RESEARCH ARTICLE

Figures and figure supplements

Antisense, but not sense, repeat expanded RNAs activate PKR/eIF2α-dependent ISR in *C9ORF72* FTD/ALS

Janani Parameswaran *et al*.

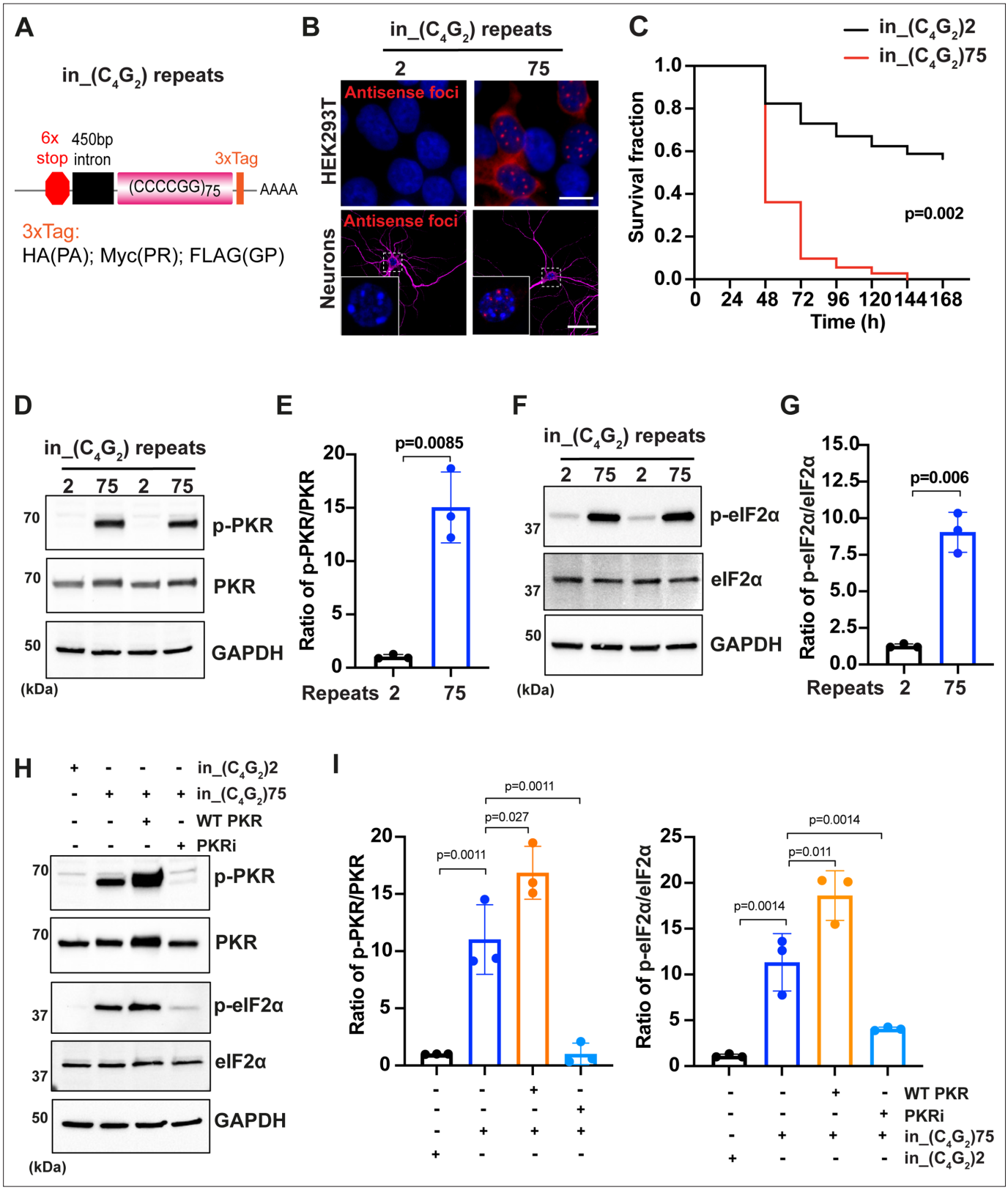

Figure 1. *C9ORF72* antisense C_4G_2 expanded repeats activate PKR/eIF2α-dependent integrated stress response and cause neuronal toxicity. (**A**) Schematic illustration of the in_(C_4G_2)75 repeat construct including 6× stop codons, 450 bp of human intronic sequences at the N-terminus and 3× protein tags at the C- terminus of the repeats to monitor the DPR proteins in each frame. (**B**) Representative images of antisense RNA foci in HEK293T cells and in primary cortical neurons expressing in_(C_4G_2)75 detected by RNA FISH. Red, foci; blue, DAPI; magenta, MAP2. (**C**) Kaplan–Meier curves

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showing increased risk of cell death in in_(C_4G_2)75-expressing primary cortical neurons compared with neurons expressing 2 repeats. Statistical analyses were performed using Mantel–Cox test (replicated three times with similar results). (**D, E**) Immunoblotting analysis of phosphorylated PKR (p-PKR) and total PKR in HEK293T cells expressing in_(C_4G_2)75 or 2 repeats. p-PKR levels were detected using anti-p-PKR (T446) and normalized to total PKR. GAPDH was used as a loading control. Error bars represent SD (n = 3 independent experiments). Statistical analyses were performed using Student's *t*-test. (**F, G**) Immunoblotting analysis of phosphorylated eIF2α (p-eIF2α) and total eIF2α in HEK293T cells expressing in_(C_4G_2)75 or 2 repeats. p-eIF2α levels were detected using anti-phosphor eIF2α (Ser51) and normalized to total eIF2α. GAPDH was used as a loading control. Error bars represent SD (n = 3 independent experiments). Statistical analyses were performed using Student's *t*-test. (**H, I**) Immunoblotting analysis of p-PKR and p-eIF2α in HEK293T cells expressing in_(C_4G_2)75, with or without co-expression of wild type PKR, or treatment of a PKR inhibitor, C16. Error bars represent SD (n = 3 independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test. Scale bars: 10 μm (neurons), 20 μm (HEK293T).

