Supplementary Information for

# CRISPR/Cas13d targeting suppresses repeat-associated non-AUG translation of C9orf72 hexanucleotide repeat RNA

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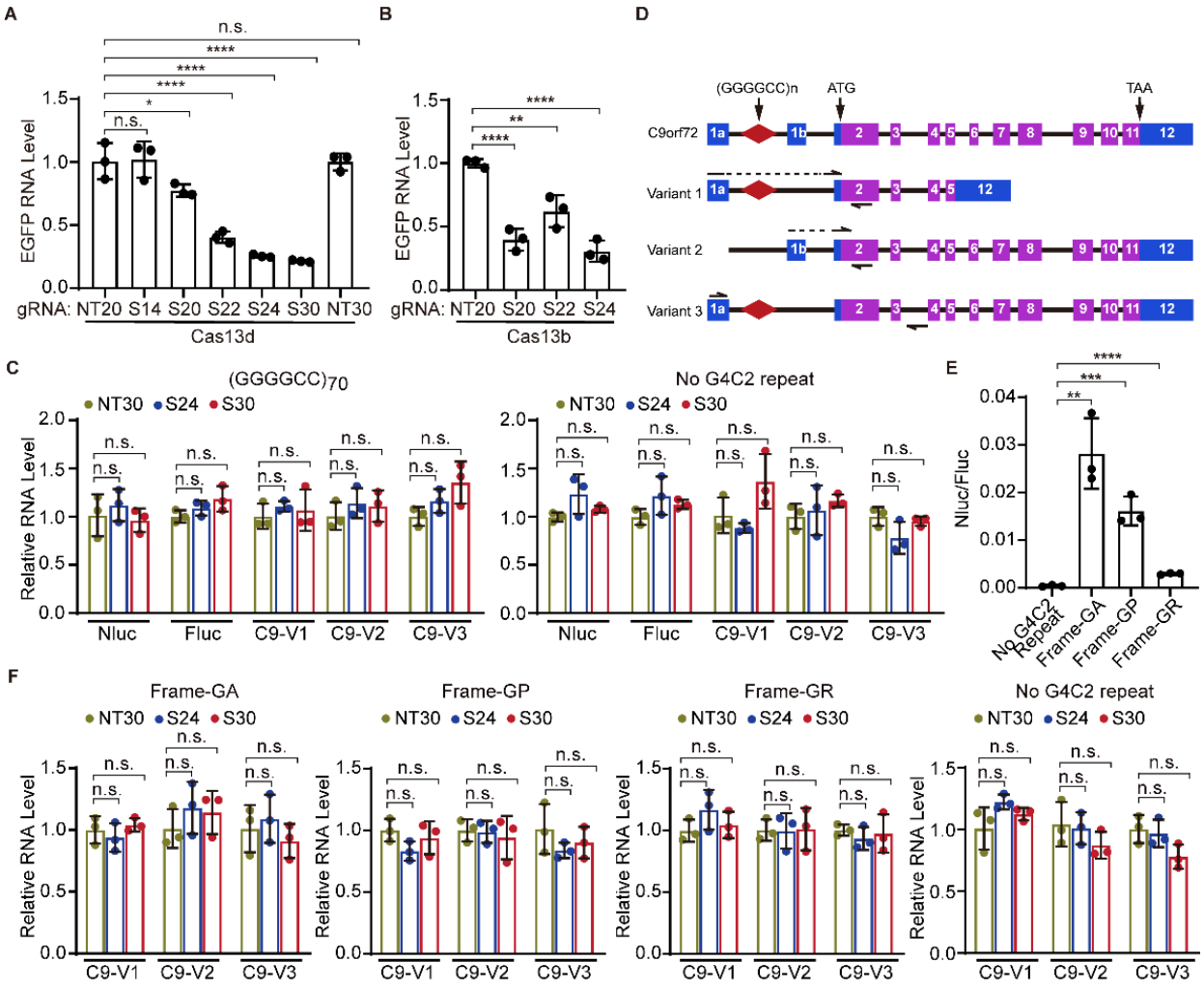
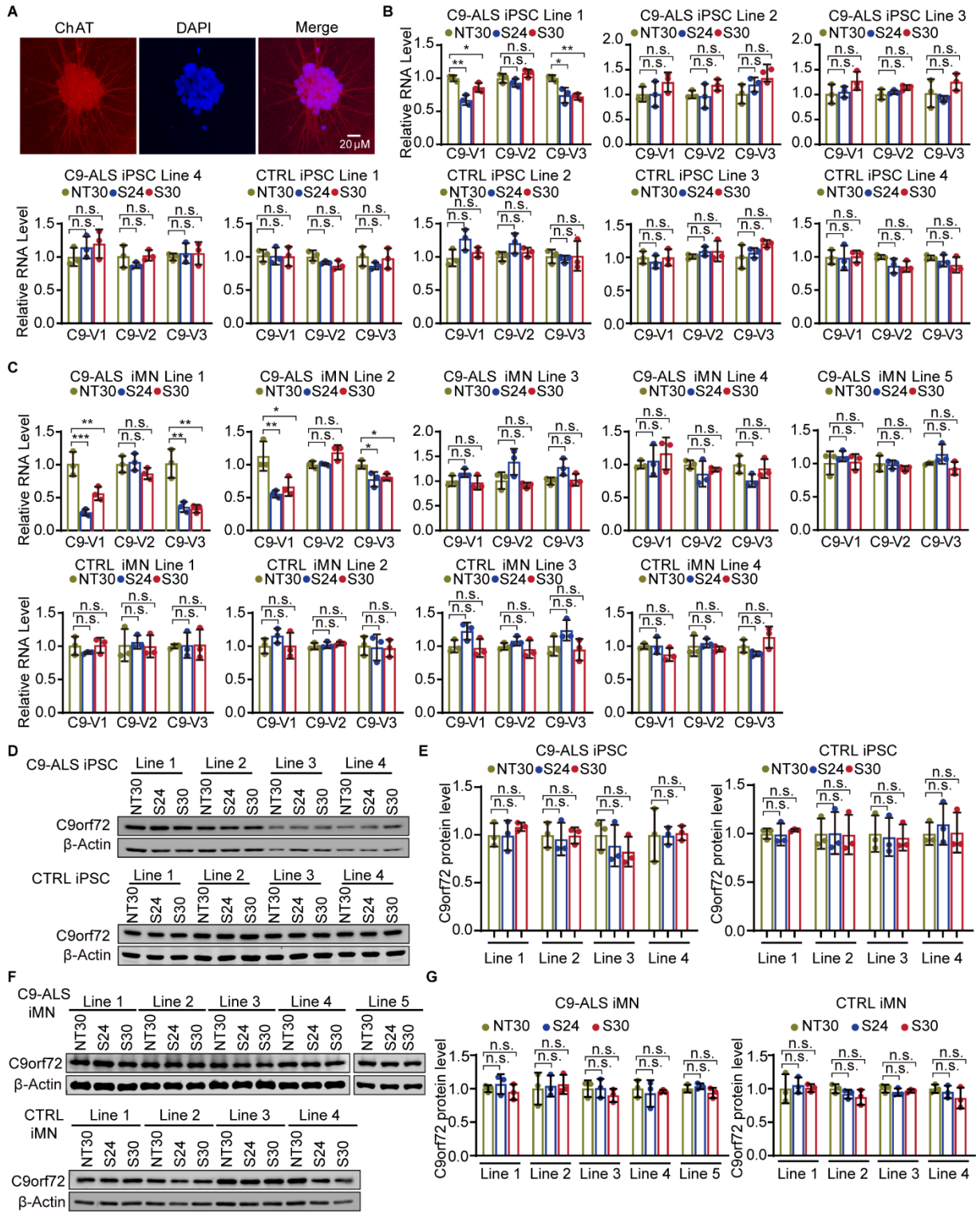
Supplemental Figure 1




