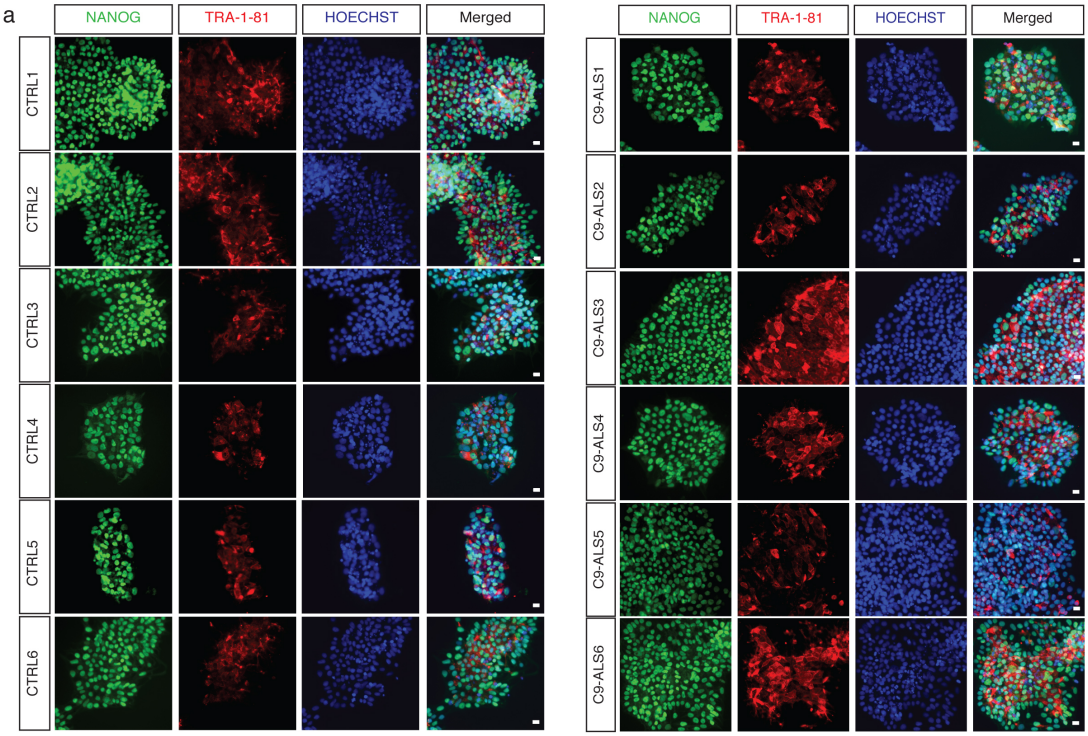
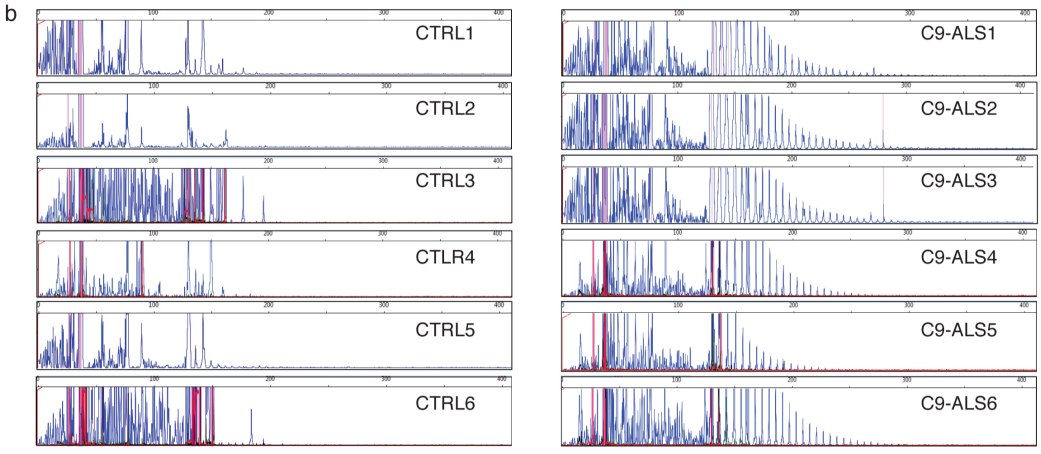
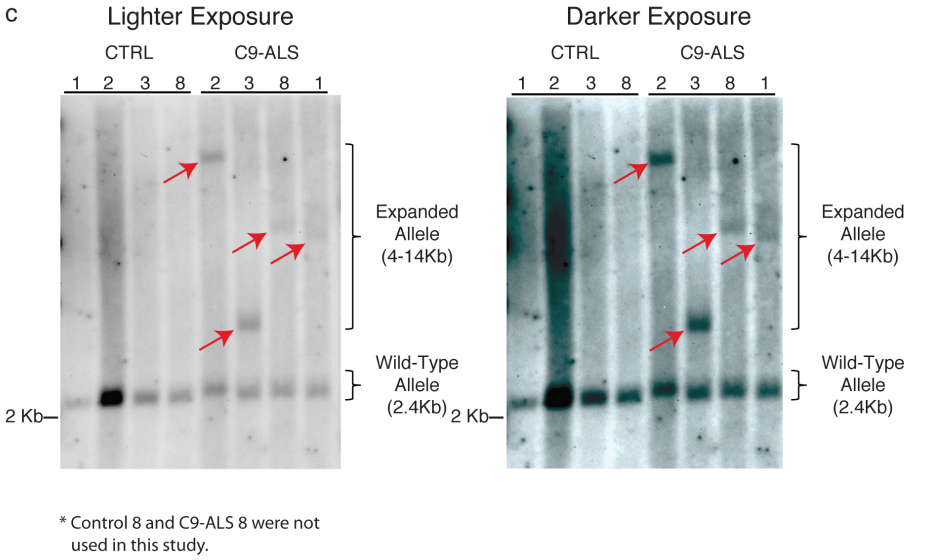
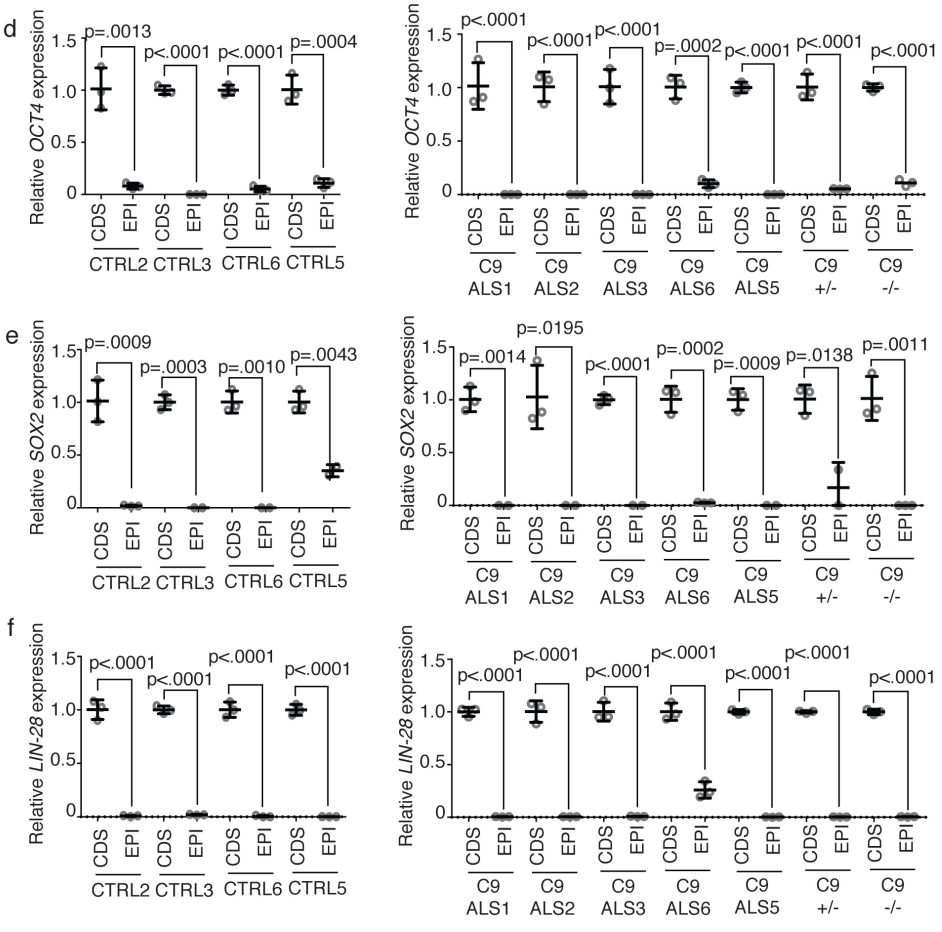
Supplementary Figure1. Derivation of iPSCs from controls and *C9ORF72* patients





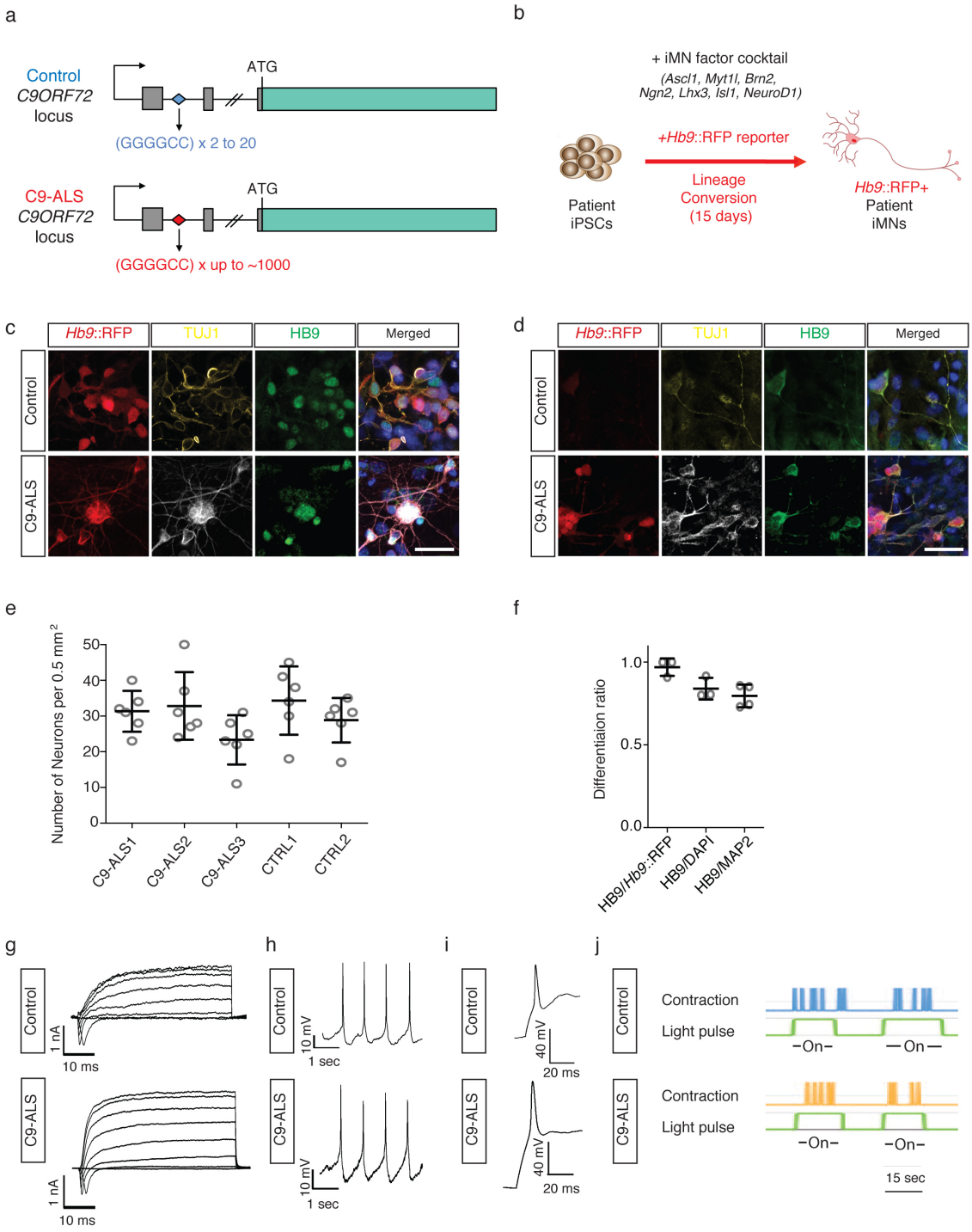
Supplementary Figure 1 (c-f). Derivation of iPSCs from controls and *C9ORF72* patients