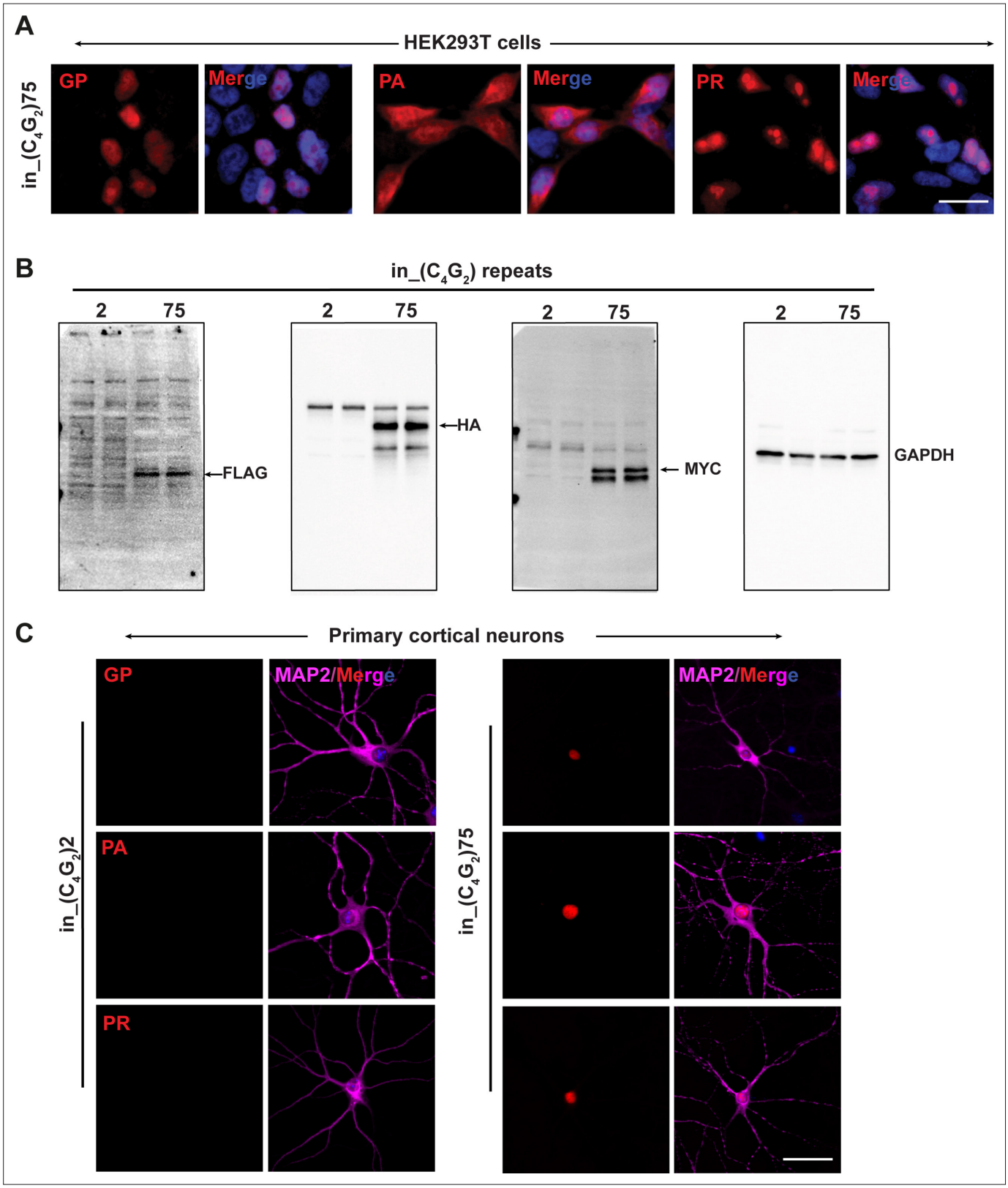

Figure 1—figure supplement 1. *C9ORF72* C_4G_2 repeat expanded repeats produce antisense DPR proteins in HEK293T cells and primary neurons. (**A**) Representative images of DPR protein staining in HEK293T expressing in_(C_4G_2)75 repeats using DPR antibodies. Red, GP, PA, and PR; blue, DAPI. (**B**) Immunoblotting of DPR proteins in HEK293T expressing in_(C_4G_2)75 repeats using TAG antibodies. GAPDH was used as a loading control. (**C**) Representative images of DPR protein staining in primary neurons expressing in_(C_4G_2)75 repeats using DPR antibodies. Red, GP, PA, and PR; blue, DAPI; MAP2, magenta. Scale bars, 10 μm (neurons), 20 μm (HEK293T).

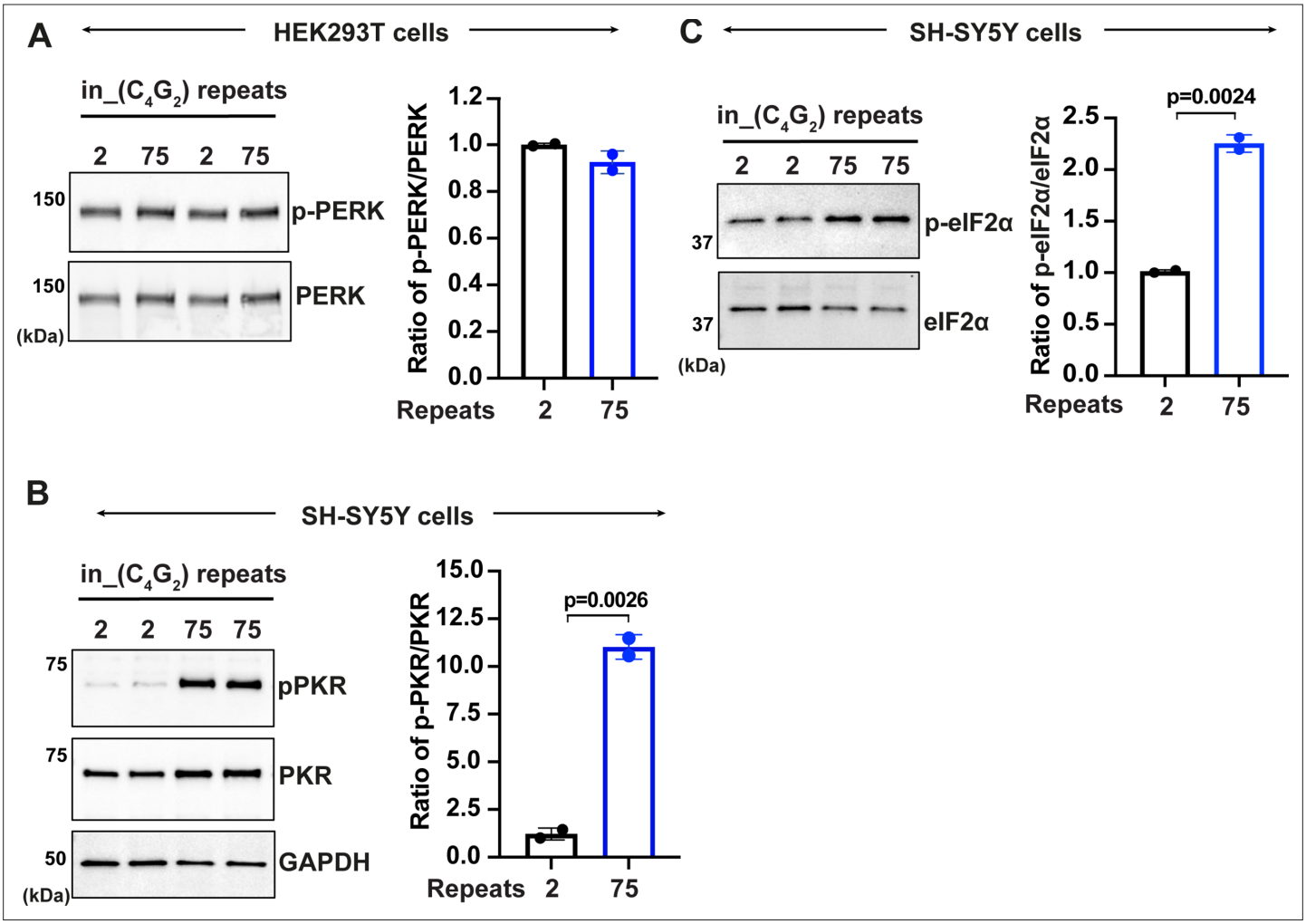

Figure 1—figure supplement 2. *C9ORF72* C_4G_2 repeat expanded repeats activate PKR/eIF2α-dependent integrated stress response in SH-SY5Y cells. (**A**) Immunoblotting of p-PERK in HEK293T cells expressing in_(C_4G_2)75 or (C_4G_2)2 repeats. Phosphorylated PERK levels were quantified and normalized to total PERK. GAPDH was used as a loading control. Error bars represent SD (n = 2 independent experiments). Statistical analyses were performed using Student's *t*-test. (**B, C**) Immunoblotting of p-PKR and p-eIF2α in SH-SY5Y cells expressing (C_4G_2)75 or (C_4G_2)2 repeats. p-PKR (T446) and p-eIF2α (Ser51) were normalized to total PKR and eIF2α, respectively. GAPDH was used as a loading control. Error bars represent SD (n = 2 independent experiments). Statistical analyses were performed using Student's *t*-test.

