing the number of adult flies by the number of eggs. More than 500 eggs per genotype were used. Data were normalized by setting the values of samples from flies expressing two copies of EGFP as 100 (Fig 2D).

**Climbing assay**

Twenty male flies were gently introduced into a glass vial. After a 5-minute adaptation period, the bottom of the vial was gently tapped and the height the flies reached in 10 seconds was recorded using a digital video camera, and scored as follows: 0 (lower than 2 cm), 1 (from 2 to 3.9 cm), 2 (from 4 to 5.9 cm), 3 (from 6 to 7.9 cm), 4 (from 8 to 9.9 cm), and 5 (higher than 10 cm). Five trials were performed in each experiment at intervals of 20 sec. The assay was performed between 8:00 and 10:00. Climbing scores were calculated as an average of five trials.

**GeneSwitch experiments**

Flies were crossed in the absence of RU486 (mifepristone) on standard fly food.
One-day old adult male flies were transferred to Formula 4-24® Instant Drosophila medium (Wako) with RU486 (100 µg/mL) for the indicated periods. Every 2 or 3 days, flies were transferred to new medium with RU486. Climbing assays were performed at 0, 7, and 14 days after the start of RU486 treatment (Figure 2E).

**RNA fluorescence in situ hybridization**

Female third instar larvae were dissected in ice-cold phosphate-buffered saline (PBS). Salivary glands were fixed with 4% paraformaldehyde (PFA) (pH 7.0) in PBS for 30 min, and incubated in 100% methanol. Fixed samples were rehydrated in 75% (v/v), 50%, and 25% ethanol in PBS, and rinsed in PBS and distilled water (DW). Samples were then treated with 0.2 N HCl/DW for 20 min at room temperature (RT), and rinsed in DW. Next, the samples were permeabilized with 0.2% Triton X-100 in PBS for 10 min, rinsed in PBS for 5 min, fixed again in 4% PFA in PBS for 20 min, then washed twice for 5 min each in PBS, and incubated twice for 15 min each in 2 mg/mL glycine/PBS. After the acetylation treatment, samples were incubated for 1 hour at 37 °C in hybridization buffer consisting of 50% formamide, 2× saline sodium citrate (SSC), 0.2 mg/mL yeast tRNA, and 0.5 mg/mL heparin. For hybridization, samples were incubated overnight at 80 °C with a 5′ end Alexa594-labeled (G₂C₄)₄ or Alexa488-labeled (C₂G₄)₄ locked nucleic acid (LNA) probe (5 nM) in hybridization buffer. These LNA probes were synthesized by GeneDesign Inc. After the hybridization, samples were washed once for 5 min in 4× SSC at 80 °C, three times for 20 min each in 2× SSC and 50% formamide at 80 °C, three times for 40 min each in 0.1× SSC at 80 °C, and once for 5 min in PBS containing 0.5% Triton X-100 (PBT) at RT. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) or 2′-(4-ethoxyphenyl)-5-(4-methyl-1-
piperazinyl)-2,5'-bi-1-H-benzimidazole, trihydrochloride (Hoechst 33342). Stained samples were mounted in SlowFade® Gold antifade reagent (Thermo Fisher Scientific) and observed under a Zeiss LSM710 or LSM880 confocal laser-scanning microscope.

After RNA FISH, samples were scanned using a Zeiss LSM710 or LSM880 confocal laser-scanning microscope along the z-axis direction. One z-stack image was taken per salivary gland using ZEN imaging software (Zeiss). RNA foci-positive nuclei in more than 30 cells per salivary gland were counted, and the percentage of nuclei containing RNA foci in the salivary gland was calculated. Ten salivary glands were analyzed for each genotype.

Immunohistochemistry

Female third instar larvae were dissected in ice-cold PBS. Eye imaginal discs and salivary glands were fixed with 4% PFA in PBS for 30 min and washed 3 times with PBT. After blocking with 5% goat serum/PBT, the samples were incubated overnight at 4 °C with a rat monoclonal anti-poly(GR) antibody (clone 5A2, MABN778, Millipore), a rabbit polyclonal anti-poly(GA) antibody (CAC-TIP-C9-P01, Cosmo Bio), a rabbit polyclonal anti-poly(GP) antibody (NBP2-25018, Novus Biologicals), or a rabbit polyclonal anti-FUS antibody (A300-302A, Bethyl Laboratories) at 1:1,000 dilution as the primary antibody. After washing 3 times with PBT, the samples were incubated with an Alexa 633-conjugated anti-rat antibody (A-21094, Thermo Fisher Scientific), or an Alexa 488-conjugated or Alexa 555-conjugated anti-rabbit antibody (A-11008 or A-21428, respectively, Thermo Fisher Scientific) at 1:500 dilution as the secondary antibody. After washing 3 times with PBT, nuclei were stained with DAPI or Hoechst 33342. Stained samples were mounted in SlowFade®.
Gold antifade reagent and observed using a Zeiss LSM710 or LSM880 confocal laser-scanning microscope.

The number of DPR aggregates in the eye discs was quantitatively measured using ZEN imaging software (Zeiss), as follows: 1) selection of photoreceptor neurons within the 13 developing ommatidia in rows 2 and 3 at the posterior end of the eye discs (Saitoh et al., 2015) by DAPI or Hoechst 33342 staining, because these ommatidia are at similar stages of development and can be easily identified; 2) counting of the number of DPR aggregates with a diameter of larger than 2 µm in the cytoplasm. Ten to 15 eye discs were analyzed for each genotype.

### Measurement of poly(GP) protein levels

The heads of 5-day-old female flies expressing both \( (G_4C_2)_{89}(H) \) and either \( EGFP, FUS, \) or \( FUS-RRMmut \) using the \( GMR-Gal4 \) driver were collected and stored at \(-80 \, ^\circ C\). Samples were prepared using a previously reported method (Tran et al., 2015). Poly(GP) levels were measured by a sandwich immunoassay that uses Meso Scale Discovery electrochemiluminescence detection technology, as described previously (Su et al., 2014). Data were normalized by setting the values of samples from flies expressing \( (G_4C_2)_{89}(H) \) and \( EGFP \) (Figure 3F) as 100.

### Western blotting

To assess the expression levels of FUS and FUS-RRMmut (Figure 2—figure supplement 1), or GR-GFP (Figures 3G–3I), 10 heads of 5-day-old female flies expressing \( FUS \) or \( FUS-RRMmut \), or both \( LDS-(G_4C_2)_{44}^{GR-GFP} \) and either \( FUS \) or \( FUS-RRMmut \) using the \( GMR-Gal4 \) driver were homogenized in 100 µL of sodium dodecyl
sulfate (SDS) sample buffer using a pestle, boiled for 5 min, and centrifuged at 10,000 × g for 3 min at 25 °C. Five μL of each supernatant were run on a 5% to 20% gradient polyacrylamide gel (Wako) and then transferred onto an Immun-Blot® polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBST) or PVDF Blocking Reagent for Can Get Signal® (TOYOBO) for 2 hours at RT, and then incubated overnight at 4 °C with a rabbit polyclonal anti-FUS antibody (A300-302A, Bethyl Laboratories), a rat monoclonal anti-poly(GR) antibody (clone 5A2, MABN778, Millipore), a Living Colors® EGFP mouse monoclonal antibody (632569, Clontech), or a mouse monoclonal anti-actin antibody (clone AC-40, A4700, Sigma-Aldrich) at 1:1,000 dilution as the primary antibody. After washing 3 times with PBST, membranes were incubated for 2 hours at RT with either HRP-conjugated anti-rat, anti-rabbit, or anti-mouse antibody (31470, 31460, or 31430, respectively, Invitrogen) at 1:5,000 dilution as the secondary antibody, washed 3 times with PBST, treated with SuperSignal™ West Dura chemiluminescent substrate (Thermo Fisher Scientific), and imaged using the LuminoGraphII imaging system (ATTO). Data were normalized by setting the average values of samples from flies expressing FUS (Figure 2—figure supplement 1B), or those expressing both LDS-(G4C2)44GR-GFP and DsRed as 100 (Figures 3H and 3I).

Filter binding assay

For preparation of FUS proteins with an N-terminal His tag (His-FUS), cDNAs of the human FUS protein (wild type or RRM mutant) from pUAST-FUS or pUAST-FUS-RRMmut were cloned into the multiple cloning site (XhoI and BamHI) of the plasmid vector pET-15b (Novagen) (Nomura et al., 2014). After transfection of the
plasmids into *E. coli* BL21 (DE3), the expression of His-FUS proteins was induced by culturing the transformed cells in the presence of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 20 °C for 20 hours. Cells were lysed by ultrasonication in PBS (pH 7.4) containing 2% (v/v) Triton X-100, 1 M NaCl, DNase I, MgSO₄, and ethylenediaminetetraacetic acid-free Complete Protease Inhibitor Cocktail (Roche Applied Science). After centrifugation at 20,000 × g for 30 min at 4 °C, the pellets were redissolved in a buffer (pH 7.0) containing 6 M guanidine hydrochloride (GdnHCl), 50 mM Tris, and 1 M NaCl. His-FUS proteins in the pellets were purified by Ni²⁺ affinity chromatography. In brief, the His-FUS proteins were mixed with Profinity IMAC Ni²⁺-charged resin (Bio-Rad) for 30 min at 20 °C. Then, FUS proteins bound to the resin were washed with wash buffer (6 M GdnHCl, 50 mM Tris, and 1 M NaCl, pH 7.0), and eluted with elution buffer (6 M GdnHCl, 50 mM Tris, 100 mM NaCl, and 250 mM imidazole, pH 7.0). For preparation of soluble, refolded FUS, FUS proteins (200 μM) in the elution buffer were diluted 20-fold with a buffer (pH 7.0) containing 50 mM Tris, 100 mM NaCl, 10% (v/v) glycerol, and 5 mM Tris (2-carboxyethyl) phosphine (buffer A), which, however, produced significant amounts of precipitate. This insoluble material was removed by centrifugation at 20,000 × g for 10 min at 4 °C, resulting in the recovery of soluble FUS proteins in the supernatant fraction. Protein concentrations were spectrosocopically determined from the absorbance at 280 nm using the following extinction coefficients: 71,630 cm⁻¹ M⁻¹ for both FUS and FUS-RRMmut.