**Supplemental Figure 1. Quantification of RNA levels of EGFP, Nluc, Fluc, and three C9orf72 transcripts showed the knockdown of EGFP by specific Cas13 protein or guide RNAs, while the Nluc, Fluc, and three C9orf72 transcripts were not affected by the Cas13 systems in the RAN translation reporter cell lines.** EGFP RNA levels were quantified in HEK293 cells transfected with the CRISPR-Cas13d (**A**) or CRISPR-Cas13b (**B**) systems, showing changes consistent with those at the EGFP protein levels presented in Figure 1C. NeoR gene in the CRISPR-Cas13b/d constructs was used as the control gene for qPCR. (**C**) The levels of Nluc, Fluc and three C9orf72 transcripts were not significantly affected in the HeLa

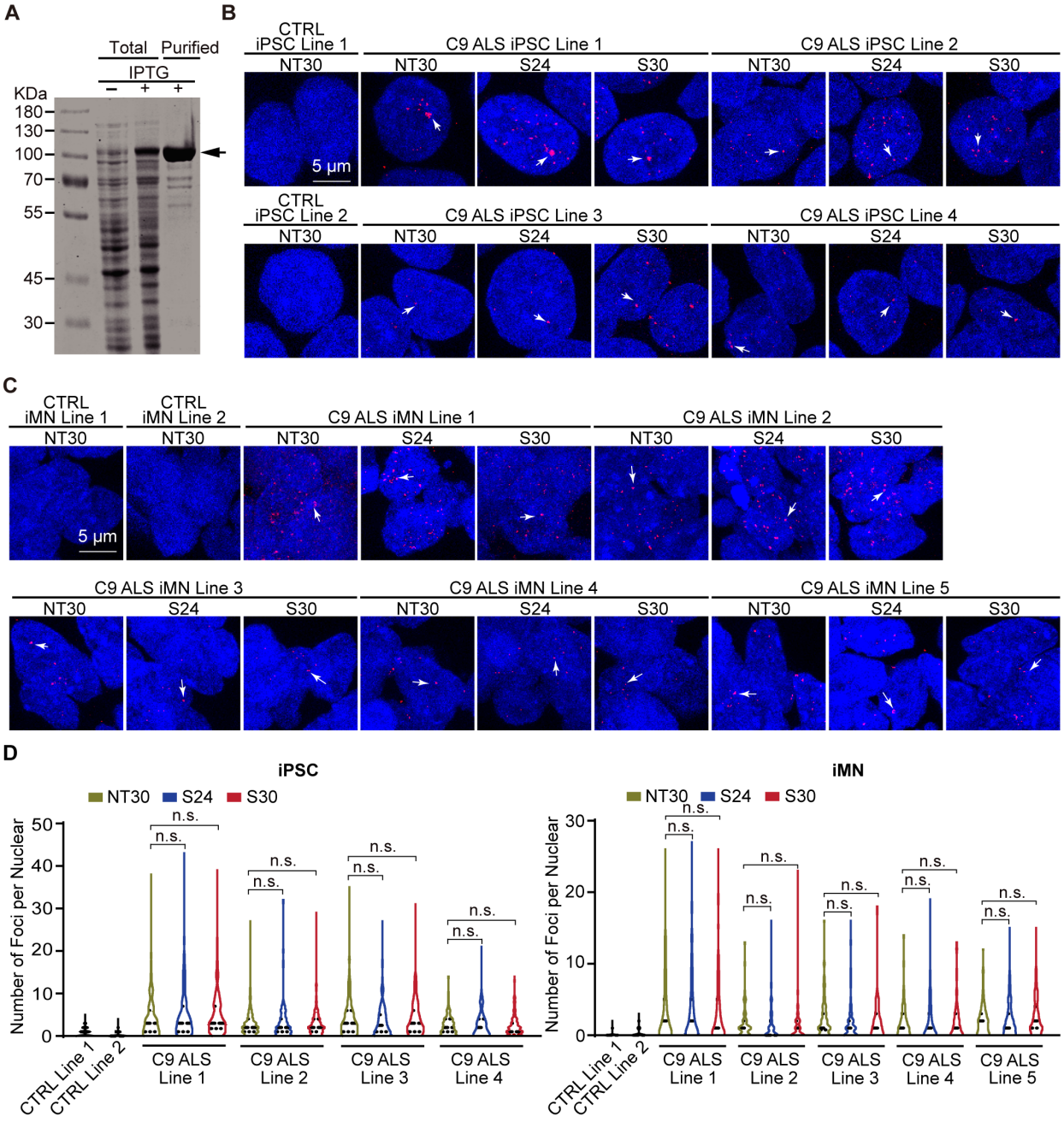
RAN translation reporter cell lines with either (GGGGCC)70 (left) or No-G4C2-repeat (right). (**D**) Schematics of the C9orf72 gene and its three transcript variants. The arrows indicate the locations of primers used for quantifying C9orf72 transcripts. (**E**) Relative RAN translation efficiency (Nano luciferase activity normalized to firefly luciferase) from the GA, GP, or GR frame was significantly higher than the No-G4C2-repeat negative control in HEK293 cells transfected with corresponding constructs. (**F**) The levels of the three C9orf72 transcripts were not significantly changed in the HEK293 cells co-transfected with CRISPR-Cas13d constructs with either the GA-frame, GP-frame, GR-frame or the No-G4C2-repeat control construct. Data are presented as means ± SD of three independent experiments and analyzed with unpaired two-tailed Student's t-test (E) and ordinary one-way ANOVA with Dunnett's multiple comparisons test (A-C and F). **P < 0.01, ***P < 0.001, ****P < 0.0001; "n.s.", no significance.