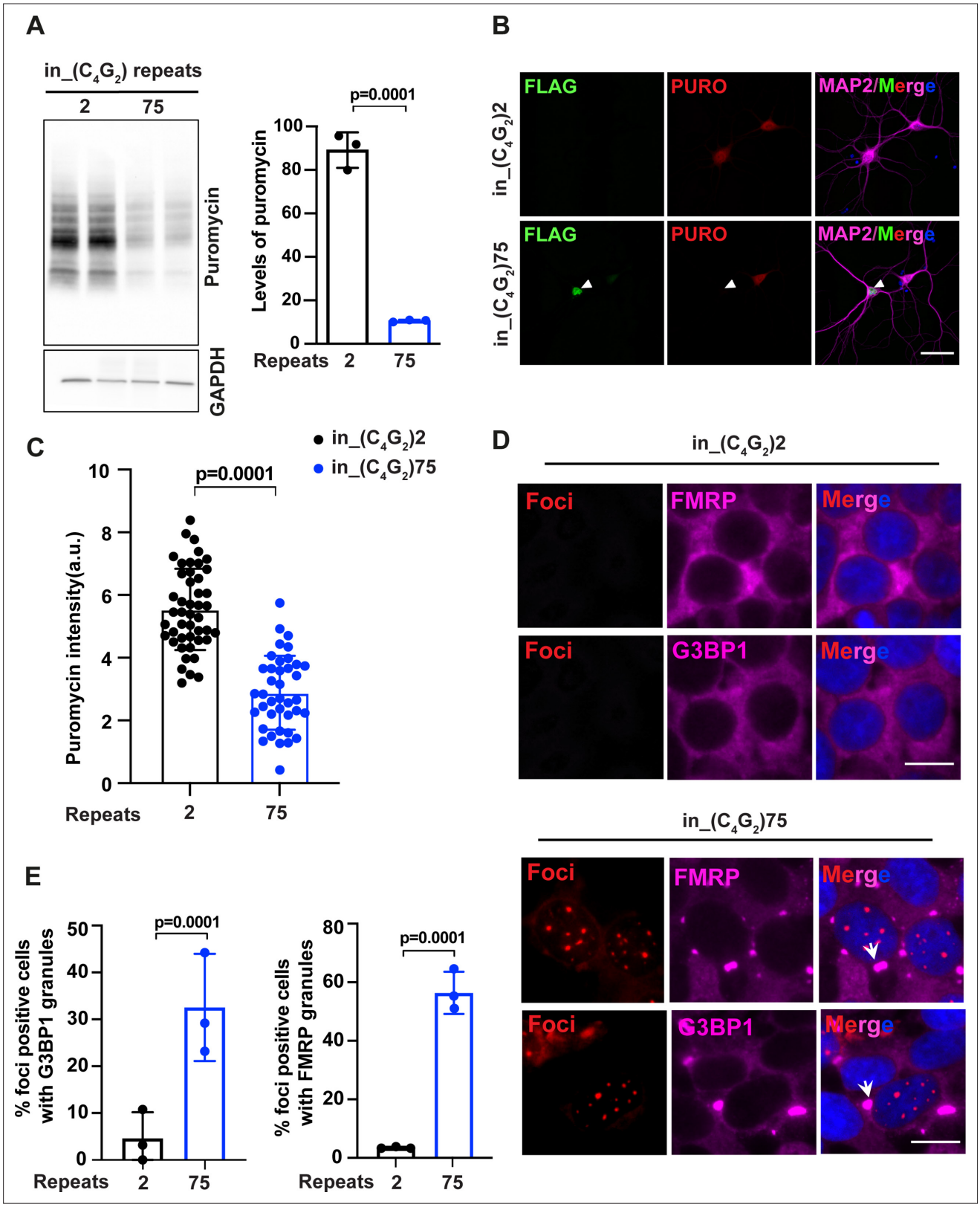

Figure 2. *C9ORF72* antisense C_4G_2 expanded repeats inhibit global protein synthesis and induce stress granule assembly. (**A**) Immunoblotting of puromycin in HEK293T cells expressing in_(C_4G_2)75 or 2 repeats. Cells were incubated with puromycin for 30 min before harvesting. The level of puromycin was normalized to GAPDH. Error bars represent SD (n = 3 independent experiments). Statistical analyses were performed using Student's *t*-test (**B**) Representative images and (**C**) quantification of primary neurons expressing either (C_4G_2)75 or 2 repeats stained with anti-puromycin (red),

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anti-FLAG (green), DAPI (blue), and MAP2 (magenta). The puromycin intensity was quantified using ImageJ. Error bars represent SD (n = 40–50 neurons/group; similar results were obtained from two independent experiments). Statistical analyses were performed using Student's *t*-test. (**D**) Representative images of G3BP1 and FMRP staining in HEK293T cells expressing in_(C_4G_2)75 and in_(C_4G_2)2 repeats. (**E**) Quantification of antisense foci-positive cells with G3BP1 and FMRP granules. Error bars represent SD (n = 150 cells/condition and three independent experiments). Statistical analyses were performed using Student's *t*-test. Scale bars, 10 μm (neurons), 20 μm (HEK293T).

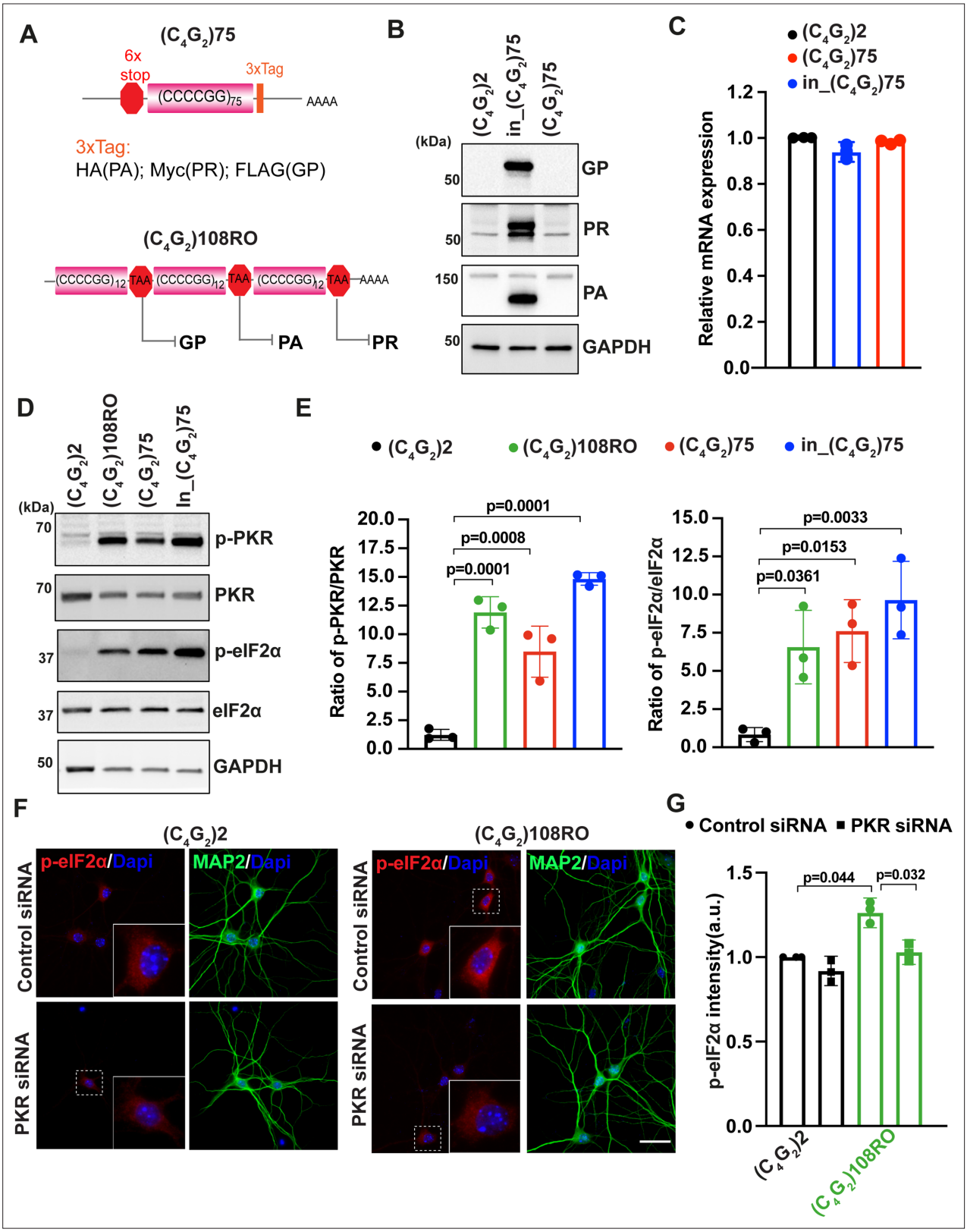

Figure 3. Antisense C_4G_2 repeat expanded RNAs activate the PKR/eIF2α pathway independent of DPR proteins. (**A**) (Top) Schematic illustration of $(C_4G_2)75$ repeats without the human intronic sequences. 3× protein tags were included at the C- terminus of the repeats to monitor the DPR proteins in each frame. (Bottom) Schematic illustration of antisense $(C_4G_2)108RO$ repeats with stop codons inserted in every 12 repeats to prevent the translation of DPR proteins from all reading frames. (**B**) Immunoblotting of DPR proteins in HEK293T cells expressing in_$(C_4G_2)75$, $(C_4G_2)75$, or 2 repeats. DPR

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protein levels were detected using anti-FLAG (frame with GP), anti-MYC (frame with PR), and anti-HA (frame with PA). GAPDH was used as a loading control. (**C**) mRNA levels were measured by quantitative qPCR in cell expressing in_(C_4G_2)75, (C_4G_2)75, or 2 repeats. Error bars represent SD (n = 3). (**D, E**) Immunoblotting of p-PKR and p-eIF2α in HEK293T cells expressing in_(C_4G_2)75, (C_4G_2)75, (C_4G_2)108RO, or 2 repeats. p-PKR (T446) and p-eIF2α (Ser51) were normalized to total PKR and eIF2α, respectively. GAPDH was used as a loading control. Error bars represent SD (n = 3 independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test. (**F, G**) Representative images (**F**) and quantitation (**G**) of p-eIF2α in primary neurons expressing antisense (C_4G_2)108RO or 2 repeats in the presence and absence of PKR siRNA. Scale bars, 10 μm.