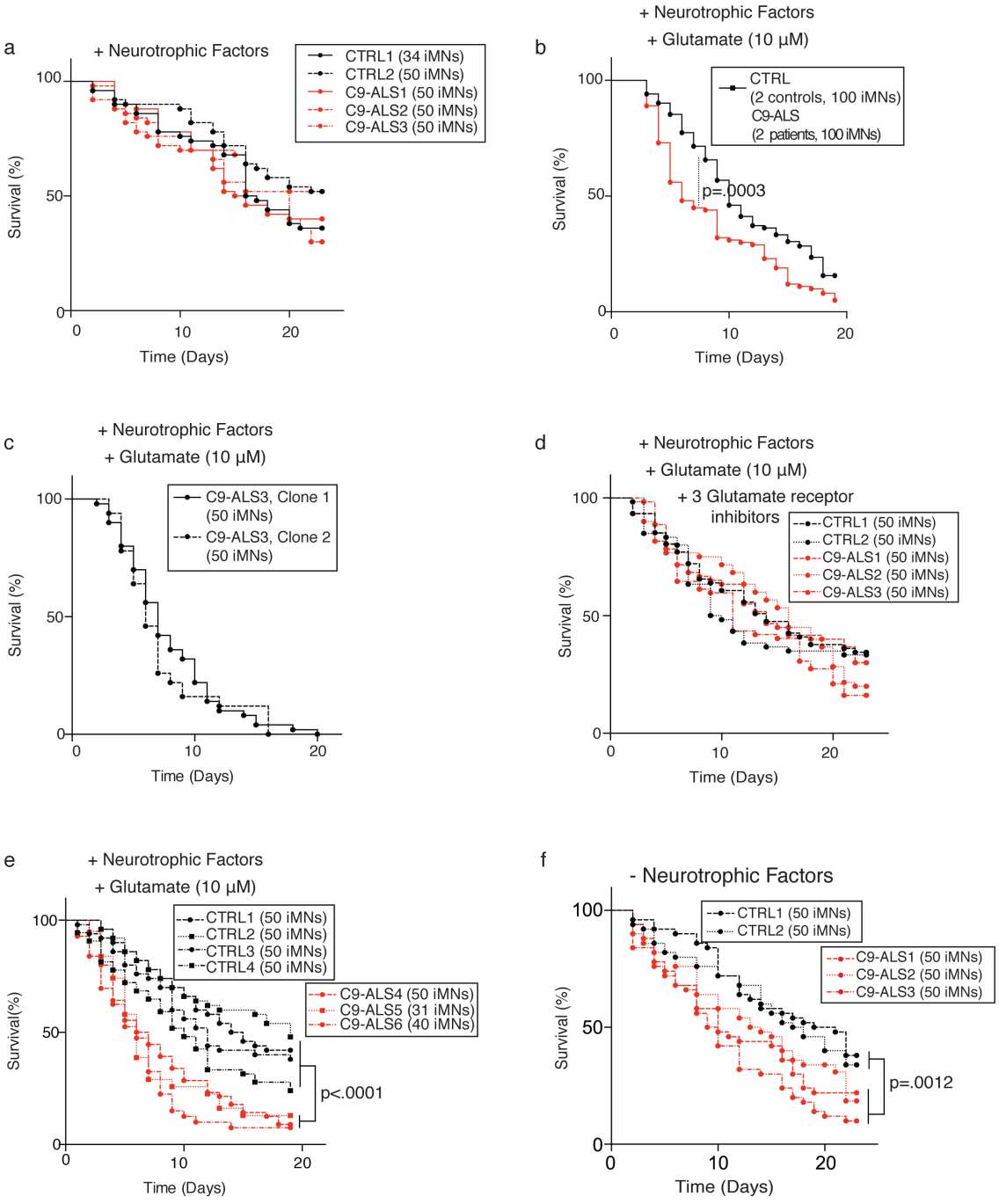
Supplementary Figure 1, related to Figure 1. Derivation of iPSCs from Controls and *C9ORF72* Patients. **a,** Control and C9-ALS patient iPSC lines express markers of pluripotency including NANOG (green) and TRA-1-81 (red). Nuclei (blue) are labeled with Hoechst. Scale bars: 40 μm. **b,** Repeat-primed PCR (RP-PCR) to quantify the intronic repeats in *C9ORF72* in control (CTRL) and C9-ALS patient iPSC lines. **c,** Southern blot to examine the *C9ORF72* repeat region in control and patient iPSC lines. The wild-type allele gives a single 2.4-kb band, while the presence of an expanded allele gives an additional high molecular weight band (4-14 kb) in all four of the heterozygous patient lines (C9-ALS 1, 2, 3, 8). Red arrows denote the repeat expanded allele. *Note – Control 8 and C9-ALS 8 were not used in this study. The experiments in (a-c) were repeated twice with similar results. **d-f,** qPCR genotyping to quantify expression of the episomal plasmid iPSC reprogramming constructs in iPSC lines. A representative genefrom each of the 3 reprogramming plasmids was tested (*OCT4* (a), *SOX2* (b), and *LIN28* (c)). CDS refers to the endogenous gene and was PCR amplified using PCR primers specific to the endogenous locus. EPI refers to the episomal gene and was PCR amplified using PCR primers specific to the episomal version. All lines tested had low or undetectable levels of the episomal factors. PCR primers and qPCR assay were designed as described previously [61]. Mean +/- s.d. Two-tailed t-test between the CDS and EPI levels for each gene for each line. n=3 biologically independent iPSC cultures per line for each gene evaluated.

Supplementary Figure 2. Generation of functional motor neurons from *C9ORF72* patients

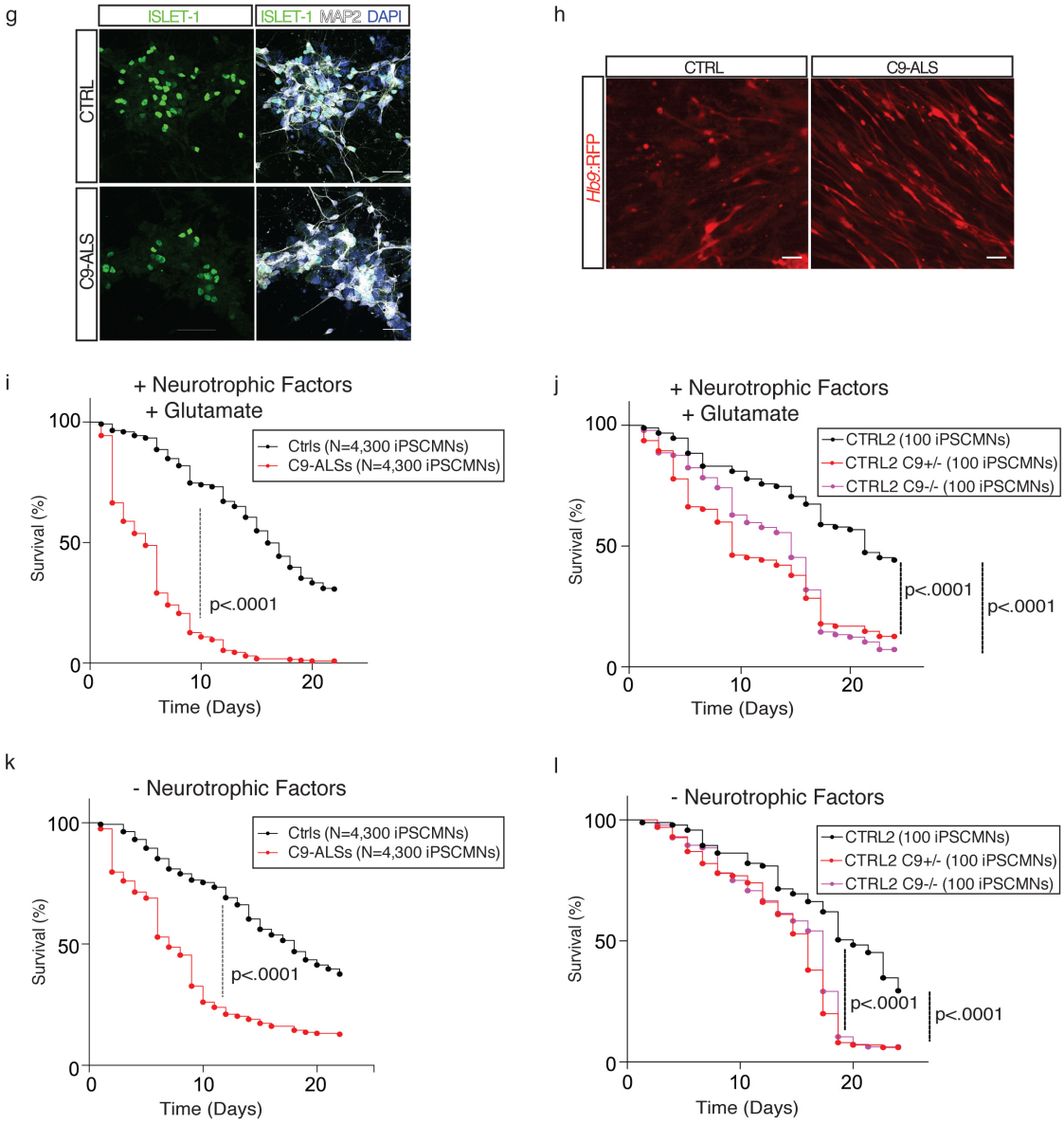


Supplementary Figure 2, related to Figure 1. Generation of Functional Motor Neurons From Controls and *C9ORF72* Patients. **a,** The *C9ORF72* locus in healthy controls and C9ORF72-ALS (C9-ALS) patients. **b,** Conversion of patient iPSCs into iMNs. iPSC, induced pluripotent stem cell; iMN, induced motor neuron. **c–d,** Immunocytochemistry shows iMNs express the *Hb9::*RFP reporter (red, **c, d**), TUJ1 (yellow, **c, d**), HB9 (green, **c**) and vesicular acetylcholine transporter (VACHT) (green, **d**). Nuclei (blue) are labeled with Hoechst. Scale bars: 125 μm. These experiments were repeated twice with similar results. **e,** Number of control and C9-ALS iMNs generated per 0.5 mm^2 of culture dish area. Mean ± s.d. n=6 biologically independent iMN conversions per line. **f,** Quantification of iMN generation, showing the amount of HB9+ cells as a proportion of *HB9::*RFP+ cells, or of DAPI-staining nuclei, or of MAP2+ cells. Mean ± s.d. n=3 (for HB9/*Hb9*::RFP and HB9/DAPI) or 4 (HB9/MAP2) biologically independent iMN conversions. **g–i**, Sample patch clamp recordings showing that iMNs possess functional sodium and potassium channels (**g**) and fire action potentials spontaneously (**h**) or in response to a current injection (**i**). These experiments were repeated twice on biologically independent iMN conversions with similar results. **j,** Channel rhodopsin-transduced iMNs actuate contractions of primary chick embryonic muscle (top) in response to light (bottom).

Supplementary figure 3 a-f. C9ORF72 iMNs Display Neurodegenerative Phenotypes

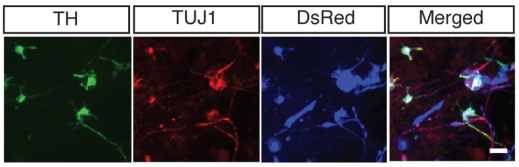


Supplementary figure 3 g-l. C9ORF72 iMNs Display Neurodegenerative Phenotypes



Supplementary figure 3m to o. C9ORF72 iMNs Display Neurodegenerative Phenotypes

m



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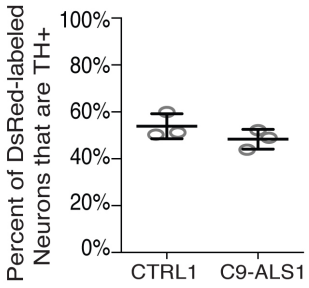

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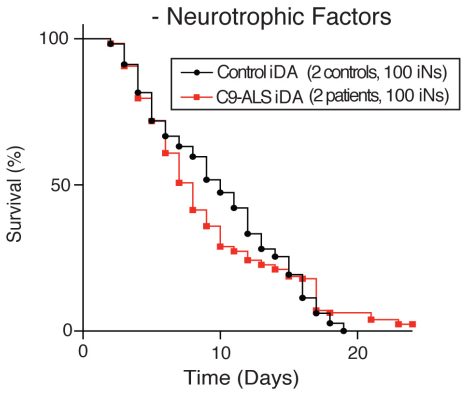
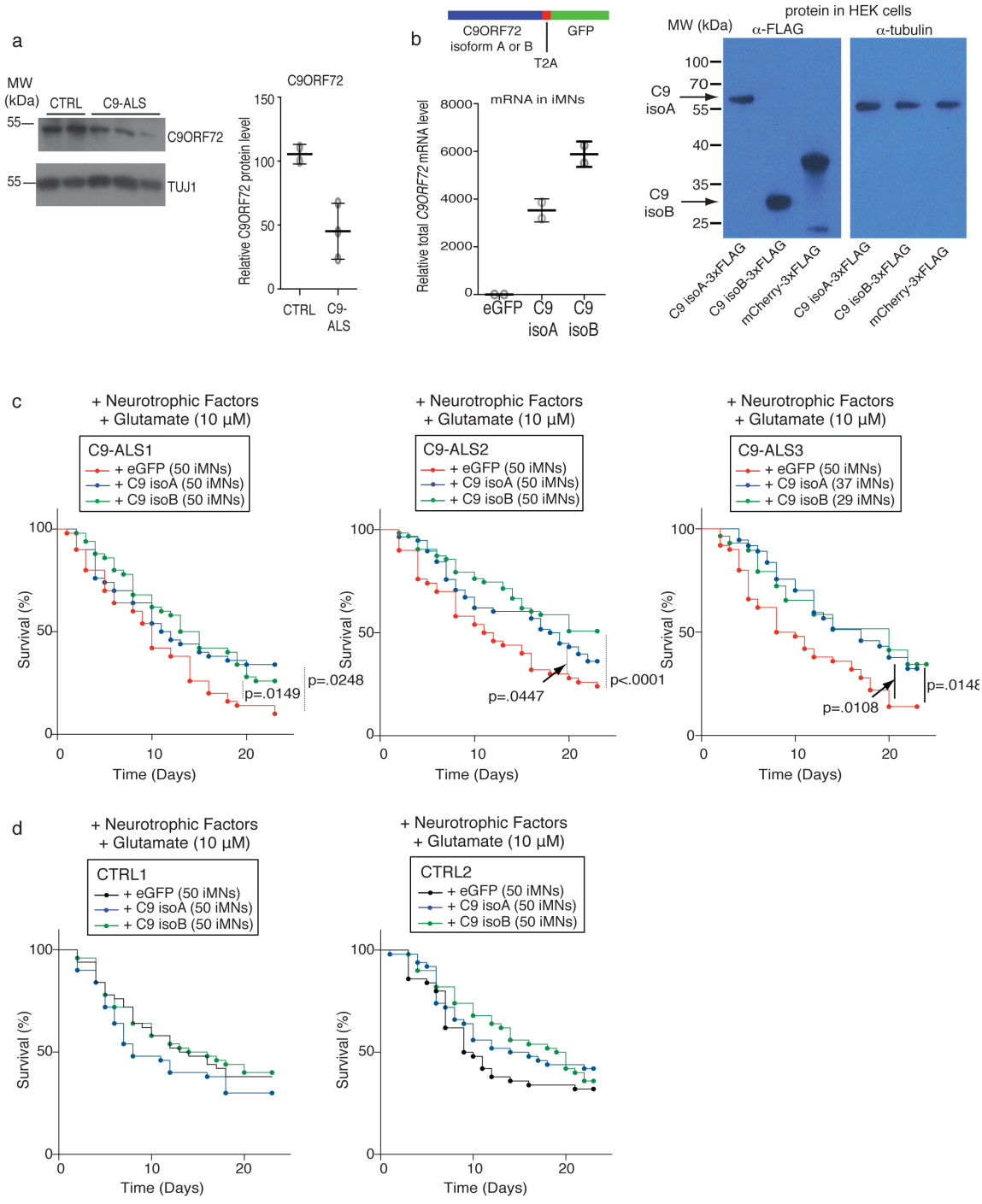