Biotinylated RNAs were synthesized by GeneDesign Inc. Ten nM biotin-(G₄C₂)₄, 10 nM biotin-(AAAAAA)₄, or 10 nM biotin-(UUAGGG)₄ (telomeric repeat-containing RNA: TERRA) were incubated with soluble FUS proteins (5, 10, or 50 nM) in buffer A with 0.4 U/μL RNase inhibitor (RNasin Plus RNase Inhibitor, Promega).
Biotinylated (AAAAAA)$_4$ and TERRA are negative and positive controls, respectively. After an hour at RT, the mixture was filtered through a nitrocellulose membrane (PROTRAN®, 0.2 μm, Amersham Biosciences) overlaid on a nylon membrane (Hybond-N+, 0.45 μm, Schleicher & Schuell) in a 96-well slot-blot apparatus (ATTO) (Furukawa et al., 2011). After extensive washing of the membranes with buffer A, the bound RNAs were crosslinked to the membranes using ultraviolet radiation (254 nm; UV Stratalinker, Stratagene) at an energy level of 0.12 J. After blocking with 3% (w/v) BSA in tris-buffered saline with 0.1% Tween-20, the membranes were incubated with streptavidin-HRP (1:5,000; Nacalai Tesque), and the biotinylated RNAs on the membranes were detected with ImmunoStar LD reagent (Wako).

**Preparation of recombinant FUS protein**

For preparation of the FUS protein, the human FUS (WT) gene flanked at the 5′ end with an Ndel recognition site and at the 3′ end with a XhoI recognition site was amplified by PCR from pUAST-FUS. PCR fragments were digested with Ndel and XhoI. These fragments were ligated into the cloning sites of the plasmid vector pET-21b (Novagen) between Ndel and XhoI. After transfection of the plasmids into E. coli BL21 (DE3), expression of the FUS protein was induced by culturing the transformed cells in the presence of 0.5 mM IPTG at 37 °C for 6 hours. Cells were harvested by centrifugation and suspended with buffer B (10% glycerol, 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid [HEPES]-NaOH [pH 7.0], 300 mM NaCl, 1 mM dithiothreitol [DTT], 1 mM ethylenediaminetetraacetic acid [EDTA], 0.1% Tween-20, and 0.1% benzamidine hydrochloride) containing 1.5 mg/mL lysozyme, and stored for 30 min on ice. Cell lysates were sonicated, and insoluble protein was collected by
centrifugation. The pellet was solubilized in buffer C (6 M urea, 10% glycerol, 20 mM HEPES-NaOH [pH 7.0], 1 mM DTT, 1 mM EDTA, and 0.1% benzamidine hydrochloride). After centrifugation, supernatants were loaded onto a DE52 (GE Healthcare) open column. The flow-through fraction was loaded onto a CM52 (GE Healthcare) open column. The flow-through fraction of DE52 was applied to a CAPTO S column (GE Healthcare), and the flow-through fraction was collected using the ÄKTAexplorer 10S/100 system (GE Healthcare). The flow-through fraction was applied to a Mono S column (GE Healthcare). Proteins were fractionated with a 0 to 500 mM linear gradient of NaCl in buffer D (6 M urea, 10% glycerol, 20 mM HEPES-NaOH [pH 7.0], 1 mM DTT, and 1 mM EDTA) using ÄKTA explorer 10 S/100 system. The FUS fraction was eluted at 150 to 200 mM NaCl. For refolding, the eluted peak fraction was diluted fivefold using refolding buffer (900 mM arginine, 100 mM N-cyclohexyl-2-hydroxyl-3-aminopropanesulfonic acid [pH 9.5], 0.3 mM reduced glutathione, 0.03 mM oxidized glutathione, and 1 mM ZnCl$_2$), and stored overnight at RT. The solution was concentrated using a centrifugal filter (Vivaspin 6-10 kDa; GE Healthcare) to 1 to 2 mg/mL, and then dialyzed against buffer E (10% glycerol, 20 mM HEPES-NaOH [pH 6.8], 300 mM NaCl, 0.1 mM EDTA, and 10 mM β-cyclodextrin), and stored frozen at −80 °C.

Surface plasmon resonance analyses

The binding of FUS to (G$_4$C$_2$)$_4$ RNA was analyzed using a Biacore T200 instrument (GE Healthcare). (G$_4$C$_2$)$_4$ RNAs biotinylated at the 5’ end in 10 mM HEPES pH 6.8 and 500 mM MCl (M = K, Na, or Li) was injected over the streptavidin-coated surface of a sensor chip SA (GE Healthcare). The amount of immobilized RNA was as
follows: 240 resonance unit (RU) in KCl, 363 RU in NaCl, or 319 RU in LiCl buffer condition. Binding experiments were performed using the single cycle kinetics method. The running buffer used was 20 mM HEPES (pH 6.8), 1 mM MgCl₂, 0.05% Tween-20, and 150 mM KCl, NaCl, or LiCl. FUS was diluted in the running buffer and injected sequentially over the RNA-immobilized sensor surface in increasing concentrations (0.016, 0.031, 0.063, 0.13, or 0.25 µM). Sensorgrams were obtained at 25 °C, 30 µL/min flow rate, 60 seconds of contact time, and 120 seconds of dissociation time.

Circular dichroism spectroscopy

CD spectra were measured at 25 °C using a spectropolarimeter model J-820 (JASCO). (G₄C₂)₄ RNA was synthesized by GeneDesign Inc. and dissolved in 20 mM HEPES (pH 6.8), 18.75 mM NaCl, 10 mM MgCl₂, 0.625% glycerol, 0.625 mM β-cyclodextrin, and 0.0625 mM EDTA with 150 mM KCl, NaCl, or LiCl. RNA samples containing 150 mM KCl were first heated at 95 °C for 5 min and then cooled to RT to form the G-quadruplex structure. The other samples were not heated. FUS (1 µM) was added to the RNA sample (4 µM) and mixed before recording the spectrum. CD spectra were recorded at a speed of 50 nm min⁻¹ and a resolution of 1 nm, and 10 scans were averaged.

Nuclear magnetic resonance spectroscopy

All one-dimensional ¹H NMR spectral data were recorded using AVANCE III 800 MHz NMR spectrometers equipped with a TXI cryogenic probe (Bruker BioSpin) at 25 °C. (G₄C₂)₄ RNA dissolved in 20 mM HEPES (pH 6.8), 150 mM KCl, 18.75 mM NaCl, 10 mM MgCl₂, 0.625% glycerol, 0.625 mM β-cyclodextrin, and 0.0625 mM
EDTA was first heated at 95 °C for 5 min and then cooled to room temperature to form the G-quadruplex structure. The RNA (10 μM) was mixed with FUS at molar ratios (RNA:FUS) of 1:0, 1:0.2, 1:0.4, and 0:1. The samples were then prepared at a final concentration of 10% D_2O before recording their spectra. ^1H NMR data were acquired using simple single 90° hard-pulse excitations following solvent signal suppression with a jump-and-return pulse scheme. Free induction decay data (1,600 points in total) were collected by repeating the scans (2,600 times) with an interscan delay of 2.5 seconds. All NMR data were processed using Topspin 3.6 software (Bruker BioSpin).

RNA synthesis for in vitro translation

For preparation of the C9-RAN reporter plasmid, the pEF6-C9orf72 intron1-(G4C2)_80 vector was digested with HindIII and NotI to obtain the fragment C9orf72 intron1-(G4C2)_80, and subcloned into the pcDNA™5/FRT vector (Thermo Fisher Scientific). To add the T7 promoter upstream of the C9orf72 intron 1 sequence in this pcDNA™5/FRT-C9orf72 intron1-(G4C2)_80 vector, a forward primer including T7 promoter sequences with the 5′-terminal region of C9orf72 intron 1 flanked at the 5′ end with an HindIII recognition site, and a reverse primer recognizing the 3′-terminal region of C9orf72 intron 1 sequences including a BssHII recognition site were designed, and used to amplify a fragment containing C9orf72 intron 1 with a T7 promoter by PCR. Then, this fragment was subcloned into the pcDNA™5/FRT-C9orf72 intron1-(G4C2)_80 vector digested by HindIII and BssHII. In addition to the T7 promoter, the Myc tag in the GA frame downstream of (G4C2)_80 was introduced into this vector.

The reporter plasmids were linearized with XbaI. Linearized DNA was in vitro transcribed using mMESSAGE mMACHINE™ T7 Transcription Kit (Invitrogen).
according to the manufacturer’s instructions. T7 reactions were carried out at 37 °C for 2 hours, treated with TURBO™ DNaseI for 15 min at 37 °C to remove the DNA template, and then polyadenylated with *E. coli* Poly-A Polymerase (NEB) for 1 hour at 37 °C. Synthesized mRNAs were purified by LiCl precipitation. The size and quality of the synthesized mRNAs were verified on a denaturing RNA gel.

**In vitro translation assay**

mRNAs of *C9orf72* intron1-(G4C2)₈₀ with a Myc tag in the GA frame were in vitro translated with Flexi® Rabbit Reticulocyte Lysate System (Promega) according to the manufacturer’s instructions. Translation reactions were performed with 10 ng/μL mRNA and contained 30% rabbit reticulocyte lysate, 10 μM amino-acid mix minus methionine, 10 μM amino-acid mix minus leucine, 0.5 mM MgOAc, 100 mM KCl, 0.8 U/μL Murine RNAse Inhibitor (NEB), and 100 ng/μL human tRNAs purified from HeLa cells. FUS or BSA at each concentration (10, 100, and 1,000 μM) was added for translation in the lysate. Samples were incubated at 30 °C for 90 min before termination by incubation on ice. Ten μL of samples were analyzed by 13% SDS-polyacrylamide gel electrophoresis and Western blotting using a mouse monoclonal anti-Myc tag antibody (clone 4A6, Sigma-Aldrich) as the primary antibody.

**Quantification and statistical analysis**

Statistical parameters including the definitions and exact values of n (e.g., number of experiments, number of flies, number of eye imaginal discs, etc.), distributions, and deviations are stated in the figures and corresponding figure legends. Multiple comparison tests using one-way ANOVA with Tukey’s post hoc analysis were
performed for Figures 1B–1E, 2B–2D, 3B, 3C, 3E, 3F, 3H, 3I, 4B, 4D, 6B, 6C, 6D, 6F, 816
Figure 1—figure supplements 1C and 1D, Figure 1—figure supplements 2B–2D, and 817
Figure 4—figure supplements 1B–1D, multiple comparison test using two-way repeated 818
measures ANOVA with Tukey’s post hoc analysis was performed for Figure 2E, and the 819
unpaired t-test was used for Figures 4F, 4G, 4I, and Figure 2—figure supplement 1B. 820
Differences in means were considered statistically significant at $P < 0.05$. All statistical 821
analyses were performed using GraphPad Prism 8 software.