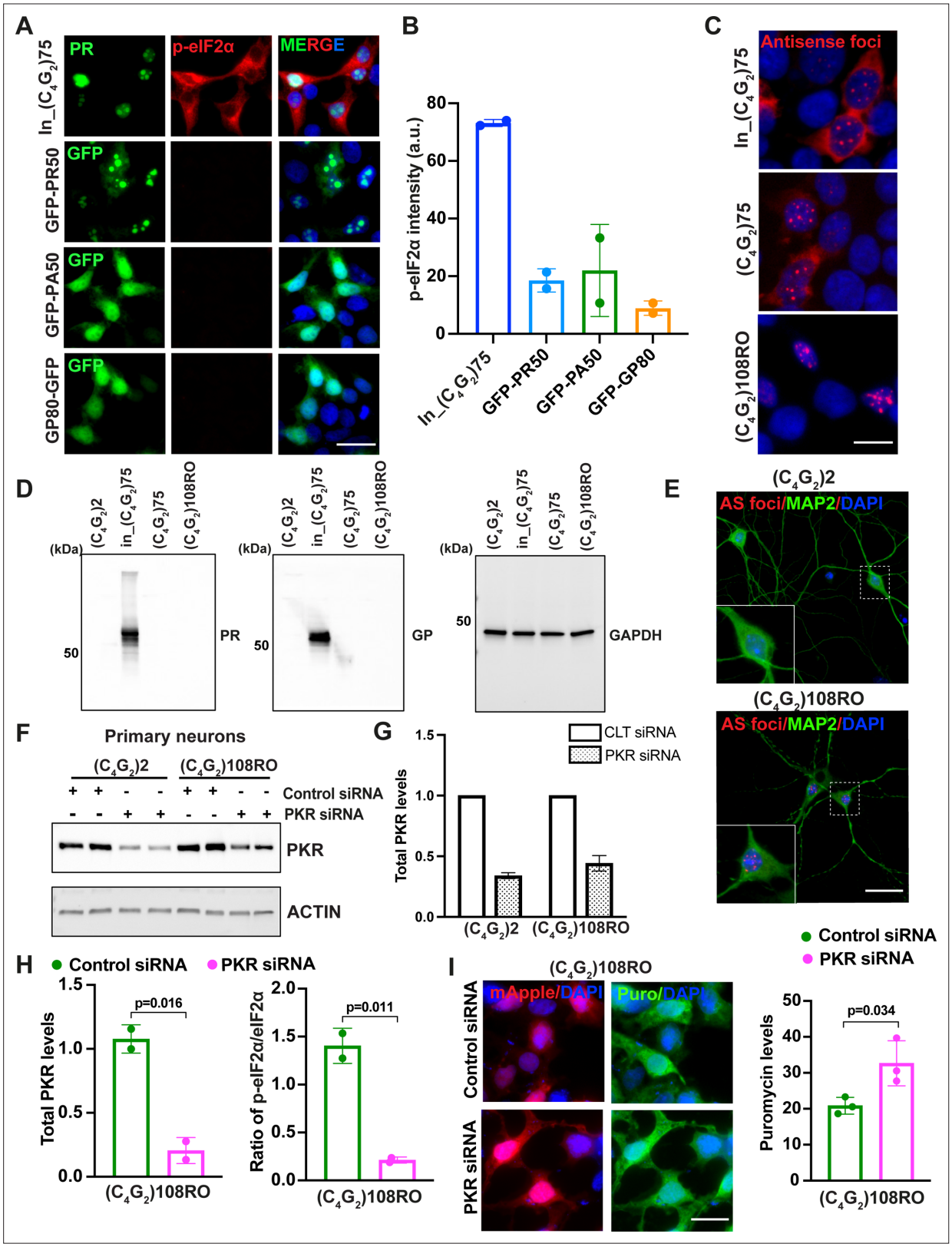

Figure 3—figure supplement 1. Antisense DPR proteins do not activate PKR/eIF2α-dependent integrated stress response. (**A**) Representative images and (**B**) quantification of p-eIF2α staining in HEK293T cells expressing in_(C_4G_2)75, PR50, PA50, or GP80. Green, GP, PA, or PR; red, p-eIF2α; blue, DAPI. Error bars represent SD (n = 2 independent experiments). (**C**) Representative images of antisense RNA foci in HEK293T cells expressing in_(C_4G_2)75, (C_4G_2)75 or (C_4G_2)108RO repeats. Foci were detected by RNA FISH. Red, foci; blue, DAPI. (**D**) Immunoblotting of DPR proteins in HEK293T cells

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expressing in_(C_4G_2)75, (C_4G_2)75 and (C_4G_2)108RO repeats. DPR protein levels were detected using anti-PR and anti-GP antibodies. GAPDH was used as a loading control. (**E**) Representative images of antisense RNA foci in primary cortical neurons expressing (C_4G_2)108 detected by RNA FISH. Red, foci; blue, DAPI; Green, MAP2. (**F**) Immunoblotting and (**G**) quantification of PKR in primary neurons expressing (C_4G_2)2 and (C_4G_2)108RO together with control or PKR siRNA. PKR were normalized to GADPH. Error bars represent SD. (**H**) The levels of PKR and p-eIF2α (Ser51) in HEK293T cells expressing (C_4G_2)108RO together with control or PKR siRNA. PKR and p-eIF2α (Ser51) were normalized to GADPH and total eIF2α, respectively. Error bars represent SD (n = 2 independent experiments). Statistical analyses were performed using Student's *t*-test. (**I**) Representative images and quantification of puromycin staining in HEK293T cells expressing (C_4G_2)108RO together with control or PKR siRNA. Error bars represent SD (n = 3 independent experiments). mApple was co-transfected to identify cells with (C_4G_2)108RO expression. Statistical analyses were performed using Student's *t*-test. Scale bars, 20 μm (HEK293T), 10 μm (neurons).

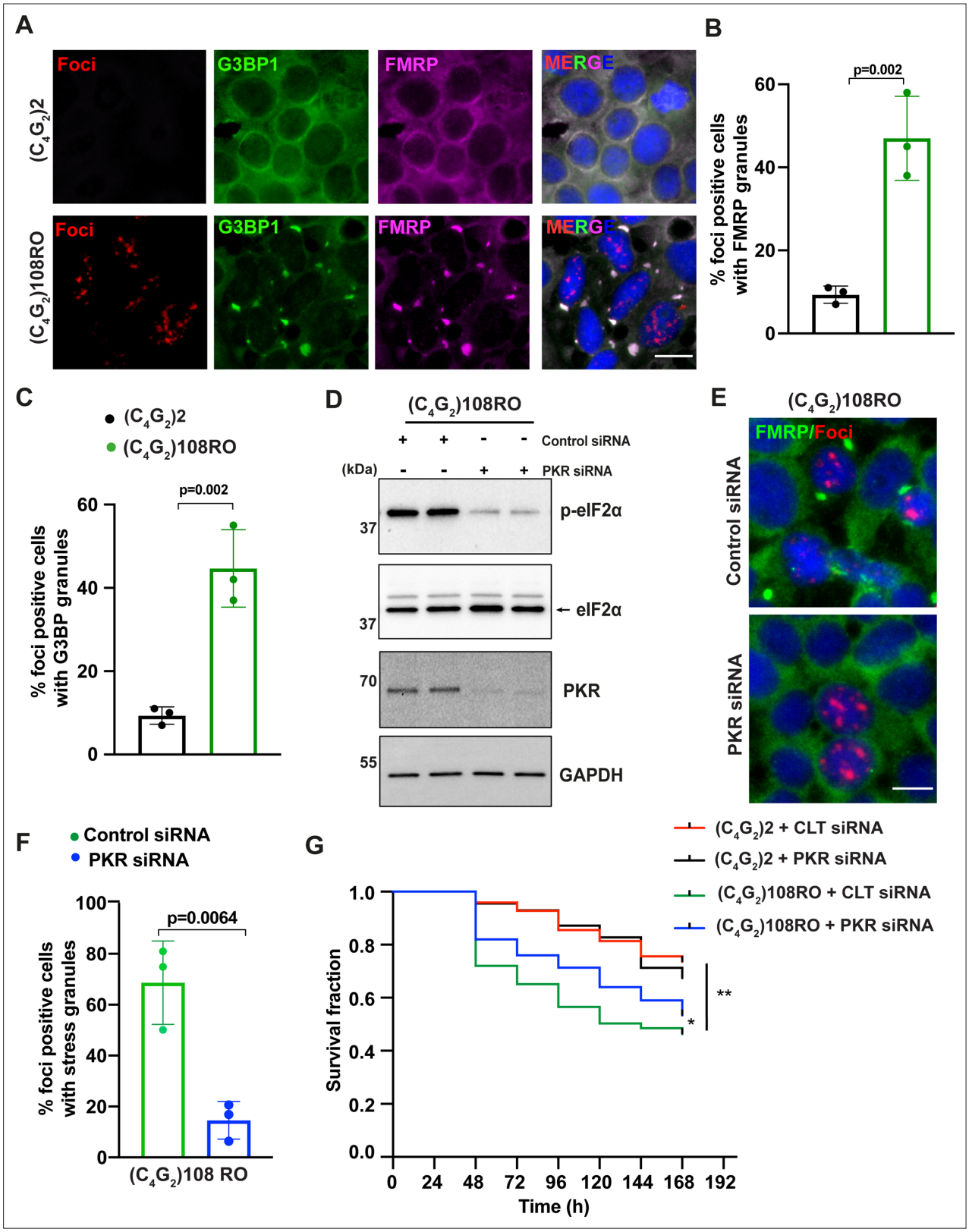

Figure 4. Antisense C_4G_2 repeat expanded RNAs themselves induce stress granules and lead to neuronal toxicity. (**A**) Representative images of FMRP and G3BP1 staining in HEK293T cells expressing (C_4G_2)108RO or 2 repeats. (**B, C**) Quantification of antisense foci-positive cells with FMRP and G3BP1 granules. Error bars represent SD (n = 180 cells/condition and three independent experiments). Statistical analyses were performed using Student's *t*-test. (**D**) Immunoblotting of PKR and p-eIF2α (Ser51) in HEK293T cells expressing (C_4G_2)108RO together with control or PKR siRNA. GAPDH was used

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as a loading control. (**E**) Representative images of FMRP staining in HEK293T cells expressing (C_4G_2)108 repeats together with either control or PKR siRNA. (**F**) Quantification of antisense foci-positive cells with FMRP granules. Error bars represent SD (n = 150 cells/condition and three independent experiments). (**F**) Kaplan–Meier curves showing the risk of cell death in (C_4G_2)108RO-expressing neurons compared with 2 repeats in the presence and absence of PKR siRNA (replicated three times with similar results). Statistical analyses were performed using Mantel–Cox test (*p<0.05, **p<0.01). Scale bars, 20 μm.