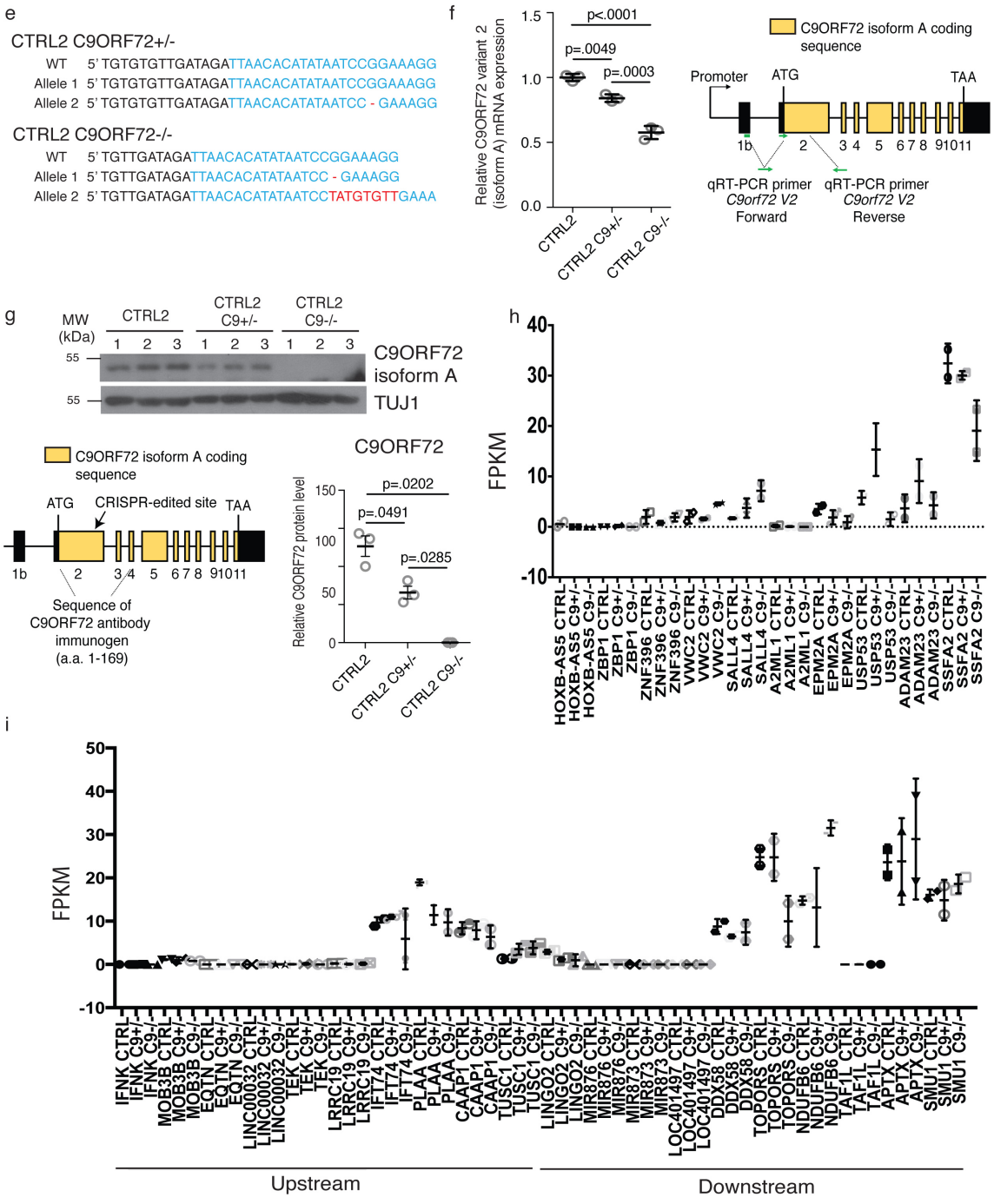
Supplementary Figure 3, related to Figures 1 and 2. *C9ORF72* iMNs Display Neurodegenerative Phenotypes. **a-f,** Representative survival curves of control and C9-ALS iMNs with neurotrophic factors (survival curves for individual lines shown) plus excess glutamate (a-c, e), with the live imaging performed in a Biostation CT for (a, d, e, f) and a Molecular Devices ImageExpress for (b) and (c), with glutamate receptor antagonists (3I) (d), or without neurotrophic factors (f). iMNs from two independent clones of the same donor (C9-ALS3) gave consistent results in this assay (**c**). iMN survival curves for individual lines cultured in excess glutamate with glutamate receptor antagonists are shown **(d)**. iMN survival curves for individual lines (including additional lines not included in Figure 1) cultured in excess glutamate **(e)**. iMN survival curves for individual lines included in Figure 1, cultured without neurotrophic factors are shown **(f)**. For (a), n=50 iMNs per line quantified from 3 biologically independent iMN conversions. For (b), n=50 iMNs per line quantified from 2 control or 2 C9-ALS lines and from 3 3 biologically independent iMN conversions per line. For (c) n=50 iMNs per line quantified from 3 biologically independent iMN conversions per line. For (d), n=50 iMNs per line quantified from 3 biologically independent iMN conversions per line. For (e), n=50 iMNs per control line, or 50, 31, or 40 iMNs per C9-ALS line quantified from 3 biologically independent iMN conversions per line. For (f), n=50 iMNs per line quantified from 3 biologically independent iMN conversions per line. **g,** ISL1+/MAP2+ iPSC-derived motor neurons generated using retinoic acid and purmorphamine. Scale bar: 30 μm. This experiment was performed 3 times with similar results. **h,** Control and C9-ALS iPSC-derived motor neurons labeled with the lentiviral *Hb9*::RFP reporter. This experiment was performed 3 times with similar results. Scale bar: 30 μm. **i-j,** Survival curves for motor neurons derived from iPSCs using soluble morphogens and cultured in excess glutamate. Survival curves of neurons from 4 controls and 4 C9-ALS patients (i) or from control and *C9ORF72*$^{+/-}$ iPSCs (j) are shown in aggregate. For (i), n=1075 motor neurons quantified from 4 lines per genotype and 3 biologically independent motor neuron cultures per line. For (j), n=100 motor neurons per line quantified from 3 biologically independent motor neuron cultures per line. **k-l,** Survival curves for motor neruons derived from iPSCs using soluble morphogens and cultured without neurotrophic factors. Survival curves of neurons from 4 controls and 4 C9-ALS patients (k) or from control and *C9ORF72*$^{+/-}$ iPSCs (l) are shown in aggregate. For (k), n=1075 motor neurons quantified from 4 lines per genotype and 3 biologically independent motor neuron cultures per line. For (l), n=100 motor neurons per line quantified from 3 biologically independent motor neuron cultures per line. **m,** Generation of TH+ dopaminergic neurons from control and *C9ORF72* patients. The induced neurons express the DA neuron marker tyrosine dehydroxylase (TH, green), as well as the neuronal marker TUJ1 (red) and DsRed (blue, co-infected with the iDA factors to mark transduced cells). Scale bar: 10 μm. This experiment was performed twice with similar results. **n,** The percentage of DsRed-labeled cells that express TH is comparable between control and C9-ALS genotypes. Mean ± s.e.m. n=3 biologically independent iDA neuron conversions, 32 cells quantified per conversions, two-tailed t-test). **o,** Survival of induced TH+ (iDA) neurons in excess glutamate. n=50 iDA neurons per line, 2 lines per genotype and 3 biologically independent iDA neuron conversions per line. All iMN survival experiments were statistically analyzed using a two-sided log-rank test. iMN survival experiments in (**a, d, e, and f)** were performed in a Nikon Biostation, and **(b, c, i-l, and o)** were performed in a

Molecular Devices ImageExpress. For iMN survival experiments, if iMNs from more than one iPSC line were combined into one curve to improve clarity, the number of iPSC lines is indicated in the legend.

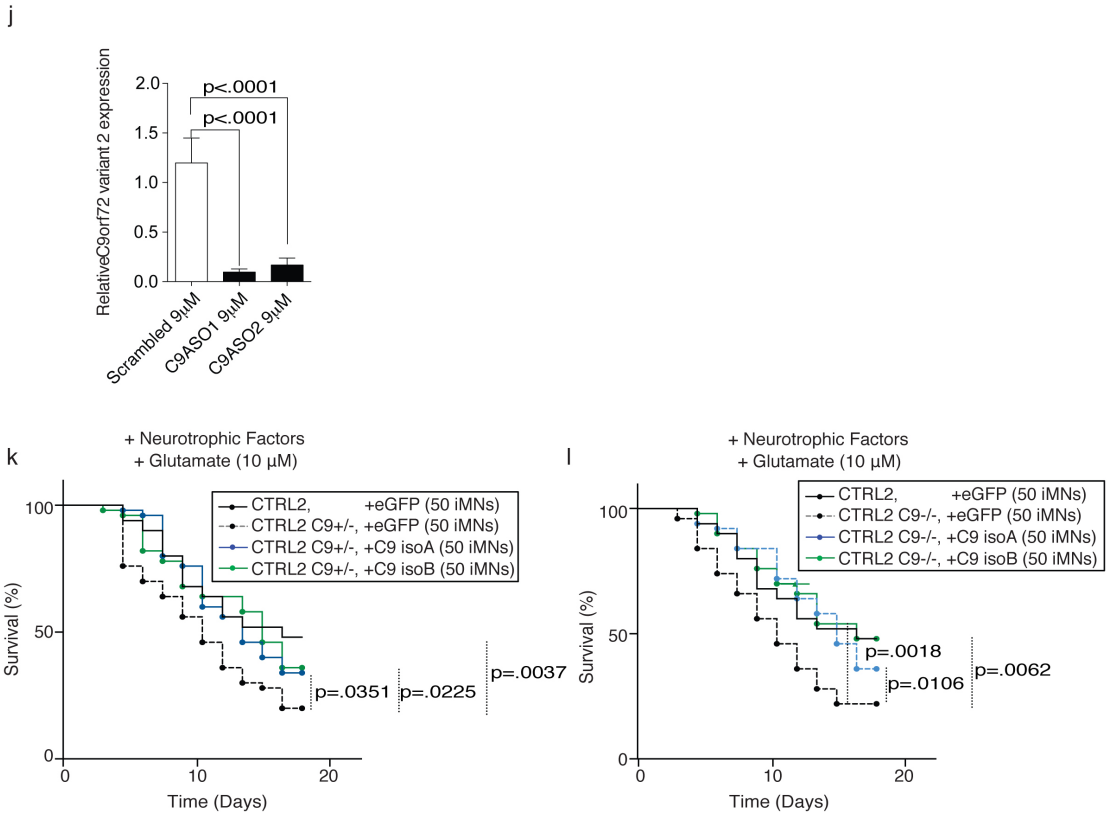
Supplementary Figure 4a-d. C9ORF72 Levels Modulate iMN Survival