As the sample sizes used in the present study were similar to previous 823
publications (Freibaum et al., 2015; Goodman et al., 2019; Mizielska et al., 2014; Xu 824
et al., 2013), statistical analyses were performed afterwards without interim data 825
analysis. Data were not excluded, and were collected and processed randomly. Sample 826
collection and analyses for the measurement of poly(GP) protein levels were performed 827
in a double-blind manner. Data collection and analyses for other experiments were not 828
performed in a blind manner regarding the conditions of the experiments.
Acknowledgements

We thank Drs E. Storkebaum (Radboud University), N. Bonini (University of Pennsylvania), and B. McCabe (École Polytechnique Fédérale de Lausanne) for kindly providing the caz mutant, UAS-LDS-(G_4C_2)_44^{GR-GFP}, and UAS-FLAG-caz and UAS-FLAG-FUS flies, respectively. We acknowledge Bloomington Drosophila Stock Center and Vienna Drosophila Resource Center for providing various fly stocks. We thank Drs H. Imataka and K. Machida (University of Hyogo) for kindly providing human tRNAs for in vitro translation. We also thank the members of our laboratory for helpful discussions, K. Yamamoto, E. Wakisaka, T. Yamashita, and A. Sugai for their technical assistance, Dr. H. Akiko Popiel for critical reading and English editing of the manuscript, and the Center for Medical Research and Education, Graduate School of Medicine, Osaka University for supplying experimental equipment. This work was supported by Grants-in-Aid for Scientific Research on Innovative Areas (Brain Protein Aging and Dementia Control) (17H05699 to Y.N. and 17H05705 to Y. Furukawa), Transformative Research Areas (A) (Multifaceted Proteins) (20H05927 to Y.N. and K.M.), and Strategic Research Program for Brain Sciences (11013026 to Y.N.), from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; by Grants-in-Aid for Scientific Research (B) (21H02840 to Y.N. and 20H03602 to K.M.), Scientific Research (C) (15K09331 and 19K07823 to M.U., 17K07291 to A.I.), Young Scientists (A) (17H05091 to K.M.), Young Scientists (B) (25860733 to M.U.), and Challenging Exploratory Research (24659438 to Y.N. and 18K19515 to K.M.) from the Japan Society for the Promotion of Science, Japan; by a Health Labor Sciences Research Grant for Research on Development of New Drugs (H24-Soyaku-Sogo-002 to Y.N.) from the Ministry of Health, Labor and Welfare, Japan; by grants for Strategic Research.
Program for Brain Sciences (JP15dm0107026 and JP20dm0107061 to Y.N.), Practical Research Projects for Rare/Intractable Diseases (JP16ek0109018, JP19ek0109222, and JP20ek0109316 to Y.N.), and Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research] (JP19am0101072 to T.F.) from Japan Agency for Medical Research and Development, Japan; Intramural Research Grants for Neurological and Psychiatric Disorders (27-7, 27-9, 30-3, 30-9, and 3-9 to Y.N.) from the National Center of Neurology and Psychiatry; an IBC Grant (H28 to Y.N.) from the Japan Amyotrophic Lateral Sclerosis Association; grants from Takeda Science Foundation (2017 to M.U. and 2016 to K.M.); and a grant from SENSHIN Medical Research Foundation (2018 to K.M.).
References

Ash, P. E., Bieniek, K. F., Gendron, T. F., Caulfield, T., Lin, W. L., Dejesus-Hernandez, M., van Blitterswijk, M. M., Jansen-West, K., Paul, J. W., 3rd, Rademakers, R., Boylan, K. B., Dickson, D. W., and Petrucelli, L. (2013). Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. Neuron 77, 639–646. https://doi.org/10.1016/j.neuron.2013.02.004

Bajc Česnik, A., Darovic, S., Prpar Mihevc, S., Štalekar, M., Malnar, M., Motaln, H., Lee, Y. B., Mazej, J., Pohleven, J., Grosch, M., Modic, M., Fonovič, M., Turk, B., Drukker, M., Shaw, C. E., and Rogelj, B. (2019). Nuclear RNA foci from C9ORF72 expansion mutation form paraspeckle-like bodies. J Cell Sci. 132, jcs224303. https://doi.org/10.1242/jcs.224303

Boivin, M., Pfister, V., Gaucherot, A., Ruffenach, F., Negroni, L., Sellier, C., and Charlet-Berguerand, N. (2020). Reduced autophagy upon C9ORF72 loss synergizes with dipeptide repeat protein toxicity in G4C2 repeat expansion disorders. EMBO J. 39, e100574. https://doi.org/10.15252/embj.2018100574

Celona, B., Dollen, J. V., Vatsavayai, S. C., Kashima, R., Johnson, J. R., Tang, A. A., Hata, A., Miller, B. L., Huang, E. J., Krogan, N. J., Seeley, W. W., and Black, B. L. (2017). Suppression of C9orf72 RNA repeat-induced neurotoxicity by the ALS-associated RNA-binding protein Zfp106. eLife 6, e19032. https://doi.org/10.7554/eLife.19032
Cheng, W., Wang, S., Mestre, A. A., Fu, C., Makarem, A., Xian, F., Hayes, L. R., Lopez-Gonzalez, R., Drenner, K., Jiang, J., Cleveland, D. W., and Sun, S. (2018). C9ORF72 GGGGCC repeat-associated non-AUG translation is upregulated by stress through eIF2α phosphorylation. Nat. Commun. 9, 51. https://doi.org/10.1038/s41467-017-02495-z

Cheng, W., Wang, S., Zhang, Z., Morgens, D. W., Hayes, L. R., Lee, S., Portz, B., Xie, Y., Nguyen, B. V., Haney, M. S., Yan, S., Dong, D., Coyne, A. N., Yang, J., Xian, F., Cleveland, D. W., Qiu, Z., Rothstein, J. D., Shorter, J., Gao, F. B., Bassik, M.C., and Sun, S. (2019). CRISPR-Cas9 Screens Identify the RNA Helicase DDX3X as a Repressor of C9ORF72 (GGGGCC)n Repeat-Associated Non-AUG Translation. Neuron 104, 885–898.e8. https://doi.org/10.1016/j.neuron.2019.09.003

Choi, S. Y., Lopez-Gonzalez, R., Krishnan, G., Phillips, H. L., Li, A. N., Seeley, W. W., Yao, W. D., Almeida, S., and Gao, F. B. (2019). C9ORF72-ALS/FTD-associated poly(GR) binds Atp5a1 and compromises mitochondrial function in vivo. Nat. Neurosci. 22, 851–862. https://doi.org/10.1038/s41593-019-0397-0

Conlon, E. G., Lu, L., Sharma, A., Yamazaki, T., Tang, T., Shneider, N. A., and Manley, J. L. (2016). The C9ORF72 GGGGCC expansion forms RNA G-quadruplex inclusions and sequesters hnRNP H to disrupt splicing in ALS brains. eLife 5, e17820. https://doi.org/10.7554/eLife.17820
Cooper-Knock, J., Walsh, M. J., Higginbottom, A., Robin Highley, J., Dickman, M. J., Edbauer, D., Ince, P. G., Wharton, S. B., Wilson, S. A., Kirby, J., Hautbergue, G. M., and Shaw, P. J. (2014). Sequestration of multiple RNA recognition motif-containing proteins by C9orf72 repeat expansions. Brain 137, 2040–2051. https://doi.org/10.1093/brain/awu120

Daigle, J. G., Lanson, N. A., Jr, Smith, R. B., Casci, I., Maltare, A., Monaghan, J., Nichols, C. D., Kryndushkin, D., Shewmaker, F., and Pandey, U. B. (2013). RNA-binding ability of FUS regulates neurodegeneration, cytoplasmic mislocalization and incorporation into stress granules associated with FUS carrying ALS-linked mutations. Hum. Mol. Genet. 22, 1193–1205. https://doi.org/10.1093/hmg/dds526

DeJesus-Hernandez, M., Mackenzie, I. R., Boeve, B. F., Boxer, A. L., Baker, M., Rutherford, N. J., Nicholson, A. M., Finch, N. A., Flynn, H., Adamson, J., Kouri, N., Wojtas, A., Sengdy, P., Hsiung, G. Y., Karydas, A., Seeley, W. W., Josephs, K. A., Coppola, G., Geschwind, D. H., Wszolek, Z. K., Feldman, H., Knopman, D. S., Petersen, R. C., Miller, B. L., Dickson, D. W., Boylan, K. B., Graff-Radford, N. R., and Rademakers, R. (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron 72(2), 245–256. https://doi.org/10.1016/j.neuron.2011.09.011 (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron 72, 245–256.

Donnelly, C. J., Zhang, P. W., Pham, J. T., Haeusler, A. R., Mistry, N. A.,
Vidensky, S., Daley, E. L., Poth, E. M., Hoover, B., Fines, D. M., Maragakis, N., Tienari, P. J., Petrucelli, L., Traynor, B. J., Wang, J., Rigo, F., Bennett, C. F., Blackshaw, S., Sattler, R., and Rothstein, J. D. (2013). RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention. Neuron 80, 415–428. https://doi.org/10.1016/j.neuron.2013.10.015

Dumas, L., Herviou, P., Dassi, E., Cammas, A., and Millevoi, S. (2021). G-Quadruplexes in RNA Biology: Recent Advances and Future Directions. Trends Biochem. Sci. 46, 270–283. https://doi.org/10.1016/j.tibs.2020.11.001

Fratta, P., Mizielsinska, S., Nicoll, A. J., Zloh, M., Fisher, E. M., Parkinson, G., and Isaacs, A. M. (2012). C9orf72 hexanucleotide repeat associated with amyotrophic lateral sclerosis and frontotemporal dementia forms RNA G-quadruplexes. Sci. Rep. 2, 1016. https://doi.org/10.1038/srep01016

Freibaum, B. D., Lu, Y., Lopez-Gonzalez, R., Kim, N. C., Almeida, S., Lee, K. H., Badders, N., Valentine, M., Miller, B. L., Wong, P. C., Petrucelli, L., Kim, H. J., Gao, F. B., and Taylor, J. P. (2015). GGGGCC repeat expansion in C9orf72 compromises nucleocytoplasmic transport. Nature 525, 129–133. https://doi.org/10.1038/nature14974