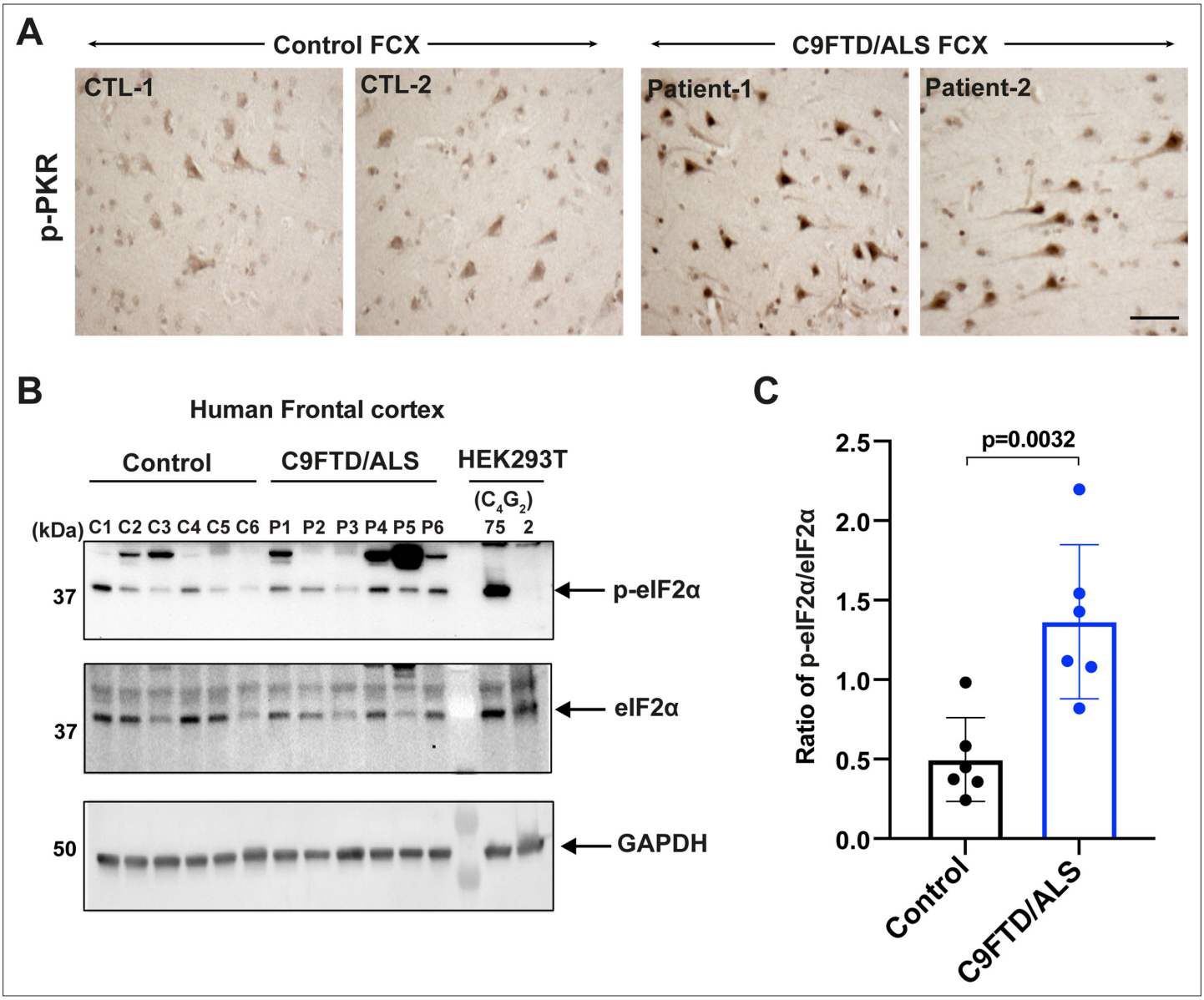

Figure 5. Increased levels of phosphorylated PKR and eIF2α in C9FTD/ALS patients. (**A**) Representative immunohistochemistry images of phosphorylated PKR staining in control and C9FTD/ALS patient's frontal cortex (FCX) using anti-p-PKR (T446) (n = 4 per genotype). (**B, C**) Immunoblotting of p-eIF2α in proteins extracted from control (C1–C6) and C9FTD/ALS patient's frontal cortex (P1–P6). p-eIF2α (Ser51) was normalized to total eIF2α. GAPDH was used as a loading control. Error bars represent SD (control n = 6 and C9FTD/ALS n = 6). Statistical analyses were performed using unpaired Student's *t*-test. Scale bars, 10 μm.

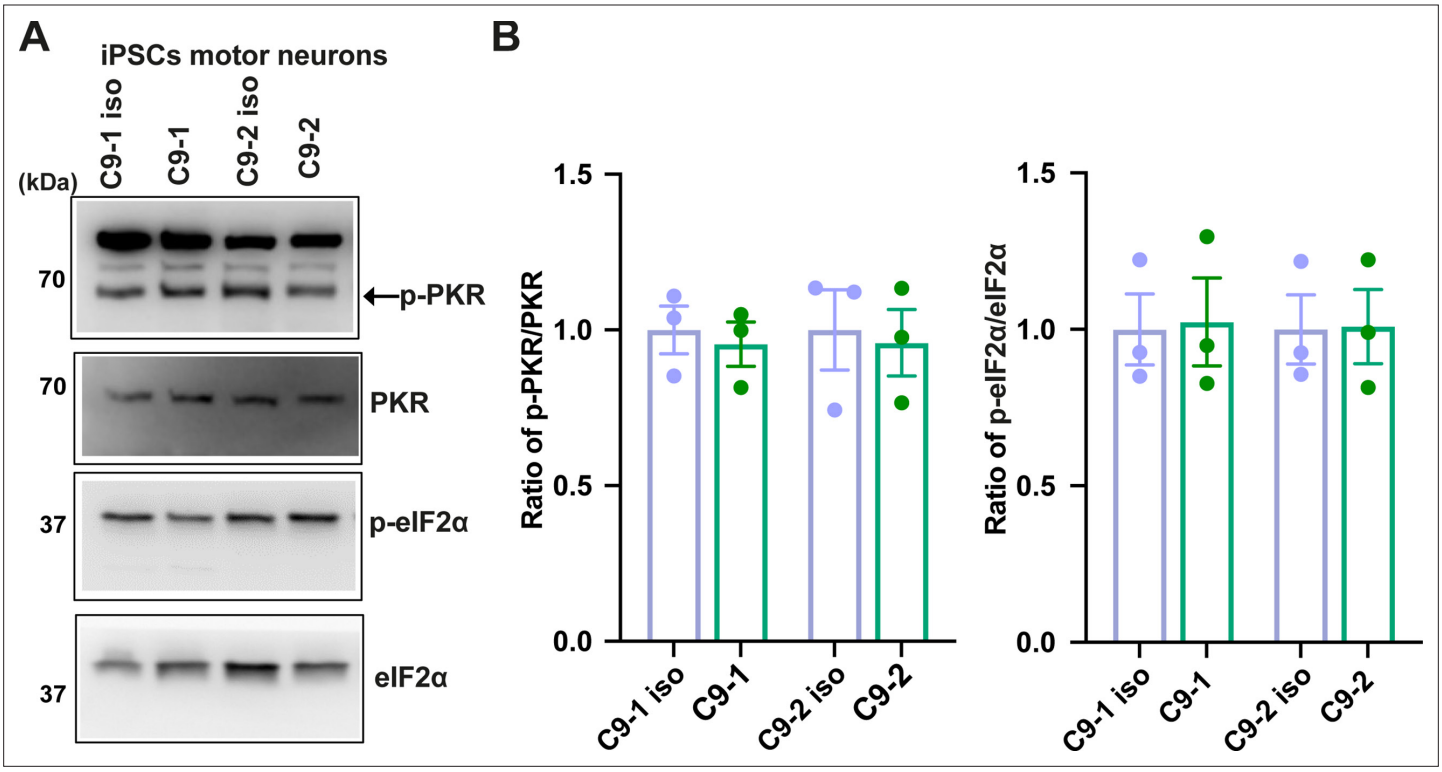

Figure 5—figure supplement 1. Phosphorylated PKR and eIF2α levels are unchanged in C9FTD/ALS iPSC-derived motor neurons. (**A**) Immunoblotting and (**B**) quantification of p-PKR and p-eIF2α in two different lines of iPSC-derived motor neurons of C9FTD/ALS patient and their isogenic controls (38 d post differentiation). P-PKR levels were detected using anti-p-PKR (T446) and normalized to total PKR. P-eIF2α levels were detected using anti-phosphor eIF2α (Ser51) and normalized to total eIF2α. Error bars represent SD (n = 3).