Supplementary Figure 4 e-i. C9ORF72 Levels Modulate iMN Survival

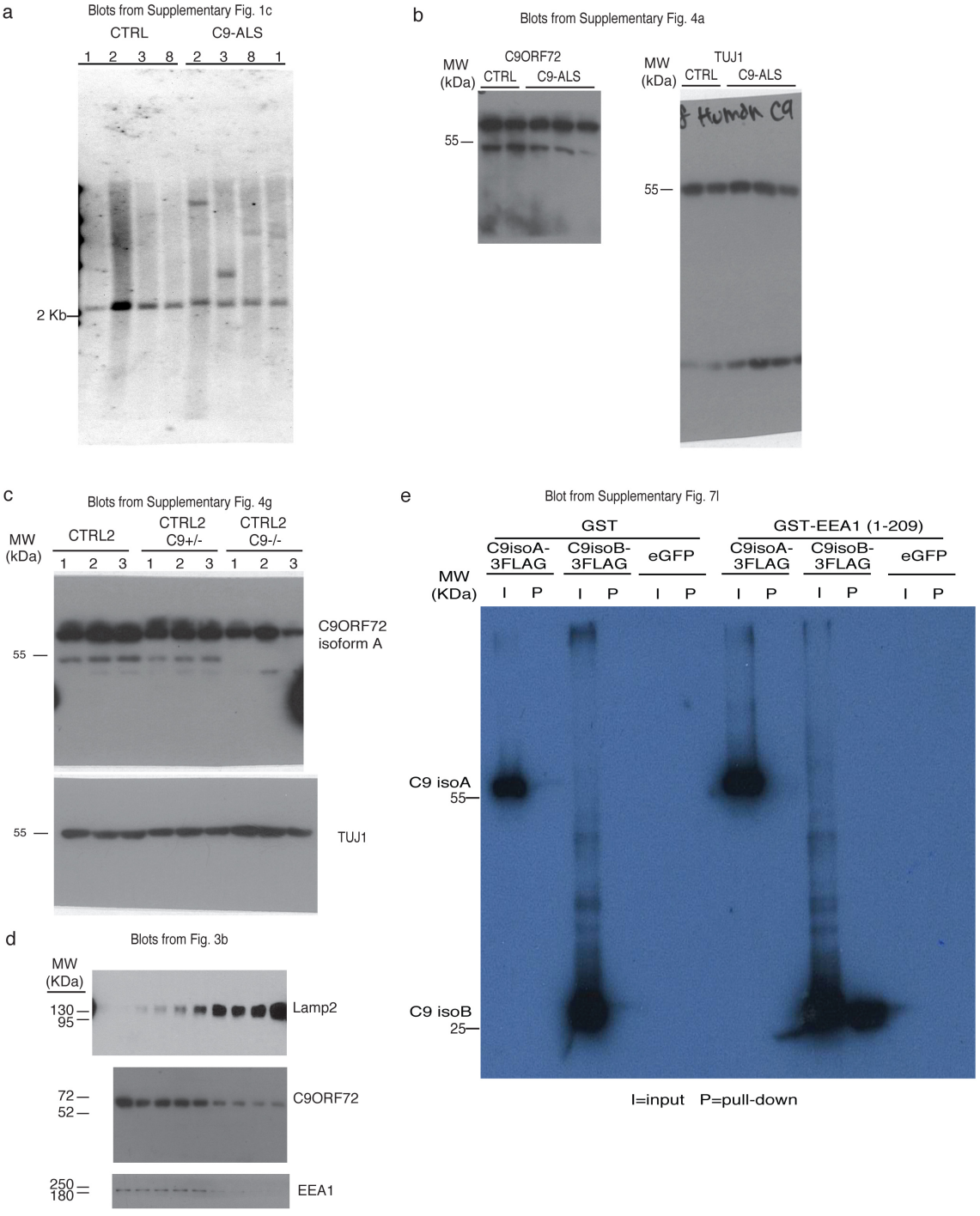


Supplementary Figure 4j to l. C9ORF72 Levels Modulate iMN Survival

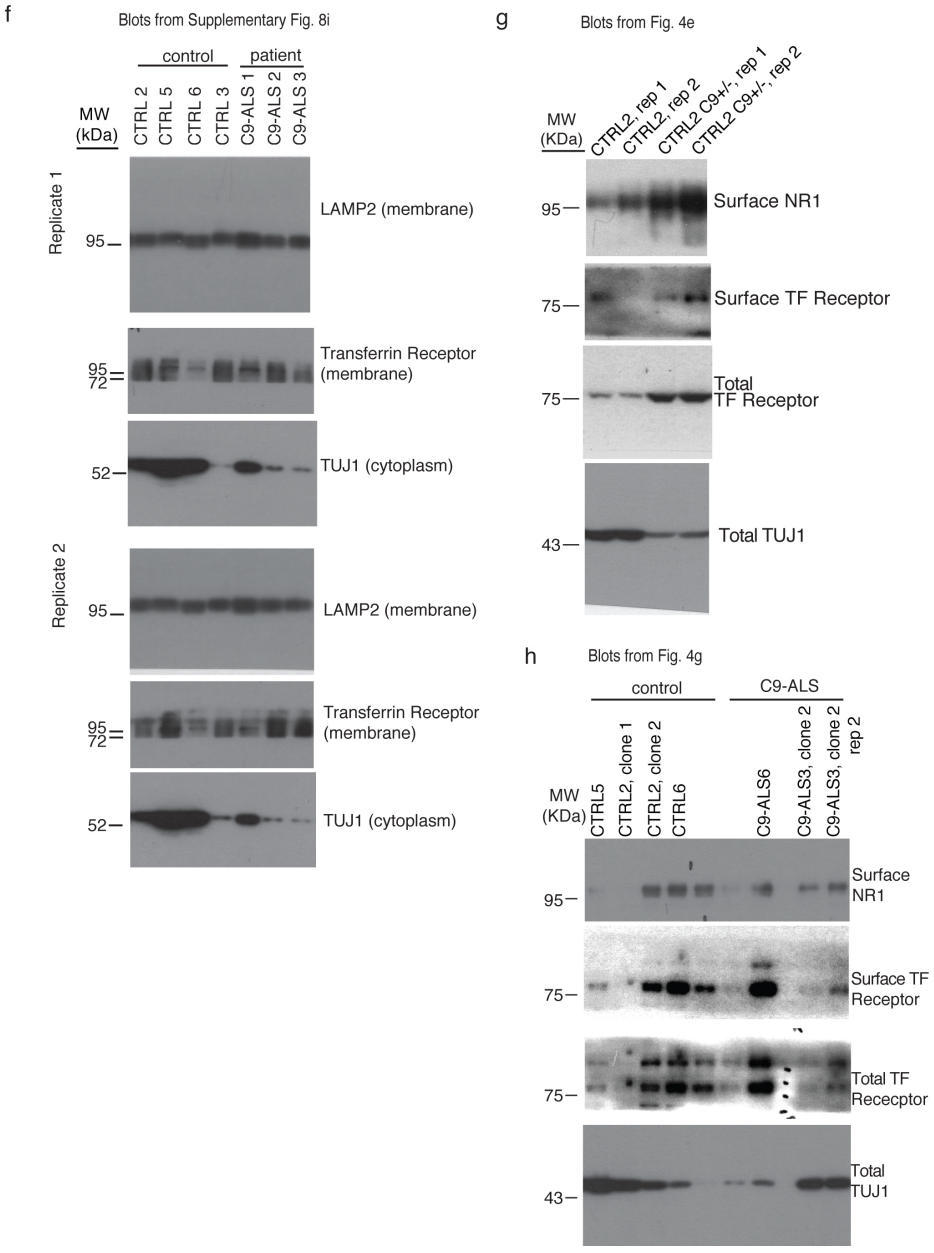


Supplementary Figure 4, related to Figure 2. C9ORF72 Levels Modulate iMN Survival. **a,** Western blot and quantification of C9ORF72 isoform A levels in control (n=2) and patient (n=3) motor neurons. Mean +/- s.d. **b,** Retroviral construct used in overexpression studies, *C9ORF72* mRNA expression levels (relative to *GAPDH*) in patient iMNs overexpressing eGFP or C9ORF72 (isoform A or B) and western blot verification of C9ORF72 protein production from retroviral constructs encoding an N-terminal FLAG-tagged version of C9ORF72 isoform A or B (to confirm specific detection of C9ORF72 protein) upon transduction into HEK cells. Full blots shown. qRT-PCR: Mean± s.e.m. n = 3 biologically independent iMN conversions per condition. **c-d,** Survival of iMNs in excess glutamate, either with C9ORF72 (isoform A or B) or eGFP overexpression. Expression of exogenous C9ORF72 does not enhance control iMN survival (2 independent lines shown) (**d**), but rescues the survival of C9-ALS2 iMNs (3 independent lines shown)(**c**). n=50 iMNs per condition except for C9-ALS3, where n=50, 37, and 29 iMNs for +GFP, +isoA, and +isoB, respectively. iMNs were quantified from 3 biologically independent iMN conversions. **e,** Sanger sequencing results of exon 2 of CRISPR/Cas9-mutated subclones of the CTRL2 iPSC line, verifying frameshift mutations. **f,** qRT-PCR analysis of the levels of *C9ORF72* variant 2 in control, *C9ORF72*$^{+/-}$, and C9ORF72$^{-/-}$ flow-sorted iMNs. The diagram depicts the qRT-PCR primer binding sites and the coding sequence for C9ORF72 isoform A. n=3 biologically independent iMN conversions per line. Mean +/- s.d. One-way ANOVA with Tukey correction. F-value (DFn, DFd): (2, 6) = 97.97. n=3 biologically independent motor neuron differentiations per line. **g,** Western blot analysis of C9ORF72 isoform A levels in motor neurons derived from control (CTRL2), *C9ORF72*$^{+/-}$, and C9ORF72$^{-/-}$ iPSCs. Quantification performed on n=3 biologically independent motor neuron differentiations for each genotype in the western blot, one-way ANOVA with Tukey correction for all comparisons. F-value (DFn, DFd): (1.528, 3.055) = 67.78. Mean +/- s.e.m. The CRISPR/Cas9 gene-edited site and the peptide sequence of C9ORF72 (aa 1-169) that was used to generate the C9ORF72 antibody (Proteintech) are shown. **h-i,** RNA-sequencing (RNA-seq) analysis of iMNs of the starting control genotype (CTRL2) and isogenic *C9ORF72* mutants (C9+/-, heterozygous; C9-/-, homozygous) generated using CRISPR/Cas9. No significant changes in gene expression are observed in the top 10 genes predicted to have possible off-target sites for the sgRNAs used (h). Likewise, expression of most genes immediately upstream or downstream of *C9ORF72* (10 genes in either direction) are not altered in the mutants (i). Values are the mean of 2 biologically independent iMN conversions. Mean ± s.d. (h, i). FPKM, fragments of kilobase of exon per million fragments mapped; n.s., not significant. **j,** qRT-PCR analysis of iMN cultures treated with a scrambled ASO or ASOs targeting *C9ORF72*. Graphs show the mean of 3 biological replicates ± s.e.m. n=3 biologically independent iMN conversions per condition. One-way ANOVA with Tukey correction for all comparisons. F-value (DFn, DFd): (2, 14) = 19.62. **k-l,** Survival of iMNs in excess glutamate, either with C9ORF72 (isoform A or B) or eGFP overexpression. Expression of exogenous C9ORF72 rescues the survival of CTRL2 iMNs with C9 mutations: CTRL2 C9ORF72$^{+/-}$ **(k)** and CTRL2 C9ORF72$^{-/-}$**(l)**. n=50 iMNs per condition quantified from 3 biologically independent iMN conversions. All iMN survival statistical analysis was performed using a two-sided log-rank test. iMN survival experiments in **(c and d)** were performed in a Nikon Biostation, and **(k and l)** were performed in a Molecular Devices ImageExpress.

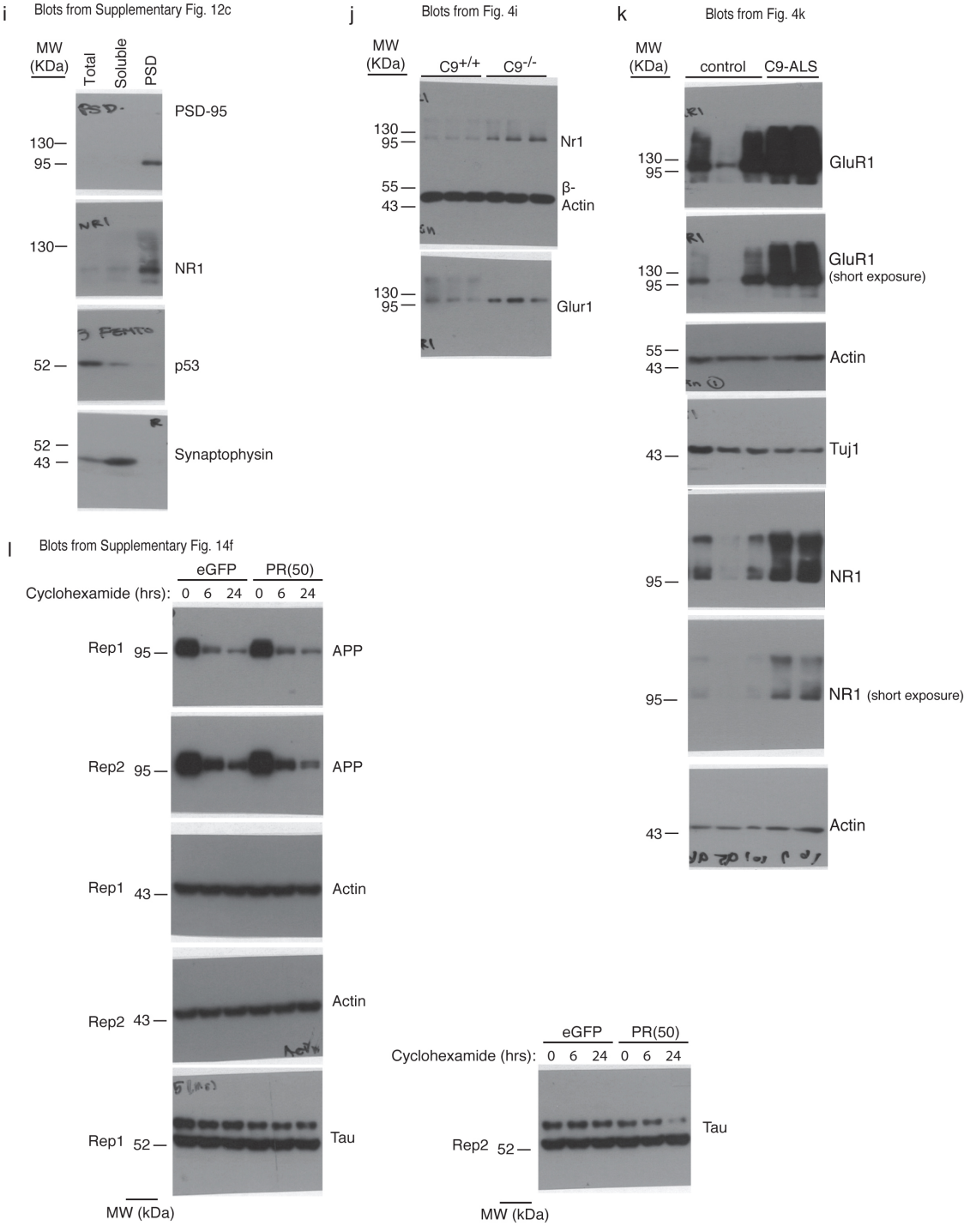
Supplementary Figure 5. Full scans of blots for all figures in which the full blot was not shown



Supplementary Figure 5. Full scans of blots for all figures in which the full blot was not shown

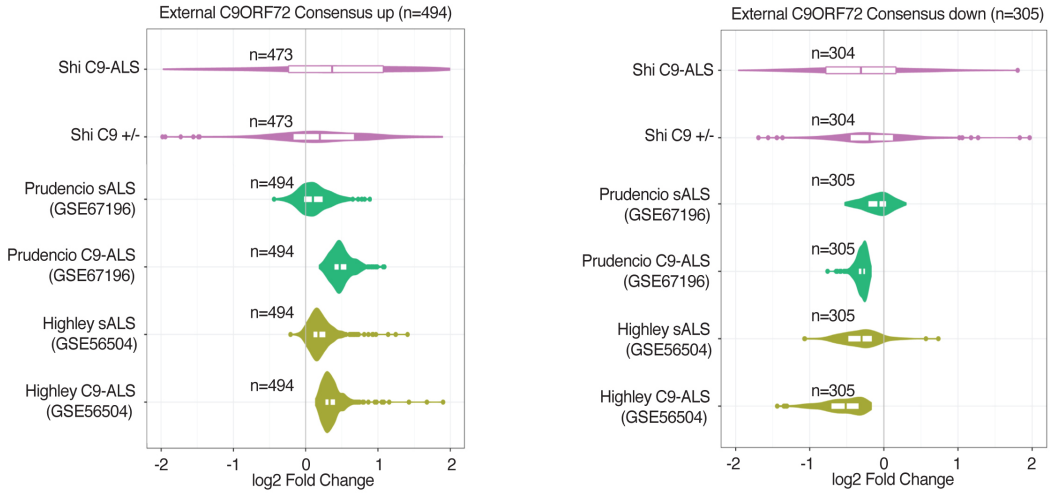


Supplementary Figure 5. Full scans of blots for all figures in which the full blot was not shown