Frickenhaus, M., Wagner, M., Mallik, M., Catinozzi, M., and Storkebaum, E. (2015). Highly efficient cell-type-specific gene inactivation reveals a key function for the Drosophila FUS homolog cabeza in neurons. Sci. Rep. 5, 9107.
A seeding reaction recapitulates intracellular formation of Sarkosyl-insoluble transactivation response element (TAR) DNA-binding protein-43 inclusions. J. Biol. Chem. 286, 18664–18672. https://doi.org/10.1074/jbc.M111.231209

Gendron, T. F., Bieniek, K. F., Zhang, Y. J., Jansen-West, K., Ash, P. E., Caulfield, T., Daughtery, L., Dunmore, J. H., Castanedes-Casey, M., Chew, J., et al. (2013). Antisense transcripts of the expanded C9ORF72 hexanucleotide repeat form nuclear RNA foci and undergo repeat-associated non-ATG translation in c9FTD/ALS. Acta Neuropathol. 126, 829–844. https://doi.org/10.1007/s00401-013-1192-8

Gendron, T. F., Bieniek, K. F., Zhang, Y. J., Jansen-West, K., Ash, P. E., Caulfield, T., Daughtery, L., Dunmore, J. H., Castanedes-Casey, M., Chew, J., Cosio, M., van Blitterswijk, M., Lee, W. C., Rademakers, R., Boylan, K. B., Dickson, D. W., and Petrucelli, L. (2017). Poly(GP) proteins are a useful pharmacodynamic marker for C9ORF72-associated amyotrophic lateral sclerosis. Sci, Transl. Med. 9, eeai7866. https://doi.org/10.1126/scitranslmed.aai7866

Gijselinck, I., Van Langenhove, T., van der Zee, J., Sleegers, K., Philtjens, S., Kleinberger, G., Janssens, J., Bettens, K., Van Cauwenberghe, C., Pereson, S., Engelborghs, S., Sieben, A., De Jonghe, P., Vandenbergh, R., Santens, P., De Bleecker, J., Maes, G., Bäumer, V., Dillen, L., Joris, G., Cuijt, I., Corsmit, E., Elinck E., Van
Dongen, J., Vermeulen, S., Van den Broeck, M., Vaerenberg, C., Mattheijssens, M.,
Peeters, K., Robberecht, W., Cras, P., Martin, J. J., De Deyn P. P., Cruts, M., and Van
Broeckhoven, C. (2012). A C9orf72 promoter repeat expansion in a Flanders-Belgian
cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral
sclerosis spectrum: a gene identification study. Lancet Neurol. 11, 54–65.

Goodman, L. D., Prudencio, M., Kramer, N. J., Martinez-Ramirez, L. F.,
Srinivasan, A. R., Lan, M., Parisi, M. J., Zhu, Y., Chew, J., Cook, C. N., Berson, A.,
Gitler, A. D., Petrucelli, L., and Bonini, N. M. (2019). Toxic expanded GGGGCC repeat
transcription is mediated by the PAF1 complex in C9orf72-associated FTD. Nat.
Neurosci. 22, 863–874. https://doi.org/10.1038/s41593-019-0396-1

Green, K. M., Glineburg, M. R., Kearse, M. G., Flores, B. N., Linsalata, A. E.,
Fedak, S. J., Goldstrohm, A. C., Barmada, S. J., and Todd, P. K. (2017). RAN
translation at C9orf72-associated repeat expansions is selectively enhanced by the
integrated stress response. Nat. Commun. 8, 2005. https://doi.org/10.1038/s41467-017-
02200-0

Haeusler, A. R., Donnelly, C. J., Periz, G., Simko, E. A., Shaw, P. G., Kim, M.
S., Maragakis, N. J., Troncoso, J. C., Pandey, A., Sattler, R., Rothstein, J. D., and Wang,
J. (2014). C9orf72 nucleotide repeat structures initiate molecular cascades of disease.
Nature 507, 195–200. https://doi.org/10.1038/nature13124

Hardin, C. C., Watson, T., Corregan, M., and Bailey, C. (1992). Cation-
dependent transition between the quadruplex and Watson-Crick hairpin forms of d(CGCG3GCG). Biochemistry 31, 833–841. https://doi.org/10.1021/bi00118a028

Hutten, S., Usluer, S., Bourgeois, B., Simonetti, F., Odeh, H. M., Fare, C. M., Czuppa, M., Hruska-Plochan, M., Hofweber, M., Polymenidou, M., Shorter, J., Edbauer, D., Madl, T., and Dormann, D. (2020). Nuclear Import Receptors Directly Bind to Arginine-Rich Dipeptide Repeat Proteins and Suppress Their Pathological Interactions. Cell Rep. 33, 108538. https://doi.org/10.1016/j.celrep.2020.108538

Ishiguro, T., Sato, N., Ueyama, M., Fujikake, N., Sellier, C., Kanegami, A., Tokuda, E., Zamiri, B., Gall-Duncan, T., Mirceta, M., Furukawa, Y., Yokota, T., Wada, K., Taylor, J. P., Pearson, C. E., Charlet-Berguerand, N., Mizusawa, H., Nagai, Y., and Ishikawa, K. (2017). Regulatory Role of RNA Chaperone TDP-43 for RNA Misfolding and Repeat-Associated Translation in SCA31. Neuron 94, 108–124.e7. https://doi.org/10.1016/j.neuron.2017.02.046

Jovičić, A., Mertens, J., Boeynaems, S., Bogaert, E., Chai, N., Yamada, S. B., Paul, J. W., 3rd, Sun, S., Herdy, J. R., Bieri, G., Kramer, N. J., Gage, F. H., Van Den Bosch, L., Robberecht, W., and Gitler, A. D. (2015). Modifiers of C9orf72 dipeptide repeat toxicity connect nucleocytoplasmic transport defects to FTD/ALS. Nat. Neurosci. 18, 1226–1229. https://doi.org/10.1038/nn.4085

Krishnan, G., Raitcheva, D., Bartlett, D., Prudencio, M., McKenna-Yasek, D., M., Douthwright, C., Oskarsson, B. E., Ladha, S., King, O. D., Barnada, S. J., Miller, T.
M., Bowser, R., Watts, J. K., Petrucelli, L., Brown, R. H., Kankel, M. W., and Gao, F. B. (2022). Poly(GR) and poly(GA) in cerebrospinal fluid as potential biomarkers for C9ORF72-ALS/FTD. Nat. Commun. 13, 2799. https://doi.org/10.1038/s41467-022-30387-4

Kanadia, R. N., Shin, J., Yuan, Y., Beattie, S. G., Wheeler, T. M., Thornton, C. A., and Swanson, M. S. (2006). Reversal of RNA missplicing and myotonia after muscleblind overexpression in a mouse poly(CUG) model for myotonic dystrophy. Proc. Natl. Acad. Sci. USA 103, 11748–11753. https://doi.org/10.1073/pnas.0604970103

Kwan, T., and Thompson, S. R. (2019). Noncanonical Translation Initiation in Eukaryotes. Cold Spring Harb. Perspect. Biol. 11, a032672. https://doi.org/10.1101/cshperspect.a032672

Kwon, I., Xiang, S., Kato, M., Wu, L., Theodoropoulos, P., Wang, T., Kim, J., Yun, J., Xie, Y., and McKnight, S. L. (2014). Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells. Science 345, 1139–1145. https://doi.org/10.1126/science.1254917

Lagier-Tourenne, C., Polymenidou, M., and Cleveland, D. W. (2010). TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. Hum. Mol. Genet. 19, R46–R64. https://doi.org/10.1093/hmg/ddq137
Lee, K. H., Zhang, P., Kim, H. J., Mitrea, D. M., Sarkar, M., Freibaum, B. D.,
Cika, J., Coughlin, M., Messing, J., Molliex, A., Maxwell, B. A., Kim, N. C., Temirov,
J., Moore, J., Kolaitis, R. M., Shaw, T. I., Bai, B., Peng, J., Kriwacki, R. W., and Taylor,
J. P. (2016). C9orf72 Dipeptide Repeats Impair the Assembly, Dynamics, and Function
of Membrane-Less Organelles. Cell 167, 774–788.e17.
https://doi.org/10.1016/j.cell.2016.10.002

Lee, Y. B., Chen, H. J., Peres, J. N., Gomez-Deza, J., Attig, J., Stalekar, M.,
Troakes, C., Nishimura, A. L., Scotter, E. L., Vance, C., Adachi, Y., Sardone, V., Miller,
J. W., Smith, B. N., Gallo, J. M., Ule, J., Hirth, F., Rogelj, B., Houart, C., and Shaw, C.
E. (2013). Hexanucleotide repeats in ALS/FTD form length-dependent RNA foci,
sequester RNA binding proteins, and are neurotoxic. Cell Rep. 5, 1178–1186.
https://doi.org/10.1016/j.celrep.2013.10.049

Lehmer, C., Oeckl, P., Weishaupt, J. H., Volk, A. E., Diehl-Schmid, J., Schroeter,
M. L., Lauer, M., Kornhuber, J., Levin, J., Fassbender, K., Landwehrmeyer, B., Schludi,
M. H., Arzberger, T., Kremmer, E., Flatley, A., Feederle, R., Steinacker, P., Weydt, P.,
Ludolph, A. C., Edbauer, D., and Otto, M. German Consortium for Frontotemporal
Lobar Degeneration (2017). Poly-GP in cerebrospinal fluid links C9orf72-associated
dipeptide repeat expression to the asymptomatic phase of ALS/FTD. EMBO Mol. Med.
9, 859–868. https://doi.org/10.15252/emmm.201607486

Li, L. B., Yu, Z., Teng, X., and Bonini, N. M. (2008). RNA toxicity is a
component of ataxin-3 degeneration in Drosophila. Nature 453, 1107–1111.
Lin, Y., Mori, E., Kato, M., Xiang, S., Wu, L., Kwon, I., and McKnight, S. L. (2016). Toxic PR Poly-Dipeptides Encoded by the C9orf72 Repeat Expansion Target LC Domain Polymers. Cell 167, 789–802.e12. https://doi.org/10.1016/j.cell.2016.10.003

Liu, H., Lu, Y. N., Paul, T., Periz, G., Banco, M. T., Ferré-D'Amare, A. R., Rothstein, J. D., Hayes, L. R., Myong, S., and Wang, J. (2021). A Helicase Unwinds Hexanucleotide Repeat RNA G-Quadruplexes and Facilitates Repeat-Associated Non-AUG Translation. J. Am. Chem. Soc. 143, 7368–7379. https://doi.org/10.1021/jacs.1c00131