Supplemental Figure 2



**Supplemental Figure 2. Quantification of C9orf72 transcripts in iPSCs and iMNs treated with CRISPR-Cas13d.** (**A**) The differentiation of iMNs was confirmed with immunofluorescence staining for the mature motor neuron marker ChAT. (**B**) qPCRs with C9-ALS patient iPSC line 1 stably expressing Cas13d-S24 or Cas13d-S30 showed significant reduction of the C9-V1 and C9-V3 transcripts but not the C9-V2 transcript, while no significant changes were found in the other three C9-ALS patient and the four control iPSC lines. (**C**) qPCRs with C9-ALS iMN line 1 and 2 treated with lentiviruses expressing Cas13d-S24 or Cas13d-S30 showed significant reduction of the C9-V1 and C9-V3 transcripts but not the C9-V2 transcript, while no significant changes were found in the C9-ALS patient iMN lines 3-5 and the control iMN lines 1-4. (**D**-**E**) Immunoblot analysis of C9orf72 protein showed that the C9orf72 protein level was unaffected in the four C9-ALS patient iPSC and four control iPSC cell lines stably expressing Cas13d-NT30, Cas13d-S24 and Cs13d-S30. (**F-G**) Immunoblot analysis of C9orf72 protein showed that the C9orf72 protein level was unaffected in the four C9-ALS patient iMN and four control iMN cell lines treated with Cas13d-NT30, Cas13d-S24 and Cs13d-S30. Data are presented as means ± SD of three independent experiments and analyzed with ordinary one-way ANOVA with Dunnett's multiple comparisons test. *P < 0.05, **P < 0.01; "n.s.", no significance.