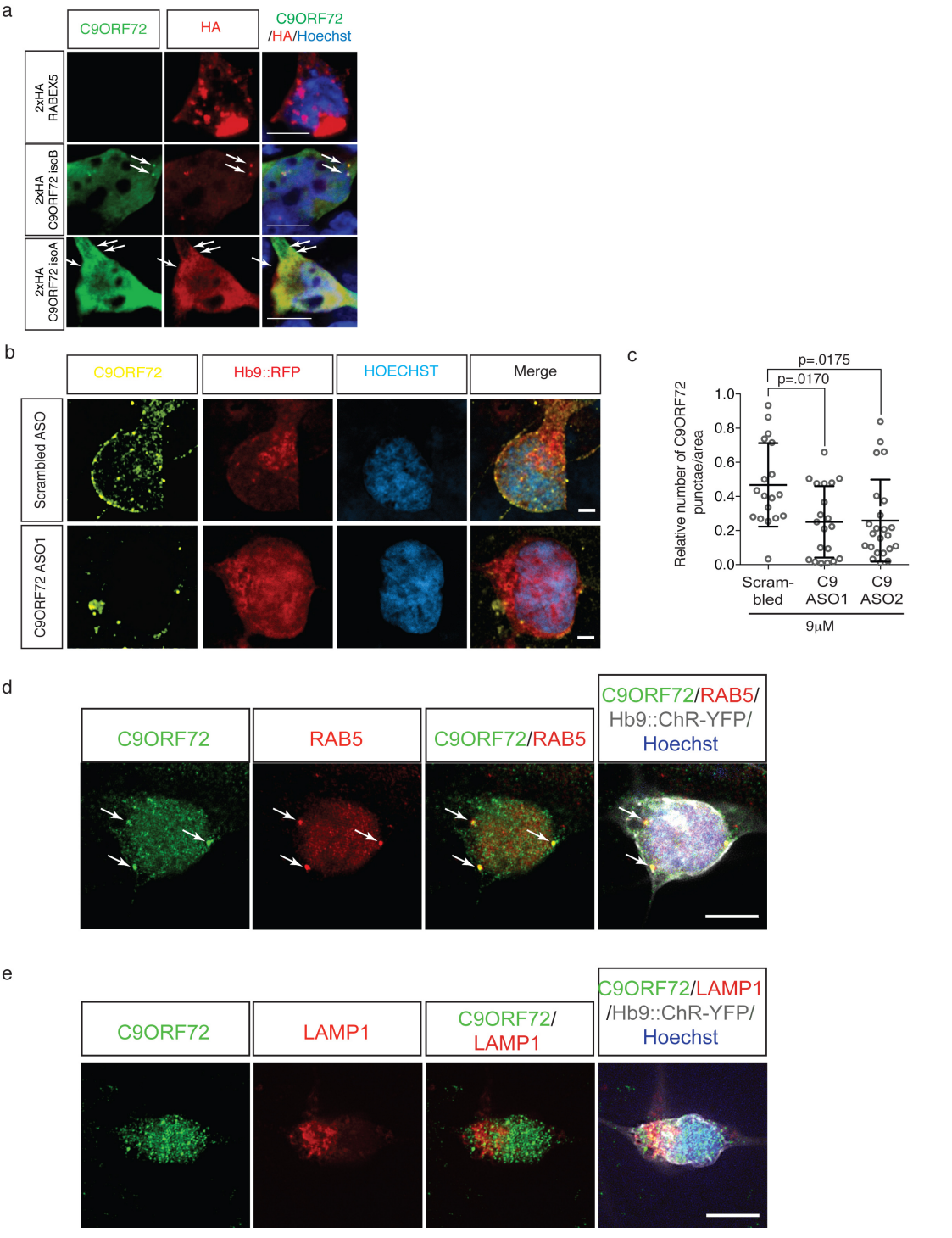
Supplementary Data Figure 5, related to Multiple Figures. Full Scans of Blots for Figures in Which the Full Blot Was Not Shown. Blots for the following figures are shown: **a,** Supplementary Fig. 1c, **b,** Supplementary Fig. 4a, **c,** Supplementary Fig. 4g, **d,** Fig. 3b, **e,** Supplementary Fig. 7l, **f,** Supplementary Fig. 8i, **g,** Fig. 4e, **h,** Fig. 4g, **i,** Supplementary Fig. 12c, **j,** Fig. 4i, **k,** Fig. 4k, **l,** Supplementary Fig. 14f.

Supplementary Figure 6. Gene expression analysis of *C9ORF72* postmortem and *C9ORF72* patient and *C9ORF72*+/- iMN samples.

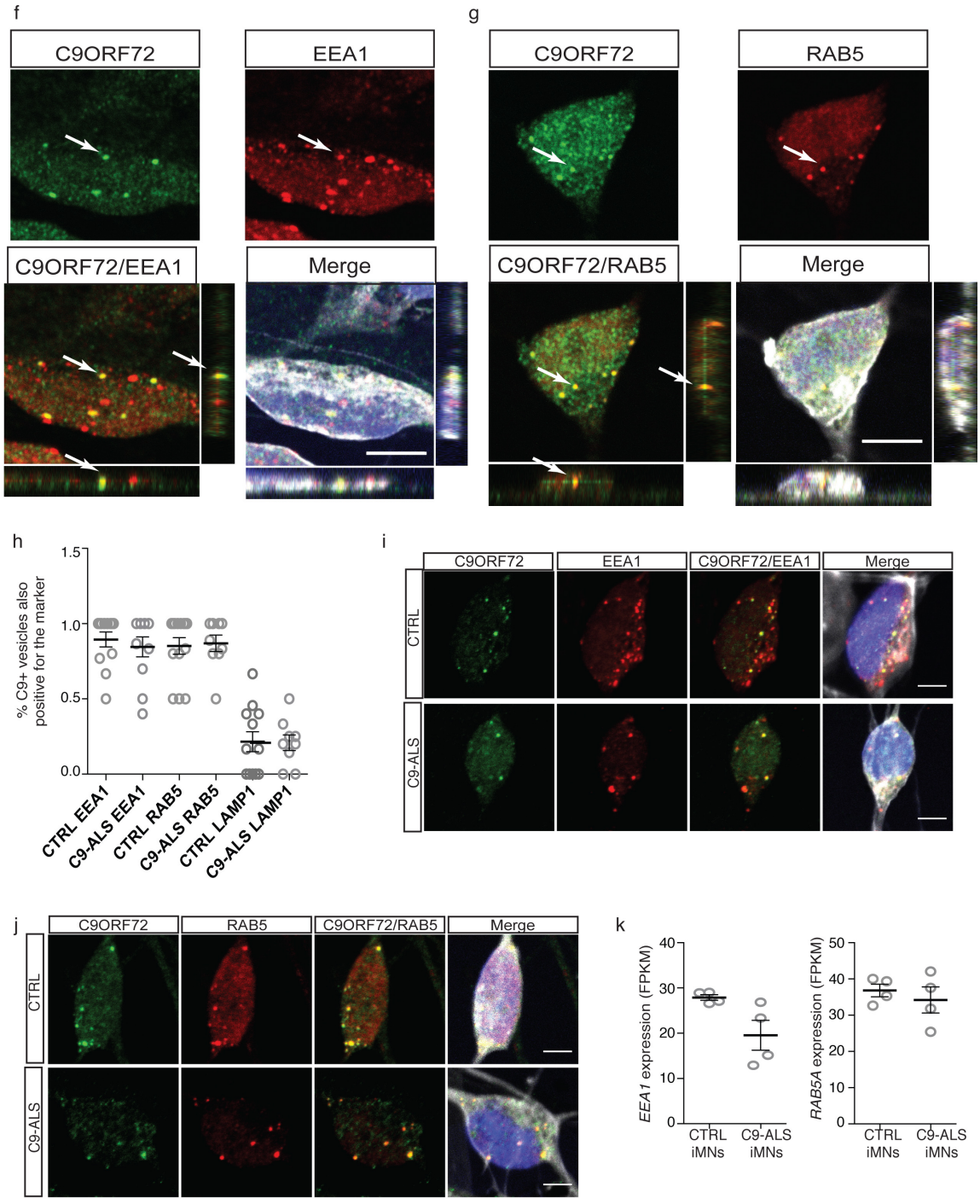


Supplementary Figure 6, related to Figure 2. Gene expression analysis of C9ORF72 postmortem and *C9ORF72* patient and *C9ORF72*$^{+/-}$ iMN samples. The samples used were iMNs from control iMNs, *C9ORF72* ALS patient iMNs, and *C9ORF72*$^{+/-}$ iMNs, frontal cortex from *C9ORF72* and sporadic ALS patients (GSE67196), and LCM motor neurons from *C9ORF72* and sporadic ALS patients (GSE56504). Sporadic patient data is included as a reference. n=number of consensus genes analyzed, which were identified by DESeq2 analysis as being significantly differentially expressed ($p<.05$) in all postmortem *C9ORF72* patient datasets. Sample numbers: iMNs, this study: N=2 biologically independent iMN conversions each for control, *C9ORF72*$^{+/-}$ and *C9ORF72*$^{-/-}$. GSE67196: N=9 healthy controls, 8 *C9ORF72* patients, 11 sporadic ALS patients. GSE56504: N=6 healthy controls, 3 *C9ORF72* patients, 3 sporadic ALS patients.

Supplementary Figure 7 (a-e). Super-Resolution Microscopy to Determine C9ORF72 Localization in iMNs

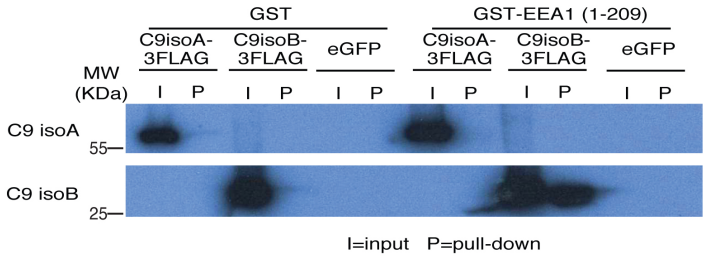


Supplementary Figure 7 (f-k). Super-Resolution Microscopy to Determine C9ORF72 Localization in iMNs



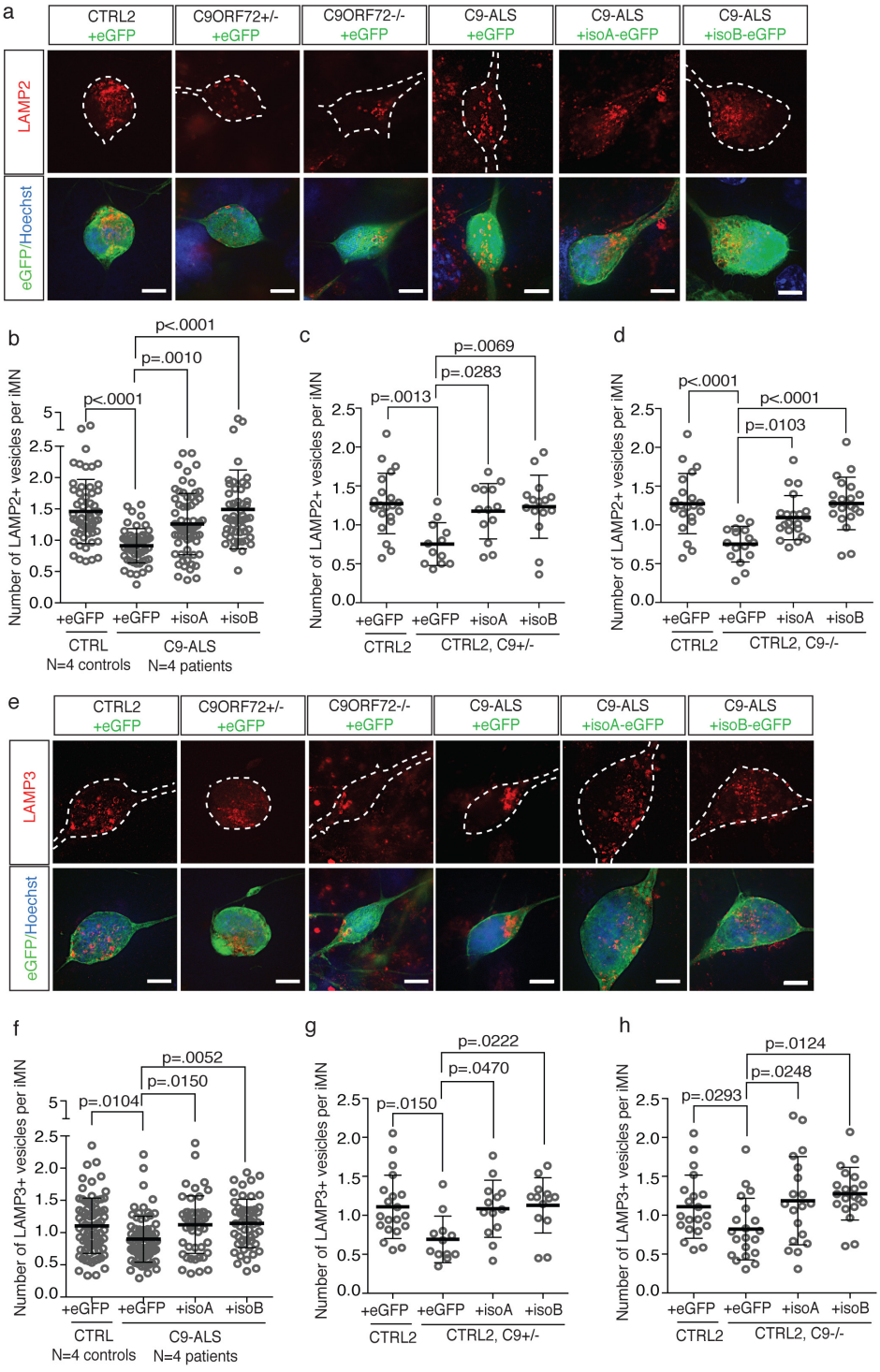
Supplementary Figure 7 (l). Super-Resolution Microscopy to Determine C9ORF72 Localization in iMNs