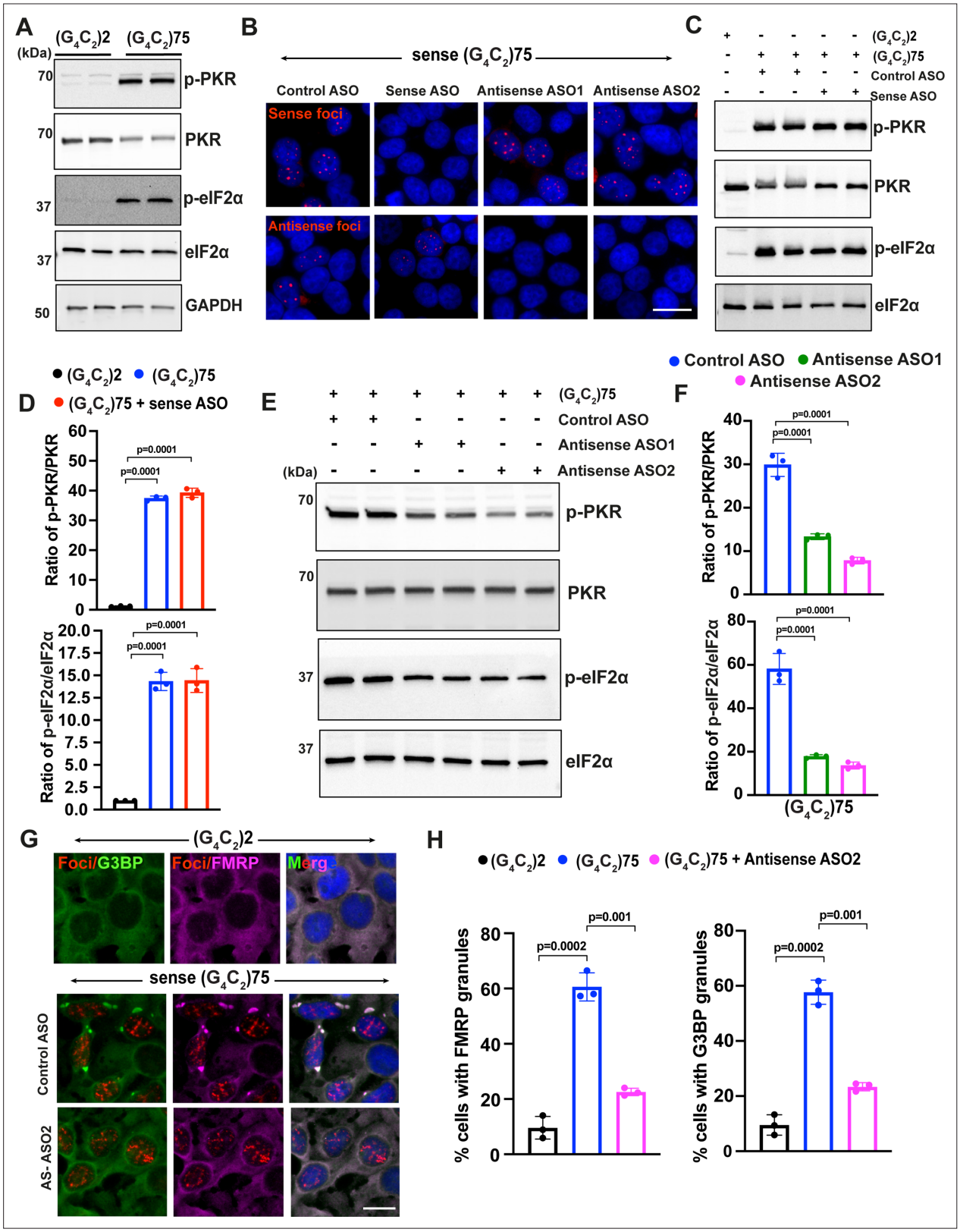

Figure 6. Sense G_4C_2 repeat expanded RNAs cannot activate the PKR/eIF2α pathway. (**A**) Immunoblotting of p-PKR and p-eIF2α in HEK293T cells expressing $(G_4C_2)75$ or $(G_4C_2)2$ repeats. (**B**) Representative images of sense and antisense RNA foci in HEK293T expressing $(G_4C_2)75$ repeats together with either control antisense oligonucleotides (ASOs), ASOs targeting sense G_4C_2RNAs or ASOs targeting antisense C_4G_2 repeat expanded RNAs. Foci were detected by RNA FISH. Red, foci; blue, DAPI. (**C**) Immunoblotting and (**D**) quantification of p-PKR and p-eIF2α in HEK293T cells expressing

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(G_4C_2)75 or (G_4C_2)2 repeats together with either control ASO or ASOs targeting sense G_4C_2 repeat expanded RNAs. Phosphorylated PKR levels were detected using anti-p-PKR (phosphor T446) and normalized to total PKR. Phosphorylated eIF2α levels were detected using anti-peIF2α (Ser51) and normalized to total eIF2α. GAPDH was used as a loading control. Error bars represent SD (n = 3 independent experiments). Statistical analyses were performed using unpaired Student's *t*-test. (**E, F**) Immunoblotting of p-PKR and p-eIF2α in HEK293T cells expressing (G_4C_2)75 or (G_4C_2)2 repeats together with either control ASO or ASOs targeting antisense G_4C_2 repeat expanded RNAs. P-PKR (T446) and p-eIF2α (Ser51) were normalized to total PKR and eIF2α, respectively. GAPDH was used as a loading control. Error bars represent SD (n = 3 independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test. (**G**) Representative images and (**H**) quantification of FMRP and G3BP1 staining in HEK293T cells expressing (G_4C_2)75 or (G_4C_2)2 repeats in the presence and absence of antisense ASO2. Error bars represent SD (n = 150 cells/condition and three independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test. Scale bars, 20 μm.