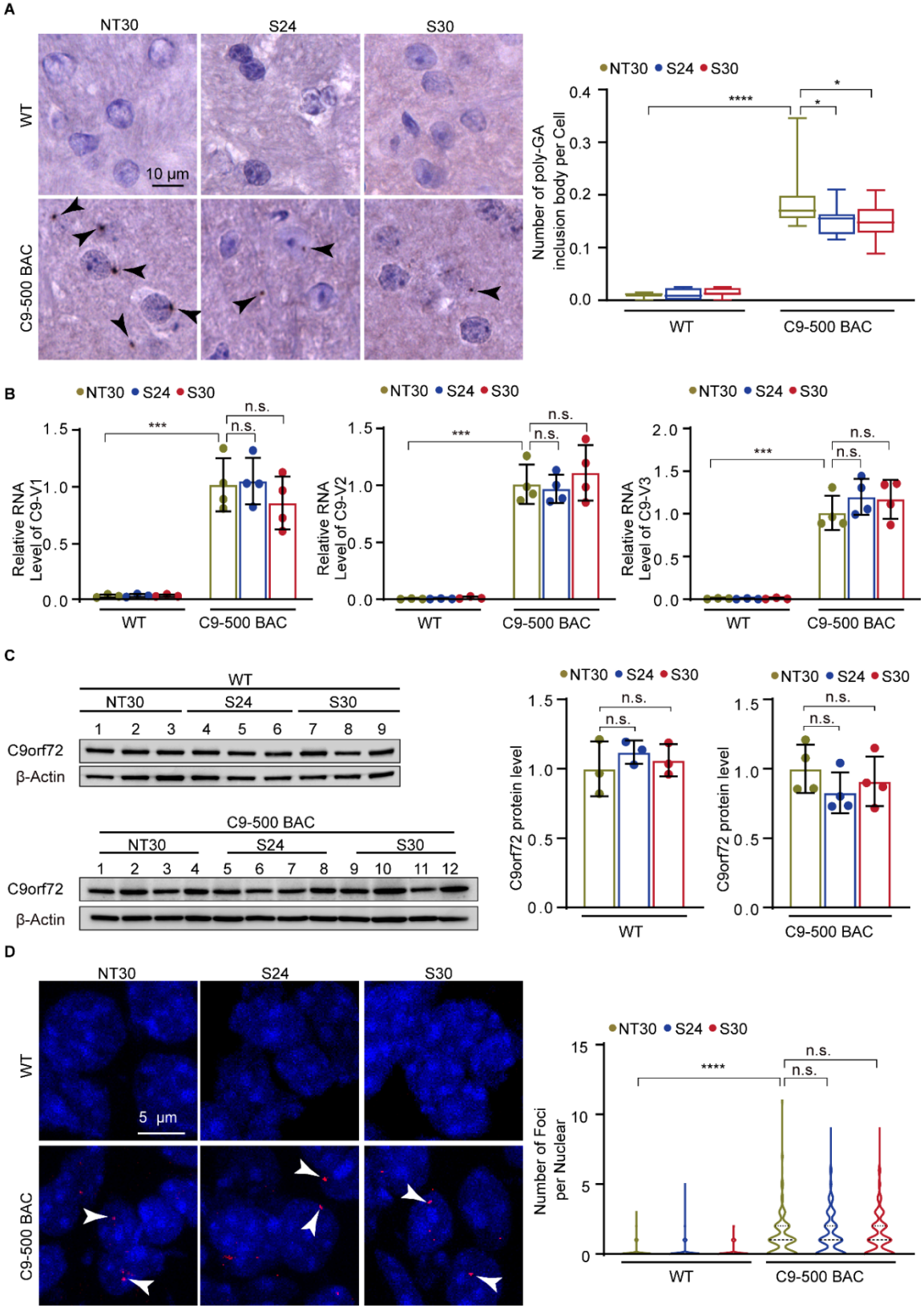
Supplemental Figure 3




**Supplemental Figure 3. CRISPR-Cas13d showed limited ability to degrade RNA foci formed by C9orf72 GGGGCC repeat RNAs**. (**A**) SDS-PAGE analysis confirmed the expression and purity of Cas13d protein. Gel was stained by Coomassie blue. (**B**) RNA FISH with a (CCCCGG)4-Cy3 probe demonstrated repeat RNA foci in the C9-ALS patient iPSC cell