l

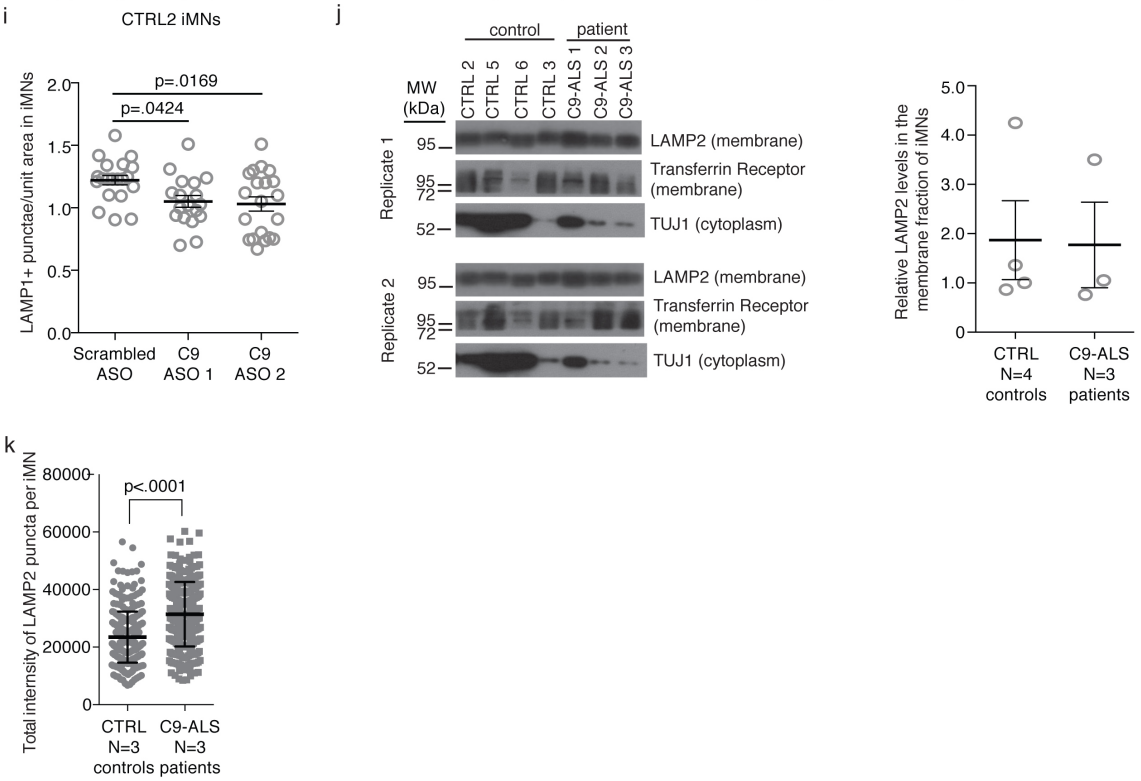


Supplementary Figure 7, related to Figure 3. Super-resolution Microscopy to Determine C9ORF72 Localization in iMNs. **a,** Confocal images of HEK293T-overexpressing HA-tagged RABEX5 or C9ORF72 (isoform A or B), showing the expression of the exogenous protein; HA (red) and C9ORF72 (green). Nuclei (blue) are labeled with Hoechst. White arrows denote areas of overlap between anti-C9ORF72 and anti-HA immunostaining. Scale bars: 10 μm **b,** Confocal images and **c,** quantification of C9ORF72+ puncta in iMNs treated with scrambled or *C9ORF72*-targeting ASOs. One way-ANOVA with Tukey correction across all comparisons F-value (DFn, DFd): (2, 58) = 5.322. n= 18, 20, 24 iMNs quantified from two biologically independent iMN conversions in the scrambled ASO, *C9ORF72* ASO1, and *C9ORF72* ASO2 conditions, respectively. Mean +/- s.d. Scale bars: 2 μm. **d–e,** Super-resolution microscopy images of control iMNs which express *Hb9::*ChR-YFP and show colocalization (arrows) of C9ORF72 (green) with RAB5 (**d**, red) but not with the lysosomal marker LAMP1 (**e**, red). Scale bars: 5 μm **f-g,** Confocal Z-axis scanning to determine C9ORF72 localization. C9ORF72 (green) colocalizes with EEA1 (**f,** red) and RAB5 (**g,** red) in 3-dimensional space. Nuclei are labeled with DAPI (blue). Scale bars: 5 μm. **h,** Quantification of the percent of $C9ORF72^{+}$ vesicles also labeled with various vesicle markers in control and C9-ALS iMNs. n=12 control iMNs and 11 C9-ALS iMNs (C9/EEA1), 14 control iMNs and 9 C9-ALS iMNs (C9/RAB5), and 12 control iMNs and 9 C9-ALS iMNs (C9/LAMP1) quantified from two biologically independent iMN conversions. Mean +/- s.e.m. **i-j,** Representative pictures showing the localization of $EEA1^{+}$ (i) and $RAB5^{+}$ (j) vesicles in control and patient iMNs. Scale bars: 5 μm. **k,** mRNA expression of *EEA1* and *RAB5A* in flow-purified control and C9-ALS iMNs. n=4 biologically independent iMN conversions per genotype. Mean +/- s.e.m. **l,** Western blot analysis of a pull-down experiment to examine the potentials of C9ORF72 isoform A and B to bind to an immobilized N-terminal fragment of EEA1. I = input, P = pull-down. The blot was probed with an anti-FLAG antibody. All immunostaining and immunoblotting assays were performed and quantified using 2 biological replicates/individual differentiations.

Supp. Figure 8(a-h). Reduced C9ORF72 activity disrupts vesicle trafficking and lysosomal biogenesis in iMNs

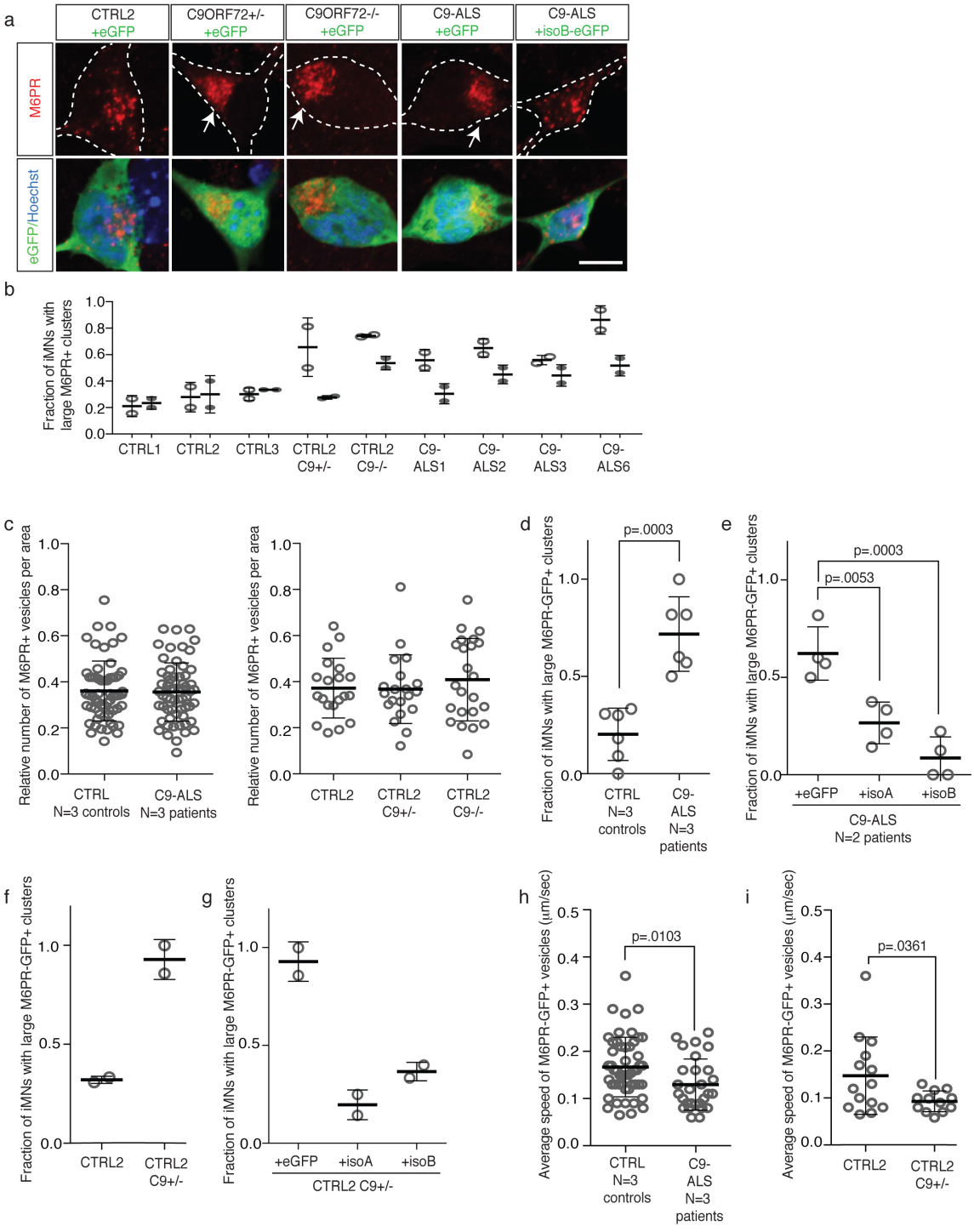


Supp. Figure 8(i-k). Reduced C9ORF72 activity disrupts vesicle trafficking and lysosomal biogenesis in iMNs