Maharana, S., Wang, J., Papadopoulos, D. K., Richter, D., Pozniakovsky, A., Poser, I., Bickle, M., Rizk, S., Guillén-Boixet, J., Franzmann, T. M., Jahnel, M., Marrone, L., Chang, Y. T., Sterneckert, J., Tomancak, P., Hyman, A. A., and Alberti, S. (2018). RNA buffers the phase separation behavior of prion-like RNA binding proteins. Science 360, 918–921. https://doi.org/10.1126/science.aar7366

Malik, I., Kelley, C. P., Wang, E. T., and Todd, P. K. (2021). Molecular mechanisms underlying nucleotide repeat expansion disorders. Nat. Rev. Mol. Cell Biol. 22, 589–607. https://doi.org/10.1038/s41580-021-00382-6

Mann, J. R., Gleixner, A. M., Mauna, J. C., Gomes, E., DeChellis-Marks, M. R.,
Needham, P. G., Copley, K. E., Hurtle, B., Portz, B., Pyles, N. J., Guo, L., Calder, C. B., Wills, Z. P., Pandey, U. B., Kofler, J. K., Brodsky, J. L., Thathiah, A., Shorter, J., and Donnelly, C. J. (2019). RNA Binding Antagonizes Neurotoxic Phase Transitions of TDP-43. Neuron 102, 321–338.e8. https://doi.org/10.1016/j.neuron.2019.01.048

May, S., Hornburg, D., Schludi, M. H., Arzberger, T., Rentzsch, K., Schwenk, B. M., Grässer, F. A., Mori, K., Kremmer, E., Banzhaf-Strathmann, J., Mann, M., Meissner, F., and Edbauer, D. (2014). C9orf72 FTLD/ALS-associated Gly-Ala dipeptide repeat proteins cause neuronal toxicity and Unc119 sequestration. Acta Neuropathol. 128, 485–503. https://doi.org/10.1007/s00401-014-1329-4

Mitra, I., Huang, B., Mousavi, N., Ma, N., Lamkin, M., Yanicky, R., Shleizer-Burko, S., Lohmueller, K. E., and Gymrek, M. (2021). Patterns of de novo tandem repeat mutations and their role in autism. Nature 589, 246–250. https://doi.org/10.1038/s41586-020-03078-7

Mizielinska, S., Grönke, S., Niccoli, T., Ridler, C. E., Clayton, E. L., Devoy, A., Moens, T., Norona, F. E., Woollacott, I., Pietrzyk, J., Cleverley, K., Nicoll, A. J., Pickering-Brown, S., Dols, J., Cabecinha, M., Hendrich, O., Fratta, P., Fisher, E., Partridge, L., and Isaacs, A. M. (2014). C9orf72 repeat expansions cause neurodegeneration in Drosophila through arginine-rich proteins. Science 345, 1192–1194. https://doi.org/10.1126/science.1256800

(Mori et al., 2013a)
Mori, K., Lammich, S., Mackenzie, I. R., Forné, I., Zilow, S., Kretzschmar, H., Edbauer, D., Janssens, J., Kleinberger, G., Cruts, M., Herms, J., Neumann, M., Van Broeckhoven, C., Arzberger, T., and Haass, C. (2013). hnRNP A3 binds to GGGGCC repeats and is a constituent of p62-positive/TDP43-negative inclusions in the hippocampus of patients with C9orf72 mutations. Acta Neuropathol. 125, 413–423. https://doi.org/10.1007/s00401-013-1088-7

(Mori et al., 2013b)

Mori, K., Arzberger, T., Grässer, F. A., Gijselinck, I., May, S., Rentzsch, K., Weng, S. M., Schludi, M. H., van der Zee, J., Cruts, M., Van Broeckhoven, C., Kremmer, E., Kretzschmar, H. A., Haass, C., and Edbauer, D. (2013). Bidirectional transcripts of the expanded C9orf72 hexanucleotide repeat are translated into aggregating dipeptide repeat proteins. Acta Neuropathol. 126, 881–893. https://doi.org/10.1007/s00401-013-1189-3

(Mori et al., 2013c)

Mori, K., Weng, S. M., Arzberger, T., May, S., Rentzsch, K., Kremmer, E., Schmid, B., Kretzschmar, H. A., Cruts, M., Van Broeckhoven, C., Haass, C., and Edbauer, D. (2013). The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTLD/ALS. Science 339, 1335–1338. https://doi.org/10.1126/science.1232927

Mori, K., Gotoh, S., Yamashita, T., Uozumi, R., Kawabe, Y., Tagami, S., Kamp, F., Nuscher, B., Edbauer, D., Haass, C., Nagai, Y., and Ikeda, M. (2021). The porphyrin
TMPyP4 inhibits elongation during the noncanonical translation of the FTLD/ALS-associated GGGGCC repeat in the C9orf72 gene. J. Biol. Chem. 297, 101120. https://doi.org/10.1016/j.jbc.2021.101120

Nomura, T., Watanabe, S., Kaneko, K., Yamanaka, K., Nukina, N., and Furukawa, Y. (2014). Intranuclear aggregation of mutant FUS/TLS as a molecular pathomechanism of amyotrophic lateral sclerosis. J. Biol. Chem. 289, 1192–1202. https://doi.org/10.1074/jbc.M113.516492

Ozdilek, B. A., Thompson, V. F., Ahmed, N. S., White, C. I., Batey, R. T., and Schwartz, J. C. (2017). Intrinsically disordered RGG/RG domains mediate degenerate specificity in RNA binding. Nucleic Acids Res. 45, 7984–7996. https://doi.org/10.1093/nar/gkx460

Rajkowitsch, L., Chen, D., Stampfl, S., Semrad, K., Waldsich, C., Mayer, O., Jantsch, M. F., Konrat, R., Bläsi, U., and Schroeder, R. (2007) RNA chaperones, RNA annealers and RNA helicases. RNA Biol. 4, 118–130. https://doi.org/10.4161/rna.4.3.5445

Reddy, K., Zamiri, B., Stanley, S., Macgregor, R. B., Jr, and Pearson, C. E. (2013). The disease-associated r(GGGGCC)n repeat from the C9orf72 gene forms tract length-dependent uni- and multimolecular RNA G-quadruplex structures. J. Biol. Chem. 288, 9860–9866. https://doi.org/10.1074/jbc.C113.452532
Renton, A.E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J. R., Schymick, J. C., Laaksovirta, H., van Swieten, J. C., Myllykangas, L., Kalimo, H., Paetau, A., Abramzon, Y., Remes, A. M., Kaganovich, A., Scholz, S. W., Duckworth, J., Ding, J., Harmer, D. W., Hernandez, D. G., Johnson, J. O., Mok, K., Ryten, M., Trabzuni, D., Guerreiro, R. J., Orrell, R. W., Neal, J., Murray, A., Pearson, J., Jansen, I. E., Sondervan, D., Seelaar, H., Blake, D., Young, K., Halliwell, N., Callister, J. B., Toulson, G., Richardson, A., Gerhard, A., Snowden, J., Mann, D., Neary, D., Nalls, M. A., Peuralinna, T., Jansson, L., Isoviita, V. M., Kaivorinne, A. L., Hölttä-Vuori, M., Ikonen, E., Sulkava, R., Benatar, M., Wuu, J., Chiò, A., Restagno, G., Borghero, G., Sabatelli, M., Heckerman, D., Rogaeva, E., Zinman, L., Rothstein, J. D., Sendtner, M., Drepper, C., Eichler, E. E., Alkan, C., Abdullaev, Z., Pack, S. D., Dutra, A., Pak, E., Hardy, J., Singleton, A., Williams, N.M., Heutink, P., Pickering-Brown, S., Morris, H. R., Tienari, P. J., and Traynor, B. J. ITALSGEN Consortium (2011). A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron 72, 257–268.

Rudich, P., Snoznik, C., Watkins, S. C., Monaghan, J., Pandey, U. B., and Lamitina, S. T. (2017). Nuclear localized C9orf72-associated arginine-containing dipeptides exhibit age-dependent toxicity in C. elegans. Hum. Mol. Genet. 26, 4916–4928. https://doi.org/10.1093/hmg/ddx372

Saitoh, Y., Fujikake, N., Okamoto, Y., Popiel, H. A., Hatanaka, Y., Ueyama, M., Suzuki, M., Gaumer, S., Murata, M., Wada, K., and Nagai, Y. (2015). p62 plays a protective role in the autophagic degradation of polyglutamine protein oligomers in
polyglutamine disease model flies. J. Biol. Chem. 290, 1442–1453.

https://doi.org/10.1074/jbc.M114.590281

Simone, R., Balendra, R., Moens, T. G., Preza, E., Wilson, K. M., Heslegrave, A., Woodling, N. S., Niccoli, T., Gilbert-Jaramillo, J., Abdelkarim, S., Clayton, E. L., Clarke, M., Konrad, M. T., Nicoll, A. J., Mitchell, J. S., Calvo, A., Chio, A., Houlden, H., Polke, J. M., Ismail, M. A., Stephens, C.E., Vo, T., Farahat, A. A., Wilson, W. D., Boykin, D. W., Zetterberg, H., Partridge, L., Wray, S., Parkinson, G., Neidle, S., Patani, R., Fratta, P., and Isaacs, A. M. (2018). G-quadruplex-binding small molecules ameliorate C9orf72 FTD/ALS pathology in vitro and in vivo. EMBO Mol. Med. 10, 22–31. https://doi.org/10.15252/emmm.201707850

Shi, Y., Lin, S., Staats, K. A., Li, Y., Chang, W. H., Hung, S. T., Hendricks, E., Linares, G. R., Wang, Y., Son, E. Y., Wen, X., Kisler, K., Wilkinson, B., Menendez, L., Sugawara, T., Woolwine, P., Huang, M., Cowan, M. J., Ge, B., Koutsodendris, N., Sandor K. P., Komberg, J., Vangoor, V. R., Senthilkumar, K., Hennes, V., Seah, C., Nelson, A. R., Cheng, T. Y., Lee, S. J., August, P. R., Chen, J. A., Wisniewski, N., Hanson-Smith, V., Belgard, T. G., Zhang, A., Coba, M., Gronseich, C., Ward, M. E., van den Berg, L. H., Pasterkamp, R. J., Trotti, D., Zlokovic, B. V., and Ichida, J. K. (2018). Haploinsufficiency leads to neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons. Nat. Med. 24, 313–325. https://doi.org/10.1038/nm.4490