lines but not in the control iPSC lines stably expressing guided Cas13d systems. (**C**) RNA FISH with a (CCCCGG)4-Cy3 probe demonstrated repeat RNA foci in the C9-ALS patient iMN lines but not in the control iMN lines treated with lentiviruses expressing guided Cas13d systems. (**D**) Quantification of RNA foci in the nuclei of iPSCs and iMNs treated with CRISPR-Cas13d showed much more foci in ALS-patient iPSCs and iMNs than in control iPSCs and iMNs, respectively, while no significant difference was observed in each cell line when comparing S24- or S30-guided Cas13d with the control NT30-guided Cas13d. For each treatment, at least 160 iPSC cells or 85 iMNs from at least three images were analyzed for the violin plot with ordinary one-way ANOVA with Dunnett's multiple comparisons test. ****$P < 0.0001$; "n.s.", no significance.

Supplemental Figure 4



**Supplemental Figure 4. Poly-GA inclusions were significantly reduced while C9orf72 transcripts and RNA foci were not significantly changed in the brain tissues of C9-500 BAC mice treated with the AAV-mediated CRISPR-Cas13d system**. (**A**) IHC staining showed that poly-GA inclusions were specifically detected in the brain tissues of transgenic C9-500 BAC mice but not the WT mice. The number of poly-GA inclusions in the brains of C9-500 BAC mice treated with Cas13d-S24 and Cas13d-S30 were significantly decreased compared to the non-targeting control treatment Cas13d-NT30. For each group, 6-10 images from two mice were analyzed. Quantitative analysis employed a one-tailed Student's t-test. (**B**) Quantification of C9-V1, C9-V2 and C9-V3 RNA levels showed much higher C9orf72 RNA levels in the transgenic C9-500 BAC mice than the wild-type mice, confirming the primer specificity and mouse genotypes. No significant differences were found in the levels of the three C9orf72 transcripts when comparing Cas13d-S24 or Cas13d-S30 with Cas13d-NT30-treated C9-500 BAC mice. The number of dots in each group indicate the number of mice in the corresponding group. (**C**) Immunoblot analysis of C9orf72 protein showed that the C9orf72 protein level was unaffected in the nine WT and twelve C9-500 BAC mice treated with Cas13d-NT30, Cas13d-S24 and Cs13d-S30. (**D**) RNA foci formed by GGGGCC repeat RNAs were found in the C9-500 BAC mice but not in the wild-type mice, and the number of foci was not significantly changed with the treatment of Cas13d-S24 or Cas13d-S30 compared to the control Cas13d-NT30. For each group, at least 315 cells in three images from three mice were analyzed. Data are presented as box plot with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values and analyzed with unpaired one-tailed t-test (A); means ± SD with unpaired two-tailed t-test (B); or ordinary one-way ANOVA with Dunnett's multiple comparisons test (C); or violin plot with unpaired two-tailed t-test (D). *P < 0.05, ***P < 0.001, ****P < 0.0001; "n.s.", no significance.

**Supplemental Table 1. Cell lines used in this study**

| iPSCs and iMNs | | | | | | | |
|---|---|---|---|---|---|---|---|
| Cell Line ID | Subject ID | Genotype | Clinical Status | Gender | Age | Source | Repeat size in WT allele |
| C9-ALS Line 1 | ND10689 | C9orf72 | Affected | F | 51 | NINDS | 5 |
| C9-ALS Line 2 | ND12099 | C9orf72 | Affected | M | 49 | NINDS | 2 |
| C9-ALS Line 3 | NDS00239 | C9orf72 | Affected | F | 64 | NINDS | 2 |
| C9-ALS Line 4 | NDS00247 | C9orf72 | At risk | M | 60 | NINDS | 15 |
| C9-ALS Line 5 | ND06769 | C9orf72 | Affected | F | 46 | NINDS | 13 |
| CTRL Line 1 | ND41865 | WT | Control | M | 64 | NINDS | 2 |
| CTRL Line 2 | GM25256 | WT | Control | M | 30 | Coriell | 2 |
| CTRL Line 3 | NDS00241 | WT | Control | M | 36 | NINDS | 2 |
| CTRL Line 4 | NDS00242 | WT | Control | F | 49 | NINDS | 2 |
| **Other Cell Lines** | | | | | | | |
| Cell Lines | Source | | | | | Repeat size in WT allele | |
| Hela | ATCC | | | | | 2 | |
| HEK 293 | ATCC | | | | | 2 | |
| Abbreviations: F (Female), M (Male), NINDS (National Institute of Neurological Disease and Stroke), WT (Wild-Type), ATCC (American Type Culture Collection). | | | | | | | |