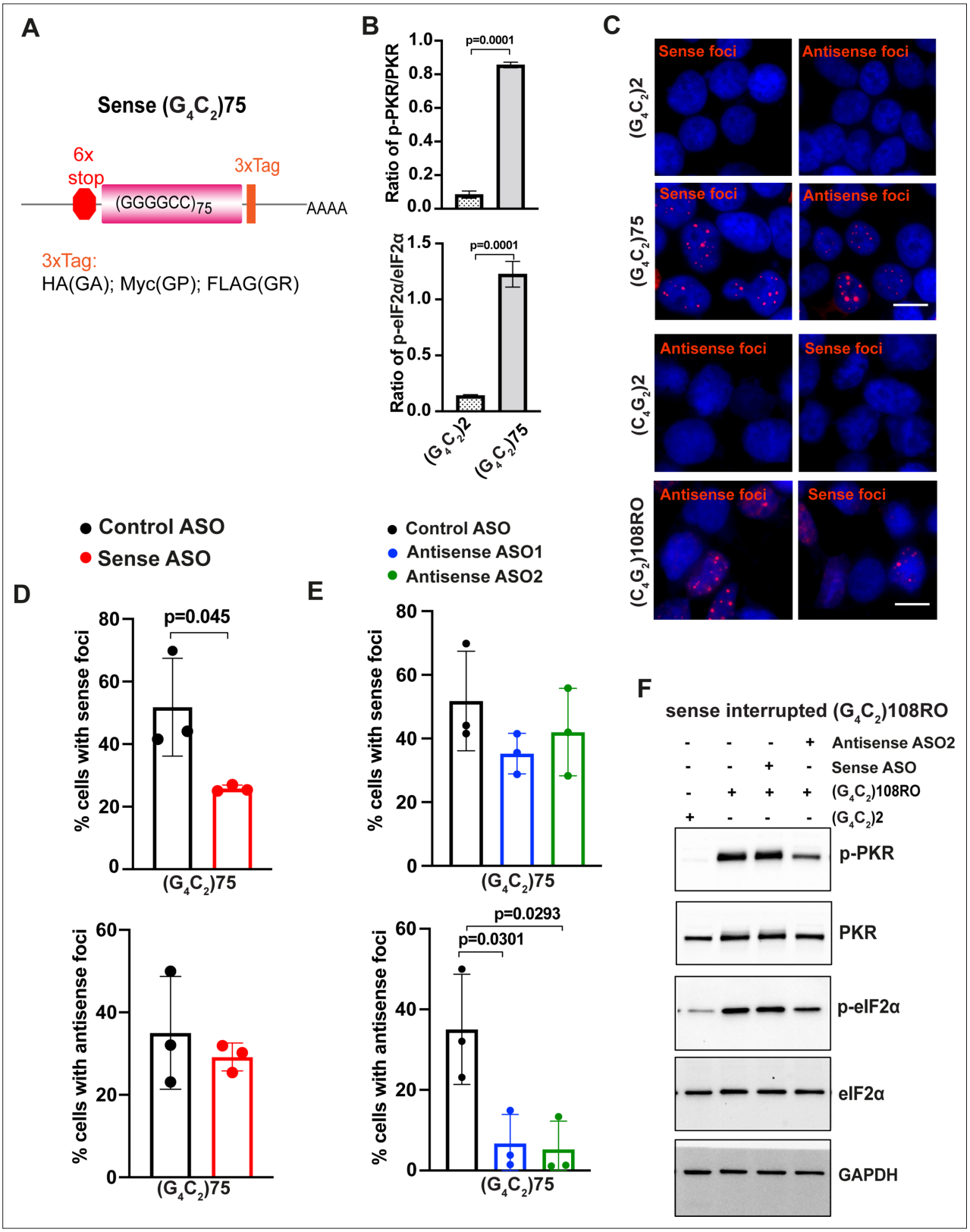

Figure 6—figure supplement 1. Sense G_4C_2 repeat expanded RNAs cannot activate the PKR/eIF2α pathway. (**A**) Schematic illustration of the sense $(G_4C_2)75$ repeat construct. (**B**) Quantification of p-PKR and p-eIF2α in HEK293T cells expressing $(G_4C_2)75$ or $(G_4C_2)2$ repeats. p-PKR (T446) and p-eIF2α (Ser51) were normalized to total PKR and eIF2α, respectively. GAPDH was used as a loading control. Error bars represent SD (n = 2 independent experiments). Statistical analyses were performed using Student's *t*-test. (**C**) Representative images of sense and antisense RNA foci in HEK293T cells

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expressing sense (G_4C_2)75 and interrupted antisense (C_4G_2)108RO repeats. Foci were detected by RNA FISH. Red, foci; blue, DAPI. (**D, E**) Quantification of RNA foci in HEK293T expressing (G_4C_2)75 together with either control antisense oligonucleotide (ASO) or ASOs targeting sense G_4C_2 and antisense C_4G_2 repeat expanded RNAs. Error bars represent SD (n = 80–100 cells/condition from three independent experiments). (**F**) Immunoblotting of p-PKR and p-eIF2α in HEK293T cells expressing interrupted sense (G_4C_2)108RO and control repeats. p-PKR levels were detected using anti-p-PKR (T446) and normalized to total PKR. P-eIF2α levels were detected using anti-phosphor eIF2α (Ser51) and normalized to total eIF2α. Error bars represent. GAPDH was used as a loading control. Error bars represent SD Scale bars, 20 μm (HEK293T).

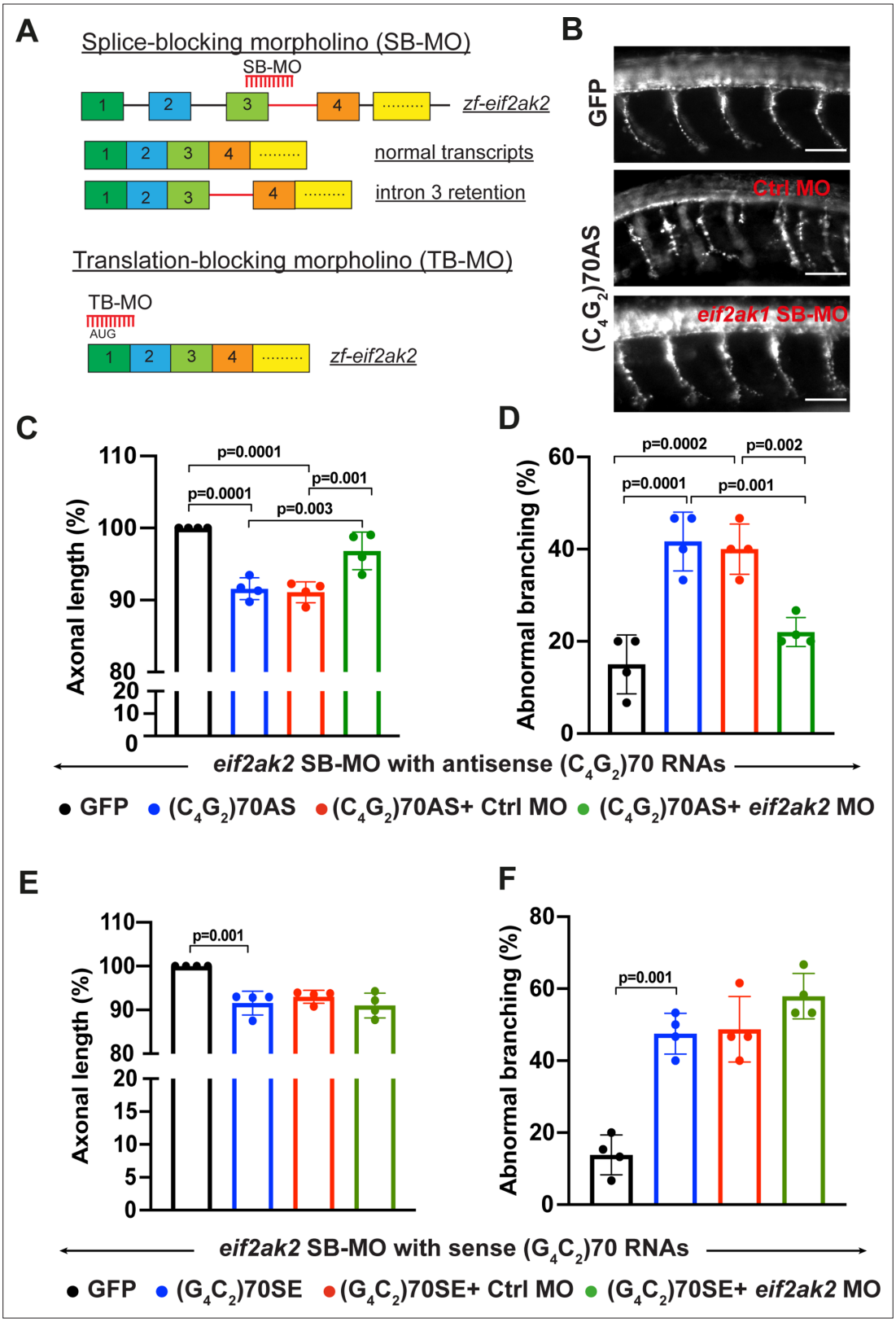

Figure 7. Reduction of PKR mitigates antisense C_4G_2, but not sense G_4C_2, RNA- mediated toxicity in zebrafish. (**A**) Schematic illustration of splice-blocking morpholino (SB-MO) targeting the exon 3/intron 3 junction in zebrafish *eif2ak2* pre-mRNA. The correctly spliced (wild type transcripts) and intron 3 retained transcripts are shown. The translation-blocking morpholino targets the AUG start codon in post-spliced *eif2ak2* mRNA. (**B**) SV2 immunostaining of motor axons in 30 hpf zebrafish embryos injected with GFP control mRNA and antisense $(C_4G_2)70$ RNAs. Scale bar = 50 µm. (**C, D**)

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Effects of SB-MO on axonal length (**C**) and branching (**D**) of zebrafish expressing antisense $(C_4G_2)70$ RNAs. Embryos were injected with 0.844 µM GFP mRNA, 0.844 µM antisense $(C_4G_2)70$ RNAs, 0.844 µM antisense $(C_4G_2)70$ RNAs plus 0.25 mM control morpholino, or 0.844 µM antisense $(C_4G_2)70$ RNAs plus 0.25 mM *eif2ak2* morpholino. Error bars represent SD (n = 4 independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test. (**E, F**) Effects of SB-MO on axonal length and branching of zebrafish expressing sense $(G_4C_2)70$ RNAs. Embryos were injected with 0.844 µM GFP mRNA, 0.844 µc sense $(G_4C_2)70$ RNAs, 0.844 µM sense $(G_4C_2)70$ RNAs plus 0.25 mM control morpholino or 0.844 µM sense $(G_4C_2)70$ RNAs plus 0.25 mM *eif2ak2* morpholino. Error bars represent SD (n = 4 independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test.