Sofola, O. A., Jin, P., Qin, Y., Duan, R., Liu, H., de Haro, M., Nelson, D. L., and Botas, J. (2007). RNA-binding proteins hnRNP A2/B1 and CUGBP1 suppress fragile X
CGG premutation repeat-induced neurodegeneration in a Drosophila model of FXTAS. Neuron 55, 565–571. https://doi.org/10.1016/j.neuron.2007.07.021

Sonobe, Y., Ghadge, G., Masaki, K., Sendoiel, A., Fuchs, E., and Roos, R. P. (2018). Translation of dipeptide repeat proteins from the C9ORF72 expanded repeat is associated with cellular stress. Neurobiol. Dis. 116, 155–165. https://doi.org/10.1016/j.nbd.2018.05.009

Su, Z., Zhang, Y., Gendron, T. F., Bauer, P. O., Chew, J., Yang, W. Y., Fostvedt, E., Jansen-West, K., Belzil, V. V., Desaro, P., Johnston, A., Overstreet, K., Oh, S. Y., Todd, P. K., Berry, J. D., Cudkowicz, M. E., Boeve, B. F., Dickson, D., Floeter, M. K., Traynor, B. J., Morelli, C., Ratti, A., Silani, V., Rademakers, R., Brown, R. H., Rothstein, J. D., Boylan, K. B., Petrucelli, L., and Disney, M. D. (2014). Discovery of a biomarker and lead small molecules to target r(GGGGCC)-associated defects in c9FTD/ALS. Neuron 83, 1043–1050. https://doi.org/10.1016/j.neuron.2014.07.041

Tabet, R., Schaeffer, L., Freyermuth, F., Jambeau, M., Workman, M., Lee, C. Z., Lin, C. C., Jiang, J., Jansen-West, K., Abou-Hamdan, H., Désaunay, L., Gendron, T., Petrucelli, L., Martin, F., and Lagier-Tourenne, C. (2018). CUG initiation and frameshifting enable production of dipeptide repeat proteins from ALS/FTD C9ORF72 transcripts. Nat. Commun. 9, 152. https://doi.org/10.1038/s41467-017-02643-5

Tran, H., Almeida, S., Moore, J., Gendron, T. F., Chalasani, U., Lu, Y., Du, X., Nickerson, J. A., Petrucelli, L., Weng, Z., and Gao, F. B. (2015). Differential Toxicity of
Nuclear RNA Foci versus Dipeptide Repeat Proteins in a Drosophila Model of C9ORF72 FTD/ALS. Neuron 87, 1207–1214. https://doi.org/10.1016/j.neuron.2015.09.015

Trost, B., Engchuan, W., Nguyen, C. M., Thiruvahindrapuram, B., Dolzhenko, E., Backstrom, I., Mirceta, M., Mojarad, B. A., Yin, Y., Dov, A., Chandrakumar, I., Prasolava, T., Shum, N., Hamdan, O., Pellecchia, G., Howe, J. L., Whitney, J., Klee, E. W., Baheti, S., Amaral, D. G., Anagnostou, E., Elsabbagh, M., Fernandez, B. A., Hoang, N., Lewis, S., Liu, X., Sjaarda, C., Smith, I. M., Szatmari, P., Zwaigenbaum, L., Glazer, D., Hartley, D., Stewart, A. K., Eberle, M. A., Sato, N., Pearson, C.E., Scherer, S.W., and Yuen, R. (2020). Genome-wide detection of tandem DNA repeats that are expanded in autism. Nature 586, 80–86. https://doi.org/10.1038/s41586-020-2579-z

Tseng, Y. J., Sandwith, S. N., Green, K. M., Chambers, A. E., Krans, A., Rainer, H. M., Sharlow, M. E., Reisinger, M. A., Richardson, A. E., Routh, E. D., Smaldino, M. A., Wang, Y. H., Vaughn, J. P., Todd, P. K., and Smaldino, P. J. (2021). The RNA helicase DHX36-G4R1 modulates C9orf72 GGGGCC hexanucleotide repeat-associated translation. J. Biol. Chem. 297, 100914. https://doi.org/10.1016/j.jbc.2021.100914

Waite, A. J., Bäumer, D., East, S., Neal, J., Morris, H. R., Ansorge, O., and Blake, D. J. (2014). Reduced C9orf72 protein levels in frontal cortex of amyotrophic lateral sclerosis and frontotemporal degeneration brain with the C9ORF72 hexanucleotide repeat expansion. Neurobiol. Aging 35, 1779.e5–1779.e13. https://doi.org/10.1016/j.neurobiolaging.2014.01.016
Wang, J. W., Brent, J. R., Tomlinson, A., Shneider, N. A., and McCabe, B. D. (2011). The ALS-associated proteins FUS and TDP-43 function together to affect Drosophila locomotion and life span. J. Clin. Invest. 121, 4118–4126. https://doi.org/10.1172/JCI57883

Wang, Z. F., Ursu, A., Childs-Disney, J. L., Guertler, R., Yang, W. Y., Bernat, V., Rzuczek, S. G., Fuerst, R., Zhang, Y. J., Gendron, T. F., Yildirim, I., Dwyer, B. G., Rice, J. E., Petrucelli, L., and Disney, M. D. (2019). The Hairpin Form of r(G4C2)exp in c9ALS/FTD Is Repeat-Associated Non-ATG Translated and a Target for Bioactive Small Molecules. Cell Chem. Biol. 26, 179–190.e12. https://doi.org/10.1016/j.chembiol.2018.10.018

Wen, X., Tan, W., Westergard, T., Krishnamurthy, K., Markandaiah, S. S., Shi, Y., Lin, S., Shneider, N. A., Monaghan, J., Pandey, U. B., Pasinelli, P., Ichida, J. K., and Trotti, D. (2014). Antisense proline-arginine RAN dipeptides linked to C9ORF72-ALS/FTD form toxic nuclear aggregates that initiate in vitro and in vivo neuronal death. Neuron 84, 1213–1225. https://doi.org/10.1016/j.neuron.2014.12.010

Xu, Z., Poidevin, M., Li, X., Li, Y., Shu, L., Nelson, D. L., Li, H., Hales, C. M., Gearing, M., Wingo, T. S., and Jin, P. (2013). Expanded GGGGCC repeat RNA associated with amyotrophic lateral sclerosis and frontotemporal dementia causes neurodegeneration. Proc. Natl. Acad. Sci. USA 110, 7778–7783. https://doi.org/10.1073/pnas.1219643110
Yamaguchi, M., Hirose, F., Inoue, Y. H., Shiraki, M., Hayashi, Y., Nishi, Y., and Matsukage, A. (1999). Ectopic expression of human p53 inhibits entry into S phase and induces apoptosis in the Drosophila eye imaginal disc. Oncogene 18, 6767–6775. https://doi.org/10.1038/sj.onc.1203113

Zhang, Y. J., Gendron, T. F., Grima, J. C., Sasaguri, H., Jansen-West, K., Xu, Y. F., Katzman, R. B., Gass, J., Murray, M. E., Shinohara, M., Lin, W. L., Garrett, A., Stankowski, J. N., Daugherty, L., Tong, J., Perkerson, E. A., Yue, M., Chew, J., Castanedes-Casey, M., Kurti, A., Wang, Z. S., Liesinger, A. M., Baker, J. D., Jiang, J., Lagier-Tourenne, C., Edbauer, D., Cleveland, D. W., Rademakers, R., Boylan, K. B., Bu, G., Link, C. D., Dickey, C. A., Rothstein, J. D., Dickson, D. W., Fryer, J. D., and Petrucelli, L. (2016). C9ORF72 poly(GA) aggregates sequester and impair HR23 and nucleocytoplasmic transport proteins. Nat. Neurosci. 19, 668–677. https://doi.org/10.1038/nn.4272

Zhang, Y. J., Gendron, T. F., Ebbert, M., O'Raw, A. D., Yue, M., Jansen-West, K., Zhang, X., Prudencio, M., Chew, J., Cook, C. N., Daugherty, L. M., Tong, J., Song, Y., Pickles, S. R., Castanedes-Casey, M., Kurti, A., Rademakers, R., Oskarsson, B., Dickson, D. W., Hu, W., Gitler, A. D., Fryer, J. D., and Petrucelli, L. (2018). Poly(GR) impairs protein translation and stress granule dynamics in C9orf72-associated frontotemporal dementia and amyotrophic lateral sclerosis. Nat. Med. 24, 1136–1142. https://doi.org/10.1038/s41591-018-0071-1
Zhu, Q., Jiang, J., Gendron, T. F., McAlonis-Downes, M., Jiang, L., Taylor, A., Diaz Garcia, S., Ghosh Dastidar, S., Rodriguez, M. J., King, P., Zhang, Y., La Spada, A., R., Xu, H., Petrucelli, L., Ravits, J., Da Cruz, S., Lagier-Tourenne, C., and Cleveland, D. W. (2020). Reduced C9ORF72 function exacerbates gain of toxicity from ALS/FTD-causing repeat expansion in C9orf72. Nat. Neurosci. 23, 615–624. https://doi.org/10.1038/s41593-020-0619-5

Zu, T., Gibbens, B., Doty, N. S., Gomes-Pereira, M., Huguet, A., Stone, M. D., Margolis, J., Peterson, M., Markowski, T. W., Ingram, M. A., Nan, Z., Forster, C., Low, W. C., Schoser, B., Somia, N. V., Clark, H. B., Schmechel, S., Bitterman, P. B., Gourdon, G., Swanson, M. S., Moseley, M., and Ranum, L. P. (2011). Non-ATG-initiated translation directed by microsatellite expansions. Proc. Natl. Acad. Sci. USA 108, 260–265. https://doi.org/10.1073/pnas.1013343108

Zu, T., Liu, Y., Bañez-Coronel, M., Reid, T., Pletnikova, O., Lewis, J., Miller, T., M., Harms, M. B., Falchook, A. E., Subramony, S. H., Ostrow, L. W., Rothstein, J. D., Troncoso, J. C., and Ranum, L. P. (2013). RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia. Proc. Natl. Acad. Sci. USA 110, E4968–E4977. https://doi.org/10.1073/pnas.1315438110

Zu, T., Cleary, J. D., Liu, Y., Bañez-Coronel, M., Bubenik, J. L., Ayhan, F., Ashizawa, T., Xia, G., Clark, H. B., Yachnis, A. T., Swanson, M. S., and Ranum, L. (2017). RAN Translation Regulated by Muscleblind Proteins in Myotonic Dystrophy Type 2. Neuron 95, 1292–1305.e5. https://doi.org/10.1016/j.neuron.2017.08.039
Figure legends

Figure 1. Screening for RBPs that suppress G₄C₂ repeat-induced toxicity in C9-ALS/FTD flies.