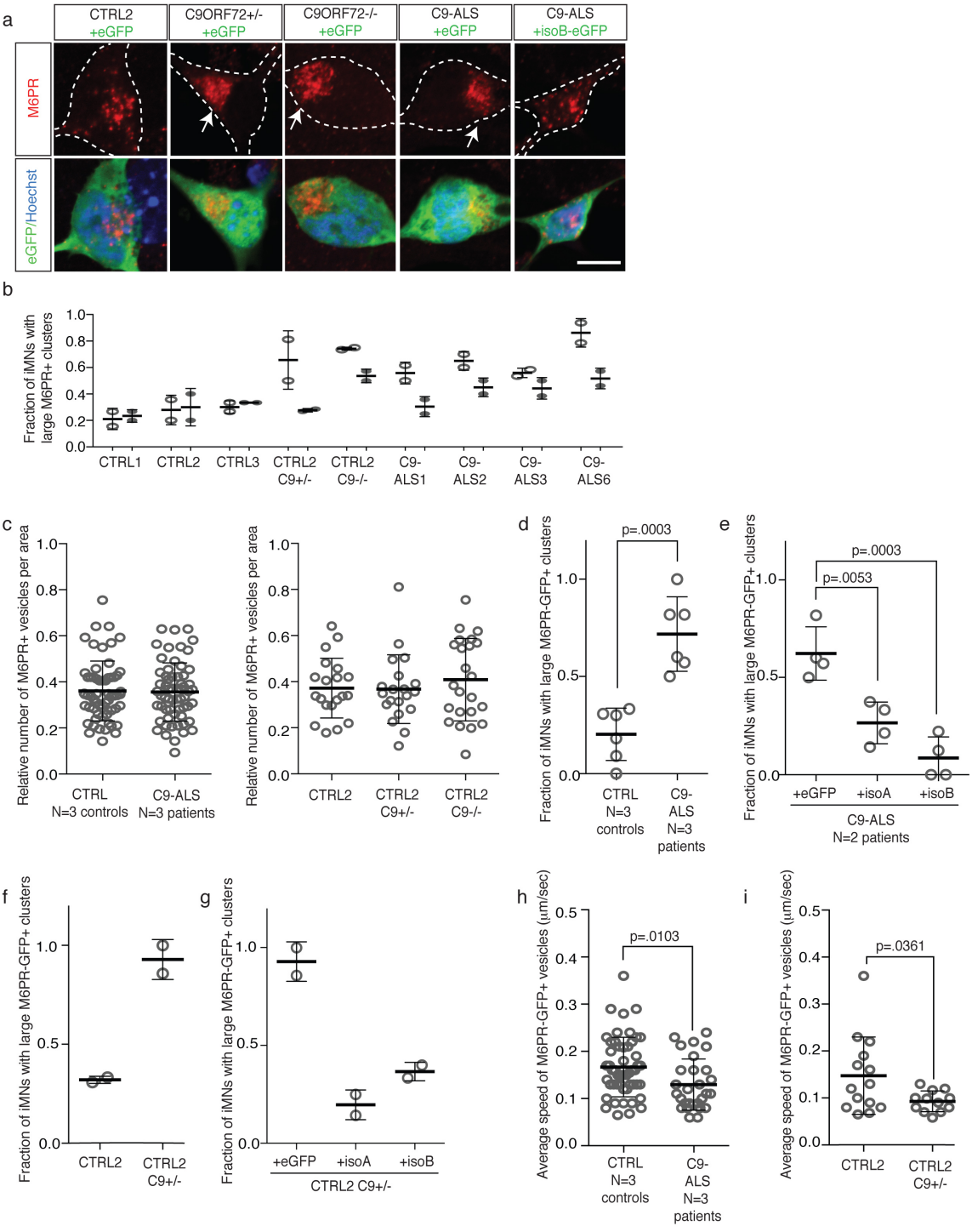


Supplementary Figure 8, related to Figure 3. Reduced C9ORF72 activity disrupts vesicle trafficking and lysosomal biogenesis in iMNs. **a,** LAMP2 immunostaining in iMNs of specified genotypes expressing eGFP or C9ORF72 (isoform A or B)-eGFP. Scale bar: 5 µm. Dotted lines outline iMNs. **(b-d)** Number of $LAMP2^{+}$ vesicles in control (b-d), patient (b), *C9ORF72*$^{+/-}$ (c), and *C9ORF72*$^{-/-}$ (d) iMNs overexpressing eGFP or C9ORF72 (isoform A or B)-eGFP. Mean ± s.d., each grey open circle represents a single iMN). n=80 iMNs per genotype from 4 different control or C9-ALS lines quantified from two biologically independent iMN conversions per condition (b), n=20 (CTRL + GFP), 12 (*C9ORF72*$^{+/-}$ + GFP), 13 (*C9ORF72*$^{+/-}$ + isoA), and 15 (*C9ORF72*$^{+/-}$ + isoB) iMNs from two biologically independent iMN conversions per condition (c), n=20 iMNs from two biologically independent iMN conversions per genotype per condition (d). For (b), one way-ANOVA with Tukey correction between control and patient conditions, F-value (DFn, DFd): (3, 122) = 15.58. For (c), one way-ANOVA with Tukey correction between CTRL2 and *C9ORF72*$^{+/-}$, F-value (DFn, DFd): (3, 57) = 5.762. For (d), one way-ANOVA with Tukey correction between CTRL2 and *C9ORF72*$^{-/-}$, F-value (DFn, DFd): (3, 74) = 10.27. **e,** LAMP3 immunostaining in iMNs of specified genotypes expressing eGFP or C9ORF72 (isoform A or B)-eGFP. Scale bar: 5 µm. Dotted lines outline iMNs. **(f-h)** Number of $LAMP3^{+}$ vesicles in control (f-h), patient (f), *C9ORF72*$^{+/-}$ (g), and *C9ORF72*$^{-/-}$ (h) iMNs overexpressing eGFP or C9ORF72 (isoform A or B)-eGFP (mean ± s.d. of iMNs from two biological replicates, each grey open circle represents a single iMN). n=80 iMNs per genotype from 4 different control or C9-ALS lines quantified from two biologically independent iMN conversions per condition (f), n=20 (CTRL + GFP), 12 (*C9ORF72*$^{+/-}$ + GFP), 13 (*C9ORF72*$^{+/-}$ + isoA), and 15 (*C9ORF72*$^{+/-}$ + isoB) iMNs from two biologically independent iMN conversions per condition (g), n=20 iMNs from two biologically independent iMN conversions per genotype per condition (h). For (f), one way-ANOVA with Tukey correction between control and patient conditions, F-value (DFn, DFd): (3, 247) = 5.329. For (g), one way-ANOVA with Tukey correction between CTRL2 and *C9ORF72*$^{+/-}$, F-value (DFn, DFd): (3, 54) = 4.112. For (h), one way-ANOVA with Tukey correction between CTRL2 and *C9ORF72*$^{-/-}$, F-value (DFn, DFd): (3, 75) = 4.11. **i,** Quantification of LAMP1 immunostaining in control iMNs treated with scrambled or *C9ORF72*-tageting ASOs. Data points represent individual cells. One way-ANOVA with Tukey correction across all comparisons F-value (DFn, DFd): (2, 55) = 4.832. n= 20 (+GFP), 18 (+isoA), and 20 (+isoB) iMNs quantified from two biologically independent iMN conversions in each ASO condition. Mean +/- s.e.m. **j,** Immunoblotting and quantification of LAMP2 levels in the membrane fraction of control and C9-ALS patient iMNs. LAMP2 levels were normalized to cytosolic TUJ1 and membrane-bound Transferrin receptor levels to account for the number of neurons harvested and the efficiency of membrane extraction. One sample each from n=4 and 3 biologically independent samples from different control and C9-ALS lines, respectively. Two-tailed t-test between all controls combined *versus* all patients combined, t-value = 0.08058, degrees of freedom = 5. Mean +/- s.d. **k,** LAMP2 immunostaining intensity in individual $LAMP2^{+}$ vesicles iMNs of specified genotypes. n = 100 $LAMP2^{+}$ vesicles quantified within a total of 10 iMNs per line, 3 lines per genotype, 2 biologically independent iMN conversions per line. Two-tailed t-test. t-value: 9.571, degrees of freedom: 563.5. Mean +/- s.d. Each data point represents a single $LAMP2^{+}$ vesicle.

Supplementary Figure 9. Reduced C9ORF72 activity disrupts M6PR+ vesicle trafficking in iMNs

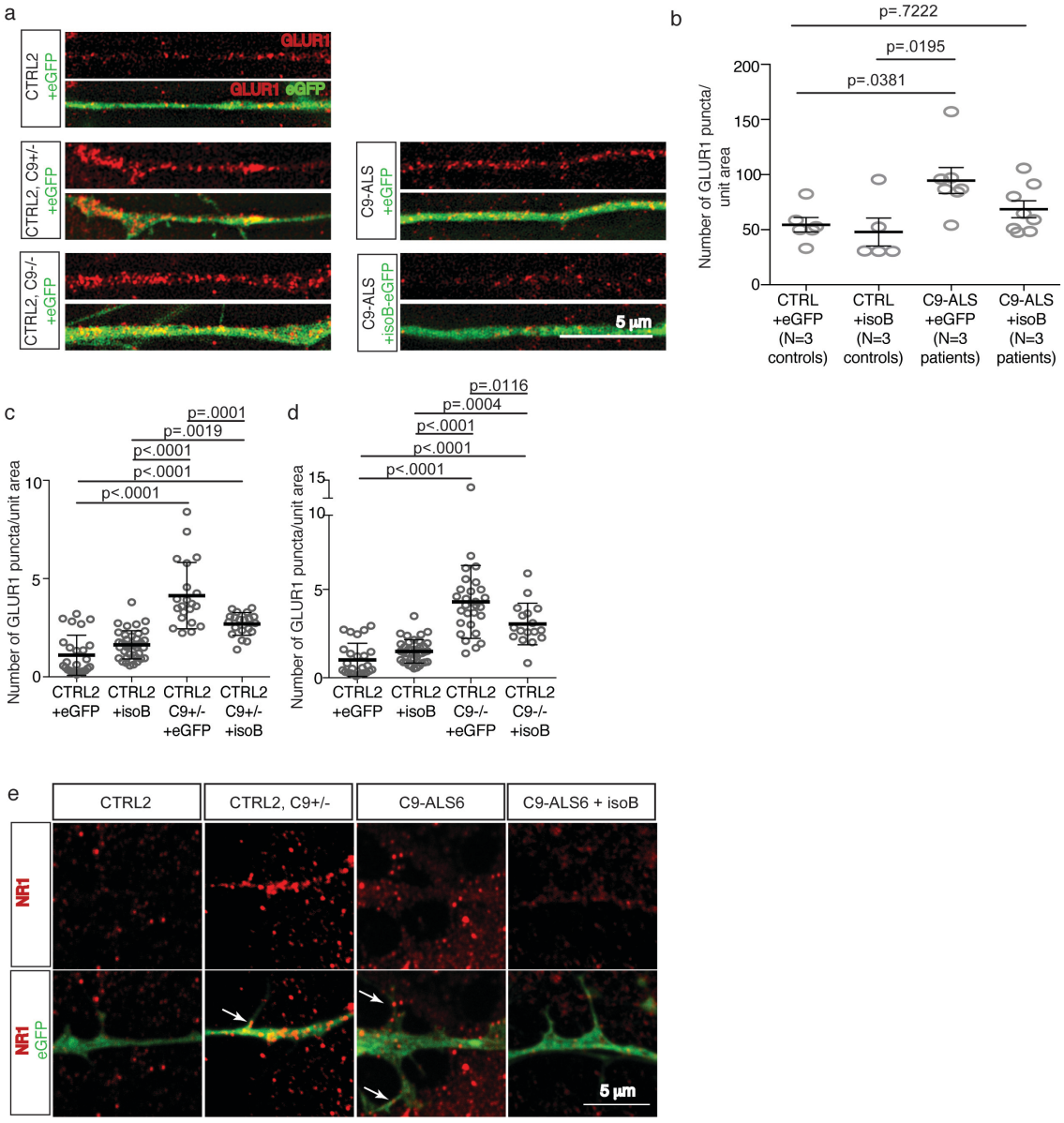


Supplementary Figure 9. Reduced C9ORF72 activity disrupts M6PR+ vesicle trafficking in iMNs

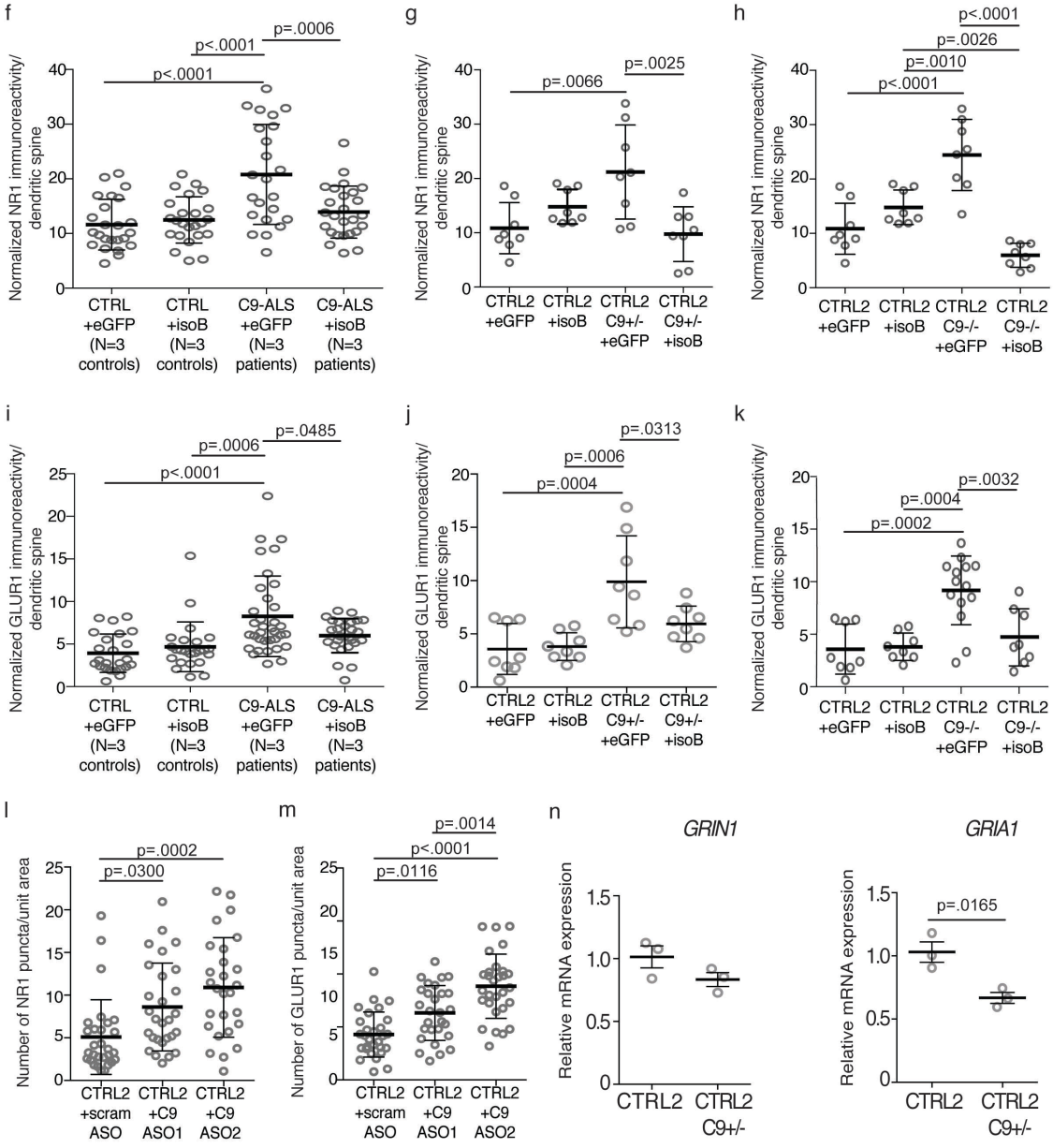


Supplementary Figure 9, related to Figure 3. Reduced C9ORF72 activity disrupts M6PR+ vesicle trafficking in iMNs. **a,** Confocal microscopy images of M6PR staining in iMNs of specified genotypes. Arrows denote large clusters of $M6PR^{+}$ vesicles. Scale bar: 5 µm. This experiment was repeated twice with similar results. Dotted lines outline iMNs. **b,** Fraction of iMNs containing large (>6 vesicles) clusters of $M6PR^{+}$ vesicles in control, patient, *C9ORF72*$^{+/-}$, and *C9ORF72*$^{-/-}$ iMNs overexpressing eGFP or C9ORF72 isoform B-eGFP. Mean ± s.d. n=2 biologically independent iMN conversions per condition, n=15 cells per conversion). **c,** The number of M6PR+ vesicles per cytoplasmic area in control, C9-ALS, *C9ORF72*$^{+/-}$, or *C9ORF72*$^{-/-}$ iMNs. n=55 iMNs from two biologically independent iMN conversions from 3 control lines from different donors and 60 iMNs from two biologically independent iMN conversions from 3 C9-ALS lines from different donors, two-tailed t-test, t-value = 0.1864, degrees of freedom = 119 for control vs C9-ALS comparison, and n=20 iMNs from two biologically independent iMN conversions from each line, one way-ANOVA with Tukey correction, F-value (DFn, DFd): (2, 60) = 0.4636 for the control vs *C9ORF72*$^{+/-}$ and *C9ORF72*$^{-/-}$ comparisons. Mean +/- s.d. **d-g,** The fraction of control (**d, f**), C9-ALS (**d, e**), and *C9ORF72*$^{+/-}$ (**f, g**) iMNs containing large M6PR-GFP+ clusters as determined by live imaging over a 1 minute time course. **e, g,** The fraction of C9-ALS or *C9ORF72*$^{+/-}$ iMNs containing large M6PR-GFP+ clusters when expressing GFP, C9ORF72 isoform A, or C9ORF72 isoform B as determined by live imaging over a 1 minute time course. (d) Two-tailed t-test with Welch's correction, t-value = 7.058, degrees of freedom = 86.89, N=6 biologically independent iMN conversions from 3 control lines and 3 C9-ALS lines from different donors (two biologically independent iMN conversions per line), 15 iMNs analyzed per conversion. (e) One-way ANOVA with Tukey correction, F-value (DFn, DFd): (2, 166) = 18.90, n=4 biologically independent iMN cultures derived from 2 different C9-ALS lines per condition (two biologically independent cultures per line), 15 iMNs analyzed per replicate, (f) n=2 biologically independent iMN cultures per genotype, 15 iMNs analyzed per culture, (g) n=2 biologically independent iMN cultures per condition, 15 iMNs analyzed per culture. Mean +/- s.d. for (d-g). **h,** The average speed of M6PR-GFP+ vesicles in control and C9-ALS iMNs as determined by live imaging over a 1 minute time course. Two-tailed t-test, t-value = 3.185, degrees of freedom = 147, n=80 iMNs per genotype from 4 different donors and two biologically independent iMN conversions per genotype. Mean +/- s.d. **i,** The average speed of M6PR-GFP+ vesicles in control and *C9ORF72*$^{+/-}$ iMNs as determined by live imaging over a 1 minute time course. Two-tailed t-test, t-value = 3.08, degrees of freedom = 30, n=20 iMNs for control and 12 iMNs for *C9ORF72*$^{+/-}$ from two biologically independent iMN conversions per genotype. Mean +/- s.d.