**Supplemental Table 2. DNA and RNA sequence**

| | | |
|---|---|---|
| Cas13b mutation | Cas13b-mut-F | GCTGCCCAAGGACCGTATCCACAGCGAGAAG |
| | Cas13b-mut-R | CTTCTCGCTGTGGATACGGTCCTTGGGCAGCT |
| | Cas13b-F | GAACTCTAGAGCCACCATGGCTCCCAAAAAGAAGAGGAAAGTGGGTTCCATGAACATCCCCGCTCTGGTG |
| | Cas13b-R | TACATGGATCCCTTCATGATGGCGTACTCC |
| Cas13b amplification for lentiviral vector | Forward Primer | GAACTCTAGAGCCACCATGGCTCCCAAAAAGAAGAGGAAAGTGGGTTCCATGAACATCCCCGCTCTGGTG |
| | Reverse Primer | TATCTGGATCCTACCTTCCTCTTCTTCTTGGGAGAGCCCTTCATGATGGCGTACTCC |
| Cas13d amplification for lentiviral vector | Forward Primer | TCATATCTAGAGCCACCATGAGCCCCAAGAAGAAG |
| | Reverse Primer | TTGACGGATCCCACCTTCCTCTTTTTCTTAGGTCCAGATCCGGAATTGCC |
| crRNA | Case3b_NT20 | GUGAUAAGUGGAAUGCCAUGGUUGUGGAAGGUCCAGUUUUGAGGGGCUAUUACAAC |
| | Case3b_S20 | CCCCGGCCCCGGCCCCGGCCGUUGUGGAAGGUCCAGUUUUGAGGGGCUAUUACAAC |
| | Case3b_S22 | CCCGGCCCCGGCCCCGGCCCCGUUGUGGAAGGUCCAGUUUUGAGGGGCUAUUACAAC |
| | Case3b_S24 | GGCCCCGGCCCCGGCCCCGGCCCCGUUGUGGAAGGUCCAGUUUUGAGGGGCUAUUACAAC |