(A) Light microscopic images of the eyes in flies expressing both (G₄C₂)₄₂ or (G₄C₂)₈₉ and the indicated RBPs using the GMR-Gal4 driver. Coexpression of FUS, IGF2BP1, or hnRNPA2B suppressed eye degeneration in both (G₄C₂)₄₂ and (G₄C₂)₈₉ flies, indicated by “Suppression (strong)”. Coexpression of hnRNPR, SAFB2, SF3B3, hnRNPA1, or hnRNPL suppressed eye degeneration in either (G₄C₂)₄₂ or (G₄C₂)₈₉ flies, indicated by “Suppression (weak)” (see also Figure 1—source data 2). Scale bar: 100 μm. (B) Quantification of eye size in (G₄C₂)₈₉ flies coexpressing the indicated RBPs (n = 5). (C), (D) Quantification of eye pigmentation in (G₄C₂)₈₉ flies (C) or (G₄C₂)₄₂ flies (D) coexpressing the indicated RBPs (n = 5). (E) Expression levels of (G₄C₂)₈₉ RNA in flies expressing both (G₄C₂)₈₉ and the indicated RBPs using the GMR-Gal4 driver (five independent experiments, n = 25 flies per genotype). The (G₄C₂)₈₉(H) fly line expresses (G₄C₂)₈₉ RNA at a high level (see also Figure 1—figure supplement 1). In (B–E), data are presented as the mean ± SEM; P < 0.0001, as assessed by one-way ANOVA; n.s.: not significant, *P < 0.05, **P < 0.01, and ***P < 0.001, as assessed by Tukey’s post hoc analysis.

Figure 1–figure supplement 1. Characterization of C9-ALS/FTD flies.

(A) (G₄C₂)ₙ constructs used in this study. These constructs do not include an ATG start codon downstream of the UAS sequence, and were expressed in a tissue-specific manner using the GAL4-UAS system. (B) Light microscopic images of the eyes in flies expressing (G₄C₂)ₙ using the GMR-Gal4 driver. Scale bar: 100 μm. (C) Expression...
levels of (G₄C₂)ₙ RNAs in flies expressing (G₄C₂)ₙ using the GMR-Gal4 driver. Strong
eye degeneration with decreased eye size and loss of pigmentation was observed in
(G₄C₂)₄₂ or ₈₉ flies, but not in (G₄C₂)₉ flies. Eye degeneration was confirmed in (G₄C₂)₄₂
and two (G₄C₂)₈₉ independent fly lines. Degree of eye degeneration in two (G₄C₂)₈₉ fly
lines was expression-level dependent [(L) vs (H) in (G₄C₂)₉] (three independent
experiments, n = 15 flies per each genotype). Expression of G₄C₂ repeat RNA of the
sense transcripts but not that of the antisense transcripts was confirmed. (D) Climbing
ability at 1 day of age in flies expressing (G₄C₂)ₙ using the elav-Gal4 driver. Flies
expressing (G₄C₂)₈₉(H) in neurons showed lethality. Decreasing climbing ability was
observed in (G₄C₂)₄₂ or ₈₉ flies compared with (G₄C₂)₉ flies (five independent
experiments, n = 100 flies per each genotype). (E) FISH analyses of G₄C₂ repeat RNA
in the salivary glands of fly larvae with two copies of GMR-Gal4 and (G₄C₂)₉ or ₈₉ (red:
G₄C₂ RNA; yellow: G₂C₄ RNA; blue [DAPI]: nuclei). RNA foci formation
(arrowheads) of the sense transcripts but not that of the antisense transcripts was
confirmed. RNA foci were observed in (G₄C₂)₈₉ flies, but not in (G₄C₂)₉ flies. Scale
bars: 100 μm (low magnification) or 20 μm (high magnification). (F)
Immunohistochemical analyses of DPRs stained with anti-DPR antibodies in the eye
imaginal discs of fly larvae with two copies of GMR-Gal4 and (G₄C₂)₉ or ₈₉ (magenta:
poly(GR); orange: poly(GA); green: poly(GP); blue [DAPI]: nuclei). Expression and
cytoplasmic aggregation (arrowheads) of three DPRs in (G₄C₂)₈₉ flies, but not in (G₄C₂)₉ flies, were confirmed. Scale bars: 50 μm (low magnification), 10 μm (middle
magnification), and 5 μm (high magnification). In (B–F), L: low-expression line; H:
high-expression line. In (C) and (D), data are presented as the mean ± SEM; P < 0.0001,
as assessed by one-way ANOVA; n.s.: not significant, and ***P < 0.001, as assessed by
Figure 1—figure supplement 2. Coexpression of FUS suppresses G₄C₂ repeat-induced toxicity in flies expressing (G₄C₂)₈₉.

(A) Light microscopic images of the eyes in flies expressing both (G₄C₂)₄₂ or ₈₉ and FUS using the GMR-Gal4 driver. FUS-2 and FUS-3 are different strains from that in Figure 1. Scale bar: 100 μm. (B) Quantification of the eye size in (G₄C₂)₈₉ flies of the indicated genotypes (n = 10). (C), (D) Quantification of eye pigmentation in (G₄C₂)₈₉ flies (C) or (G₄C₂)₄₂ flies (D) of the indicated genotypes (n = 10). In (B–D), data are presented as the mean ± SEM; P < 0.0001, as assessed by one-way ANOVA; ***P < 0.001, as assessed by Tukey’s post hoc analysis.

Figure 2. FUS suppresses G₄C₂ repeat-induced toxicity via its RNA-binding activity.

(A) Light microscopic images of the eyes in flies expressing both (G₄C₂)₈₉ and either FUS or FUS-RRMmut using the GMR-Gal4 driver. Scale bar: 100 μm. (B) Quantification of eye size in the flies of the indicated genotypes (n = 10). (C) Quantification of eye pigmentation in the flies of the indicated genotypes (n = 10). (D) Egg-to-adult viability in flies expressing both (G₄C₂)₄₂ and either FUS or FUS-RRMmut using the GMR-Gal4 driver (> 500 flies per genotype). (E) Climbing ability in flies expressing both (G₄C₂)₄₂ and FUS using the elav-GeneSwitch driver (five independent experiments, n = 100 flies per each genotype). In (B–E), data are presented as the mean ± SEM. In (B) and (C), P < 0.0001, as assessed by one-way ANOVA; n.s.: not significant, and ***P < 0.001, as assessed by Tukey’s post hoc analysis. In (D), n.s.: not
significant and ***$P < 0.001$, as assessed by Tukey’s multiple comparison test using wholly significant difference. In (E), n.s.: not significant, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, as assessed by two-way repeated measures ANOVA with Tukey’s post hoc analysis.

Figure 2–figure supplement 1. Western blot analysis showing expression levels of FUS and FUS-RRMmut proteins.

(A) Western blot analysis of the FUS and FUS-RRMmut proteins in the heads of adult flies expressing EGFP, FUS or FUS-RRMmut using the GMR-Gal4 driver, with an anti-FUS antibody. The arrowhead indicates bands from the FUS and FUS-RRMmut proteins, whereas the asterisk indicates bands resulting from nonspecific antibody binding. (B) Quantification of the FUS and FUS-RRMmut proteins from the Western blot analysis in (A) (n = 3).

In (B), data are presented as the mean ± SEM; n.s.: not significant, as assessed by the unpaired $t$-test.

Figure 3. FUS suppresses RNA foci formation and RAN translation from G$_4$C$_2$ repeat RNA.

(A) FISH analyses of G$_4$C$_2$ repeat RNA in the salivary glands of fly larvae expressing both (G$_4$C$_2$)$_{89}$ and either FUS or FUS-RRMmut using two copies of the GMR-Gal4 driver (red: G$_4$C$_2$ RNA; blue [DAPI]: nuclei). Arrowheads indicate RNA foci. Scale bar: 20 μm. (B) Quantification of the number of nuclei containing RNA foci from the FISH analyses in (A) (n = 10). (C) Expression levels of (G$_4$C$_2$)$_{89}$ RNA in fly larvae expressing both (G$_4$C$_2$)$_{89}$ and either FUS or FUS-RRMmut using the GMR-Gal4 driver (10
independent experiments, n = 50 flies per each genotype). (D) Immunohistochemical analyses of DPRs stained with anti-DPR antibodies in the eye imaginal discs of fly larvae expressing both \((G_4C_2)_89\) and either FUS or FUS-RRM\text{mut} using two copies of the GMR-Gal4 driver (magenta: poly(GR); orange: poly(GA); green: poly(GP); blue [DAPI]: nuclei). Arrowheads indicate cytoplasmic aggregates. Scale bars: 20 μm (low magnification) or 5 μm (high magnification). (E) Quantification of the number of DPR aggregates from the immunohistochemical analyses in (D) \([n = 14 \text{ or } 15 \text{ (GR), or } 10 \text{ (GA or GP)}]\). (F) Immunoassay to determine poly(GP) levels in flies expressing both \((G_4C_2)_89\) and either FUS or FUS-RRM\text{mut} using the GMR-Gal4 driver (three independent experiments, n = 30 flies per each genotype). (G) Western blot analysis of the heads of adult flies expressing both LDS-\((G_4C_2)_{44}\)\text{GR-GFP} and any of DsRed, FUS or FUS-RRM\text{mut} using the GMR-Gal4 driver, using either an anti-GFP (upper panel) or anti-GR antibody (middle panel). (H and I) Quantification of GR-GFP protein levels from the Western blot analysis in (G) (nine independent experiments, n = 90 flies per each genotype). In (B), (C), (E), (F), (H), and (I), data are presented as the mean ± SEM. In (B), (E), and (F), \(P < 0.0001\), as assessed by one-way ANOVA; n.s.: not significant, \(^*P<0.05\), \(^{**}P<0.01\), and \(^{***}P<0.001\), as assessed by Tukey’s post hoc analysis. In (C), \(P = 0.452\), as assessed by one-way ANOVA; n.s.: not significant, as assessed by Tukey’s post hoc analysis. In (H), \(P = 0.0148\), as assessed by one-way ANOVA; n.s.: not significant and \(^*P<0.05\), as assessed by Tukey’s post hoc analysis. In (I), \(P = 0.0072\), as assessed by one-way ANOVA; n.s.: not significant and \(^*P<0.05\), as assessed by Tukey’s post hoc analysis.