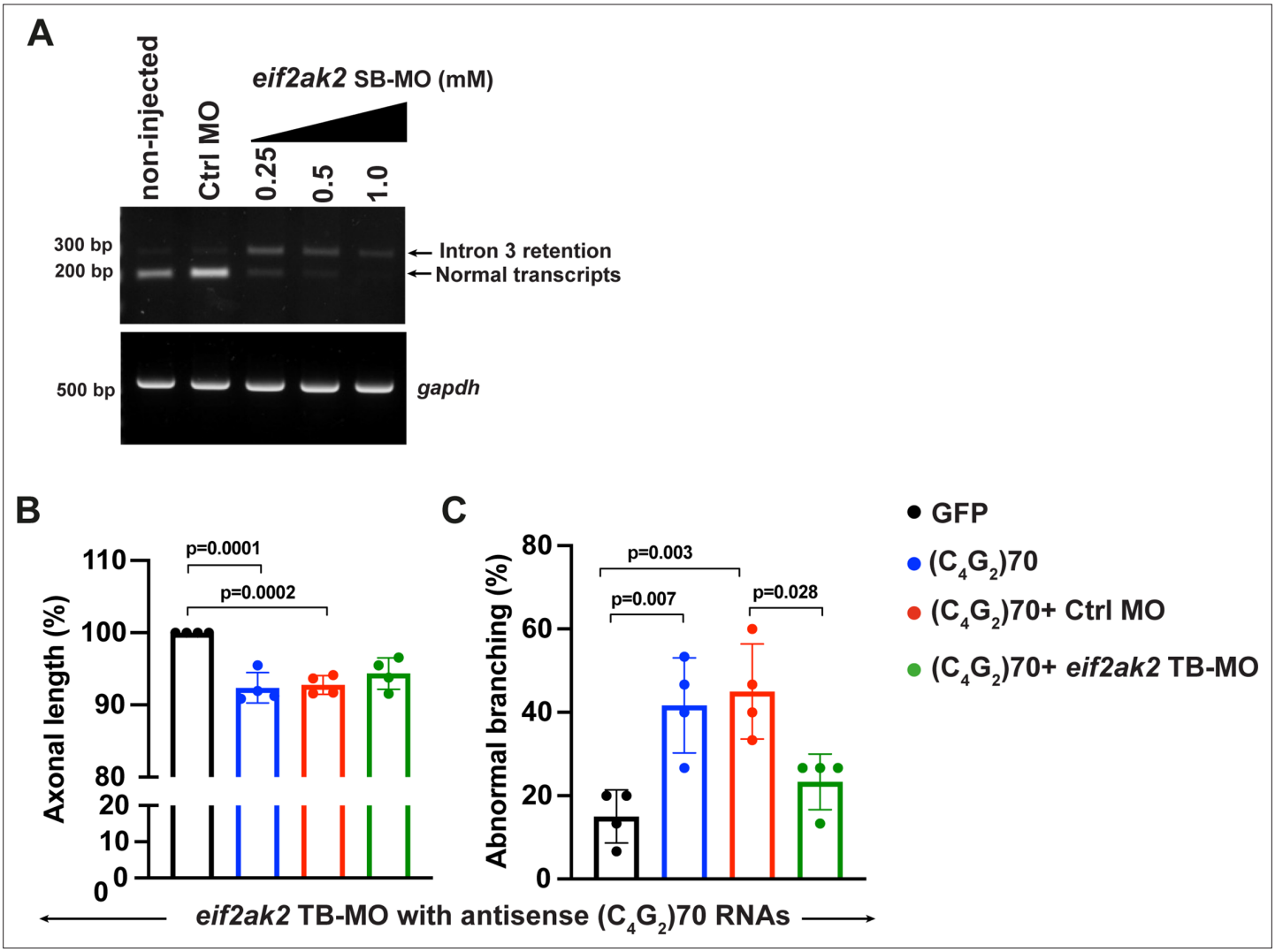

Figure 7—figure supplement 1. Translation-blocking morpholino of PKR mitigates antisense C_4G_2 repeat RNA-induced axonopathy in zebrafish. (**A**) Dose-dependent knockdown of WT transcripts and increase of transcripts with intron 3 retention as determined by RT-PCR analysis. *Eif2ak2* splice variants in non-injected, standard control and *eif2ak2* targeting MO-injected embryos 30 hpf are shown. (**B, C**) Effect of translation-blocking morpholino (TB-MO)-mediated reduction of Eif2ak2 on axonal length (**B**) and branching (**C**), using a dose of 0.25 mM for TB-MO and standard control MO in GFP and antisense $(C_4G_2)70$ RNAs expressing zebrafish. Error bars represent SD (n = 4 independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey's post hoc test.

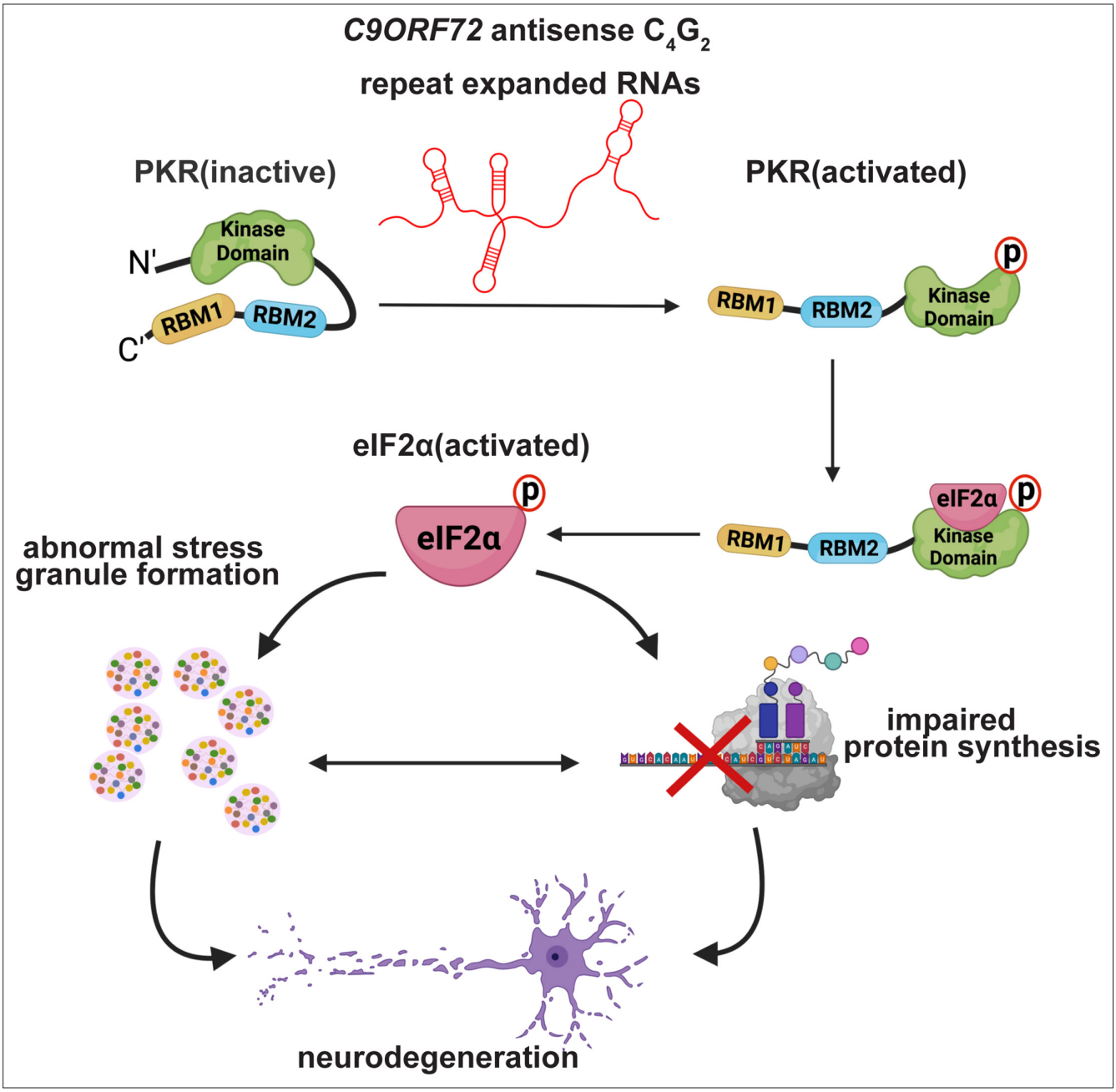

Figure 8. Proposed pathogenic mechanisms caused by *C9ORF72* antisense C_4G_2 repeat expanded RNAs. *C9ORF72* antisense C_4G_2 repeat expanded RNAs activate PKR/eIF2α-dependent integrated stress response and lead to neurotoxicity independent of DPR proteins.