| | | |
|---|---|---|
| | Cas13d_NT20 | AACCCCUACCAACUGGUCGGGGUUUGAAACGUGAUAAGUGGAAUGCCAUG |
| | Cas13d_S14 | AACCCCUACCAACUGGUCGGGGUUUGAAACCCCCGGCCCCGGCC |
| | Cas13d_S20 | AACCCCUACCAACUGGUCGGGGUUUGAAACCCCCGGCCCCGGCCCCGGCC |
| | Cas13d_S22 | AACCCCUACCAACUGGUCGGGGUUUGAAACCCCCGGCCCCGGCCCCCGGCCCC |
| | Cas13d_S24 | AACCCCUACCAACUGGUCGGGGUUUGAAACCCGGCCCCGGCCCCCGGCCCCCGGCC |
| | Cas13d_S30 | AACCCCUACCAACUGGUCGGGGUUUGAAACCCGGCCCCGGCCCCCGGCCCCCGGCCCCCGGCC |
| GR-frame construct | GA-F | TAGATTCGCGGCCGCGTCTTCACACTCGAAGATTTCG |
| | GA-R | GGTATCTTGTCGTCGTCGTCCTTGT |
| Repeat size PCR | Forward Primer | GCCCACGTAAAAGATGACGC |
| | Reverse Primer | AGTCGCTAGAGGCGAAAGC |
| Cas13d amplification for AAV vector construction | MluI-U6-F | ACTAGACGCGTGAGGGCCTATTTCCCATGATTC |
| | HindIII-Cas13d-R | ATGAGAAGCTTTTACACCTTCCTCTTTTTCTTAGGTCCAG |
| gRNA DNA oligonucleotides | T7-NT30-F | GACCTCTAATACGACTCACTATAGGAACCCCTACCAACTGGTCGGGGTTTGAAACTCACCAGAAGCGTACCATACTCACGAACAG |
| | T7-NT30-R | CTGTTCGTGAGTATGGTACGCTTCTGGTGAGTTTCAAACCCCGACCAGTTGGTAGGGGTTCCTATAGTGAGTCGTATTAGAGGTC |
| | T7-S24-F | GACCTCTAATACGACTCACTATAGGAACCCCTACCAACTGGTCGGGGTTTGAAACCCGGCCCCGGCCCCGGCCCCGGCC |
| | T7-S24-R | GGCCGGGGCCGGGGCCGGGGCCGGGTTTCAAACCCCGACCAGTTGGTAGGGGTTCCTATAGTGAGTCGTATTAGAGGTC |
| | T7-S30-F | GACCTCTAATACGACTCACTATAGGAACCCCTACCAACTGGTCGGGGTTTGAAACCCGGCCCCGGCCCCGGCCCCGGCCCCGGCC |
| | T7-S30-R | GGCCGGGGCCGGGGCCGGGGCCGGGGCCGGGTTTCAAACCCCGACCAGTTGGTAGGGGTTCCTATAGTGAGTCGTATTAGAGGTC |
| | T7-S24-M-F | GACCTCTAATACGACTCACTATAGGAACCCCTACCAACTGGTCGGGGTTTGAAACGGACACGGACACGGACACGGACAC |
| | T7-S24-M-R | GTGTCCGTGTCCGTGTCCGTGTCCGTTTCAAACCCCGACCAGTTGGTAGGGGTTCCTATAGTGAGTCGTATTAGAGGTC |
| | T7-S24-M2-F | GACCTCTAATACGACTCACTATAGGAACCCCTACCAACTGGTCGGGGTTTGAAACTGGCATCTTCAGAATTCTCTACCA |
| | T7-S24-M2-R | TGGTAGAGAATTCTGAAGATGCCAGTTTCAAACCCCGACCAGTTGGTAGGGGTTCCTATAGTGAGTCGTATTAGAGGTC |
| qPCR primers | EGFP-qF | GACGTAAACGGCCACAAGTT |
| | EGFP-qR | AAGTCGTGCTGCTTCATGTG |
| | NeoR-qF | ACCTTGCTCCTGCCGAGAAAGTAT |

| | | |
|---|---|---|
| | NeoR-qR | ATGTTTCGCTTGGTGGTCGAATGG |
| | C9_V1_qF | CCACGTAAAAGATGACGCTTGATA |
| | C9_V2_qF | CGGTGGCGAGTGGATATCTC |
| | C9_V3_qF | GCAAGAGCAGGTGTGGGTTT |
| | C9_V123_qR | TGGGCAAAGAGTCGACATCA |
| | GAPDH-qF | GTCTCCTCTGACTTCAACAGCG |
| | GAPDH-qR | ACCACCCTGTTGCTGTAGCCAA |
| | Cas13d-qF | GATGTTTCCGCCTTCAGCA |
| | Cas13d-qR | CCACGAACTTAGCGTTGACT |
| | Ms_GAPDH-qF | CATCACTGCCACCCAGAAGACTG |
| | Ms_GAPDH-qR | ATGCCAGTGAGCTTCCCGTTCAG |