Figure 3–figure supplement 1. Overexpression of FUS does not suppress eye
degeneration in DPR-only flies expressing DPRs translated from non-G₄C₂ RNAs.

Light microscopic images of the eyes in DPR-only flies coexpressing either the poly(GR) or poly(GA) protein, and either FUS or FUS-RRMmut using the GMR-Gal4 driver. Overexpression of FUS did not suppress the eye degeneration in flies expressing either (GR)₃₆ or (GR)₁₀₀. Overexpression of FUS also caused mild eye degeneration in flies expressing EGFP, (GA)₃₆, or (GA)₁₀₀, likely due to FUS toxicity.

Figure 4. Reduction of endogenous caz expression enhances G₄C₂ repeat-induced toxicity, RNA foci formation, and DPR aggregation.

(A) Light microscopic images of the eyes in flies expressing (G₄C₂)₈₉ using the GMR-Gal4 driver, with knockdown of caz. Scale bar: 100 μm. (B) Quantification of eye size in flies of the indicated genotypes shown in (A) (n = 10). (C) Light microscopic images of the eyes in flies expressing (G₄C₂)₈₉ using the GMR-Gal4 driver, with a hemizygous deletion of caz. Scale bar: 100 μm. (D) Quantification of eye size in the flies of the indicated genotypes shown in (C) (n = 10). (E) FISH analyses of G₄C₂ repeat RNA in the salivary glands of fly larvae expressing (G₄C₂)₈₉ using the GMR-Gal4 driver, with knockdown of caz (red: G₄C₂ RNA; blue [DAPI]: nuclei). Arrowheads indicate RNA foci. Scale bar: 20 μm. (F) Quantification of the number of nuclei containing RNA foci from the FISH analyses in (E) (n = 10). (G) Expression levels of (G₄C₂)₈₉ RNA in fly larvae expressing (G₄C₂)₈₉ using the GMR-Gal4 driver, with knockdown of caz (four independent experiments, n = 20 flies per each genotype). (H) Immunohistochemical analyses of DPRs stained with anti-DPR antibodies in the eye imaginal discs of fly larvae expressing (G₄C₂)₈₉ using two copies of the GMR-Gal4 driver, with the knockdown of caz. (magenta: poly(GR); orange: poly(GA); green: poly(GP); blue
Figure 4–figure supplement 1. Endogenous caz is a functional homologue of FUS for the suppression of G4C2 repeat-induced toxicity.

(A) Light microscopic images of the eyes in flies expressing both (G4C2)42 or 89 and either caz (FLAG-caz) or FUS-4 (FLAG-FUS) using the GMR-Gal4 driver. FUS-4 is a different strain from those used in Figure 1 and Figure 1–figure supplement 2. Scale bar: 100 μm. (B) Quantification of the eye size in (G4C2)89 flies of the indicated genotypes (n = 10). (C), (D) Quantification of eye pigmentation in (G4C2)89 flies (C) or (G4C2)42 flies (D) of the indicated genotypes (n = 10). In (B–D), data are presented as the mean ± SEM; P < 0.0001, as assessed by one-way ANOVA; ***P < 0.001, as assessed by Tukey’s post hoc analysis. In (F), (G), and (I), n.s.: not significant, *P < 0.05, **P < 0.01, and ***P < 0.001, as assessed by the unpaired t-test.

Figure 5. FUS directly binds to and modulates the G-quadruplex structure of G4C2 repeat RNA, resulting in the suppression of RAN translation in vitro

(A) Analysis of the binding of His-tagged FUS proteins to biotinylated (G4C2)4 RNA by the filter binding assay. The nitrocellulose membrane (left) traps RNA-bound FUS proteins, whereas unbound RNAs are recovered on the nylon membrane (right), and
then the RNAs trapped on each of the membranes was probed with streptavidin-
horseradish peroxidase (HRP). Biotinylated (AAAAAA)$_4$ and (UUAGGG)$_4$ were used as negative and positive controls, respectively. (B–D) CD spectra of (G$_4$C$_2$)$_4$ RNA incubated with or without FUS in the presence of 150 mM KCl (B), NaCl (C), or LiCl (D). The CD spectrum of FUS alone was subtracted from that of (G$_4$C$_2$)$_4$ RNA incubated with FUS. The original data are shown in Figure 5—figure supplements 2B-2D. (E) Schema of the template RNA containing the (G$_4$C$_2$)$_{80}$ sequence and 113 nucleotides of the 5′-flanking region of intron 1 of the human C9orf72 G$_4$C$_2$ repeat sequence. A Myc tag in the GA frame was introduced downstream of the (G$_4$C$_2$)$_{80}$ repeat sequence. (F) Western blot analysis of samples from in vitro translation using rabbit reticulocyte lysate in the presence or absence of increasing concentrations of FUS or BSA. The GA-Myc fusion protein was detected by Western blotting using the anti-Myc antibody. Quantifications are shown below each band, using the condition in the absence of each protein as a reference.

**Figure 5—figure supplement 1. FUS colocalizes with G$_4$C$_2$ RNA foci.**
Combined FISH and immunohistochemical analyses of G$_4$C$_2$ repeat RNA and FUS in the salivary glands of flies expressing both (G$_4$C$_2$)$_{89}$ and FUS using two copies of the GMR-Gal4 driver. Arrows indicate colocalization of FUS with RNA foci. Scale bar: 10 μm.

**Figure 5—figure supplement 2. FUS modulates the G-quadruplex structure of G$_4$C$_2$ repeat RNA.**
(A–C) CD spectra of (G$_4$C$_2$)$_4$ RNA incubated with or without FUS in the presence of...
1540 150 mM KCl (B), NaCl (C), or LiCl (D). CD spectra of (G₄C₂)₄ RNA alone (black),
1541 FUS alone (green), sum of (G₄C₂)₄ RNA and FUS (blue), and the spectra of their
1542 coinubation (magenta) are shown. FUS interacts with (G₄C₂)₄ RNA under KCl or NaCl
1543 buffer conditions. (D) Imino proton NMR spectra of (G₄C₂)₄ RNA incubated with
1544 increasing amounts of FUS in the presence of 150 mM KCl.
1545
1546 Figure 6. Identification of G-quadruplex-targeting RBPs that suppress G₄C₂
1547 repeat-induced toxicity in C9-ALS/FTD flies.
1548 (A) Light microscopic images of eyes in flies expressing both (G₄C₂)₈₉ and the indicated
1549 G-quadruplex-targeting RBPs using the GMR-Gal4 driver. Scale bar: 100 µm. (B)
1550 Quantification of eye size in the flies of the indicated genotypes (n = 10). (C)
1551 Quantification of eye pigmentation in the flies of the indicated genotypes (n = 10). (D)
1552 Expression levels of (G₄C₂)₈₉ RNA in flies expressing both (G₄C₂)₈₉ and the indicated
1553 G-quadruplex-targeting RBPs using the GMR-Gal4 driver (five independent
1554 experiments, n = 25 flies per each genotype). In (B–D), data are presented as the mean ±
1555 SEM; P < 0.0001, as assessed by one-way ANOVA; n.s.: not significant, *P < 0.05, and
1556 ***P < 0.001, as assessed by Tukey’s post hoc analysis.
### Tables

**Table 1.** Association \( (k_a) \) and dissociation \( (k_d) \) rate and dissociation constants \( (K_D) \) between FUS and \((G_4C_2)_4\) RNA in different buffers as assessed by SPR analysis.

| Buffer | \( k_a \) \( (M^{-1}s^{-1}) \times 10^6 \) | \( k_d \) \( (s^{-1}) \times 10^{-3} \) | \( K_D \) \( (M) \) |
|--------|---------------------------------|---------------------------------|-----------------|
| KCl    | 1.4                             | 22                              | \( 1.5 \times 10^{-8} \) |
| NaCl   | 0.41                            | 54                              | \( 1.3 \times 10^{-7} \) |
| LiCl   | 0.0018                          | 25                              | \( 1.4 \times 10^{-5} \) |
Source data files and supplementary files list

Figure 1—source data 1. RNA-binding proteins and their cDNA accession numbers screened in the genetic analyses in Figure 1.

Figure 1—source data 2. Summary of the genetic analyses in Figure 1.

Figure 1—source data 3. Statistical data related to Figures 1B, 1C, 1D, and 1E.

Figure 1—figure supplement 1—source data 1. Statistical data related to Figure 1—
figure supplements 1C and 1D

Figure 1—figure supplement 2—source data 1. Statistical data related to Figure 1—
figure supplement 2B, 2C and 2D

Figure 2—source data 1. Statistical data related to Figures 2B, 2C, 2D, and 2E.

Figure 2—figure supplement 1—source data 1. Statistical data related to Figure 2—
figure supplement 1B

Figure 3—source data 1. Statistical data related to Figures 3B, 3C, 3E, 3F, 3H, and 3H

Figure 3—source data 2. Source data related to Figure 3G
Figure 4—source data 1. Statistical data related to Figures 4B, 4D, 4F, 4G and 4I.

Figure 4—figure supplement 1—source data 1. Statistical data related to Figure 4—figure supplements 1B, 1C, and 1D

Figure 5—source data 1. Source data related to Figure 5F

Figure 6—source data 1. RNA-binding proteins and their cDNA accession numbers screened in the genetic analyses in Figure 6.

Figure 6—source data 2. Statistical data related to Figures 6B, 6C, and 6D.

Supplementary file 1. Full genotypes of the fly lines and their cultured temperatures.
A

EGFP  caz  FUS-4

\((G_4C_2)_{89}(H)\)

\((G_4C_2)_{42}\)

EGFP

B

\begin{align*}
\text{Eye size} & \\
\hline
\text{EGFP, EGFP} & \text{EGFP, caz} & \text{EGFP, FUS-4} & \text{EGFP, FUS-4} & \text{EGFP, FUS-4} \\
\end{align*}

C

\begin{align*}
\text{Eye pigmentation} & \\
\hline
\text{EGFP, EGFP} & \text{EGFP, caz} & \text{EGFP, FUS-4} & \text{EGFP, FUS-4} & \text{EGFP, FUS-4} \\
\end{align*}

D

\begin{align*}
\text{Eye pigmentation} & \\
\hline
\text{EGFP, EGFP} & \text{EGFP, caz} & \text{EGFP, FUS-4} & \text{EGFP, FUS-4} & \text{EGFP, FUS-4} \\
\end{align*}
GMR×2 > (G₄C₂)₈₉(H), FUS