Supplementary Figure 10 (a-e). Reduced *C9ORF72* Expression Sensitizes iMNs to Glutamate



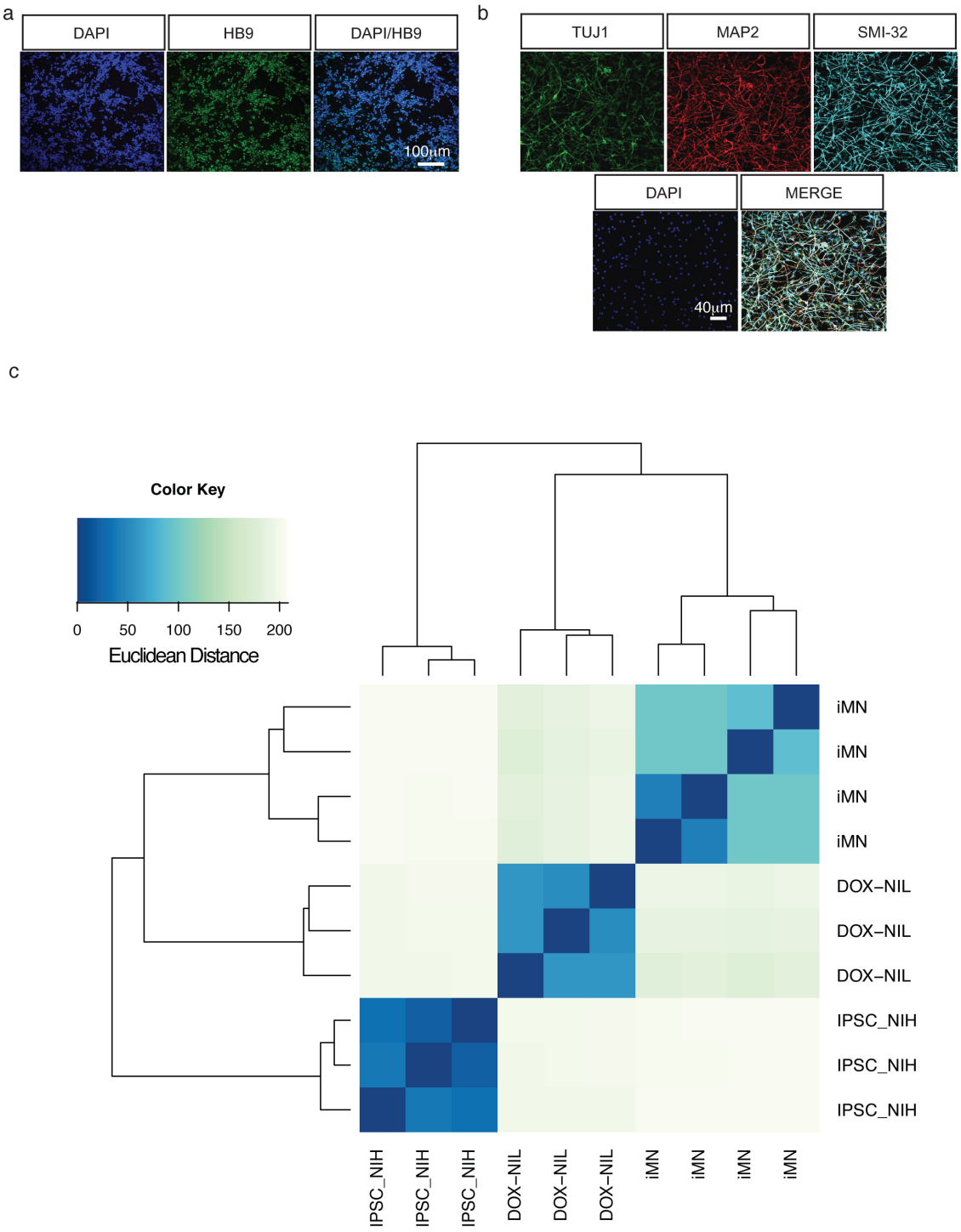
Supplementary Figure 10 (f-n). Reduced *C9ORF72* Expression Sensitizes iMNs to Glutamate



Supplementary Figure 10, related to Figure 4. Reduced C9ORF72 Expression Sensitizes iMNs to Glutamate. **a,** Super-resolution microscopy images of immunofluorescence shows GLUR1+ puncta on neurites of iMNs overexpressing eGFP or C9ORF72 isoform B-eGFP. Scale bar: 5 μm. **(b-d**) Number of GLUR1+ puncta per unit area in control (b-d), patient (b), *C9ORF72*$^{+/-}$ (c), and *C9ORF72*$^{+/-}$ (d) iMNs. For (b), n=6 biologically independent iMN conversions for control + GFP, 5biologically independent iMN conversions for control + isoB, 7 biologically independent iMN conversions for C9-ALS + GFP, and 8 biologically independent iMN conversions for C9-ALS + isoB, each from 3 different donor lines per genotype, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 22) = 4.361. Mean +/- s.e.m. For (c), n=26 iMNs for ctrl2 + GFP, 38 iMNs for ctrl2 + isoB, 21 iMNs for *C9ORF72*$^{+/-}$ + GFP, and 21 iMNs for *C9ORF72*$^{+/-}$ + isoB from two biologically independent iMN conversions per line, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 101) = 39.5. Mean +/- s.d. For (d), n=26 iMNs for ctrl2 + GFP, 38 iMNs for ctrl2 + isoB, 28 iMNs for *C9ORF72*$^{-/-}$ + GFP, and 17 iMNs for *C9ORF72*$^{-/-}$ + isoB from two biologically independent iMN conversions per line, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 105) = 37.65. Mean ± s.d., each grey open circle represents the number of GLUR1+ puncta per unit area on a single neurite (one neurite quantified per iMN). **e,** Super-resolution microscopy images of immunofluorescence shows NR1+ puncta (arrows) on dendritic spines of iMNs overexpressing eGFP or C9ORF72 isoform B-eGFP. Scale bar: 5 μm. **(f-h**) Flourescence intensity of NR1 per unit area on dendritic spines in control (f-h), patient (f), *C9ORF72*$^{+/-}$ (g), and *C9ORF72*$^{+/-}$ (h) iMNs. For (f), n=24 iMNs for control + GFP, 24 iMNs for control + isoB, 23 iMNs for C9-ALS + GFP, and 26 iMNs for C9-ALS + isoB from 3 different donor lines per genotype, two biologically independent iMN conversions per line, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 93) = 11.37. For (g), n=8 iMNs per condition from two biologically independent iMN conversions, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 28) = 6.418. For (h), n=8 iMNs per condition from two biologically independent iMN conversions, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 28) = 24.45. Mean ± s.d., each grey open circle represents the mean intensity of NR1+ puncta per unit area on spines from 3-9 dendrites on a single iMN. **i-k,** Flourescence intensity of GLUR1 per unit area on dendritic spines in control (i-k), patient (i), *C9ORF72*$^{+/-}$ (j), and *C9ORF72*$^{+/-}$ (k) iMNs. For (i), n=24 iMNs for control + GFP, 24 iMNs for control + isoB, 23 iMNs for C9-ALS + GFP, and 26 iMNs for C9-ALS + isoB from 3 different donor lines per genotype, two biologically independent iMN conversions per line, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 105) = 9.679. For (j), n=8 iMNs for control + GFP, 8 iMNs for control + isoB, 9 iMNs for *C9ORF72*$^{+/-}$ + GFP, and 8 iMNs for *C9ORF72*$^{+/-}$ + isoB from two biologically independent iMN conversions, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 28) = 9.453. For (k), n=8 iMNs for control + GFP, 8 iMNs for control + isoB, 14 iMNs for *C9ORF72*$^{-/-}$ + GFP, and 8 iMNs for *C9ORF72*$^{-/-}$ + isoB from two biologically independent iMN conversions, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 34) = 11.2. Mean ± s.d., each grey open circle represents the mean intensity of GLUR1+ puncta per unit area on spines from 3-9 dendrites on a single iMN. **l-m,** Number of NR1+ (l) and GLUR1+ (m) puncta per unit area on neurites on iMNs treated with a scrambled ASO or *C9ORF72*-targeting ASOs.

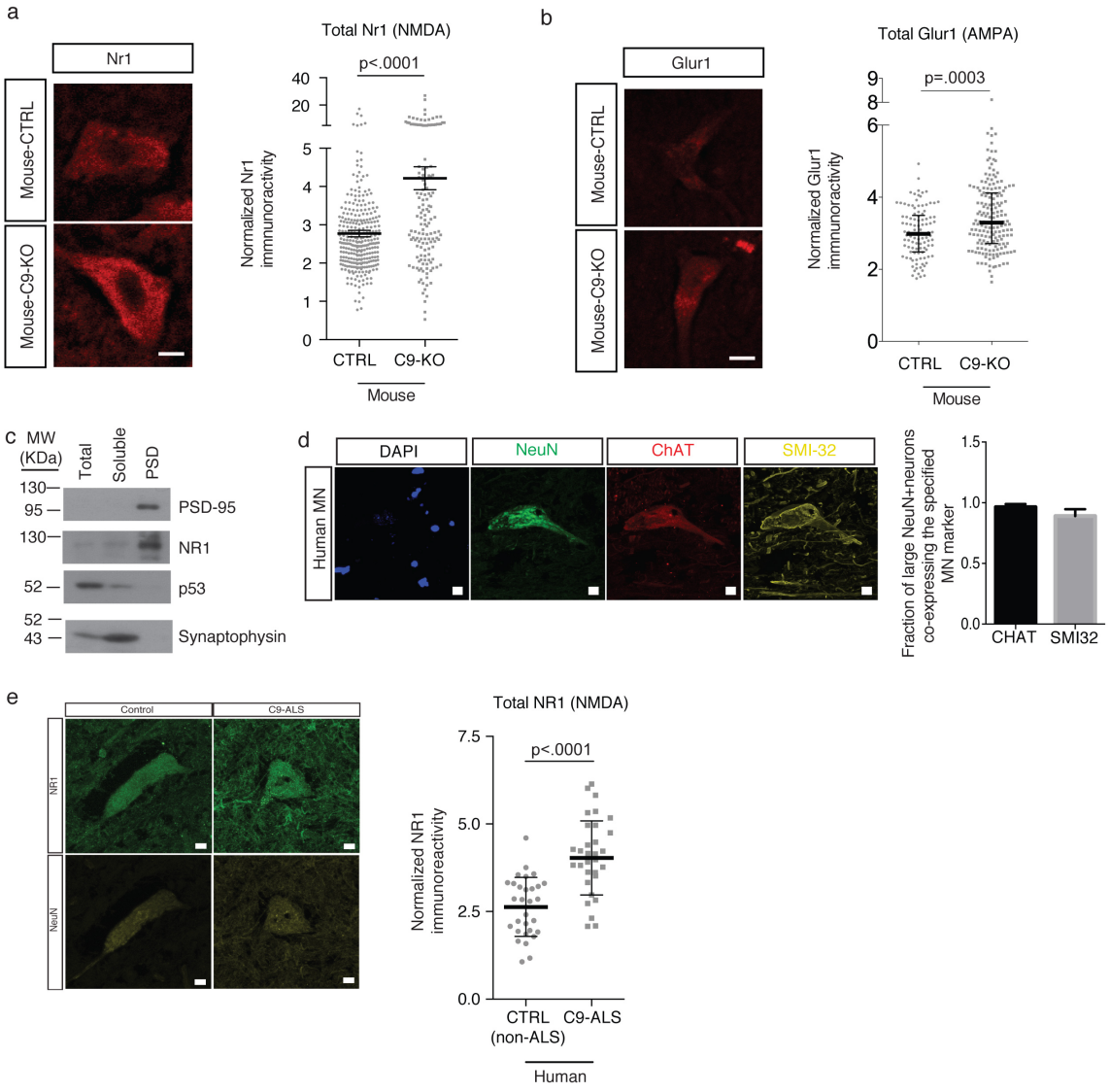
One way-ANOVA with Tukey correction across all comparisons. For (l), F-value (DFn, DFd): (2, 81) = 9.167. n= 29 (Scrambled ASO), 28 (C9 ASO1), and 27 (C9 ASO2) iMNs quantified from two biologically independent iMN conversions in each ASO condition. Mean +/- s.d. For (m), F-value (DFn, DFd): (2, 32) = 22.12. n= 29 (Scrambled ASO), 28 (C9 ASO1), and 30 (C9 ASO2) iMNs quantified from two biologically independent iMN conversions in each ASO condition. Mean +/- s.d. (Each grey open circle represents the number of NR1+ or GLUR1+ puncta per unit area on a single neurite (one neurite quantified per iMN). **n,** qRT-PCR analysis of *GRIN1* (NR1) and *GRIA1* (GLUR1) mRNA levels in *C9ORF72* wild-type and heterozygous iMNs. Mean ± s.e.m. n=3 biologically independent iMN conversions. Two-tailed t-test. t-value = 3.975, degrees of freedom = 4.

Supplementary Figure 11. Gene Expression Profile of Dox-NIL Motor Neurons



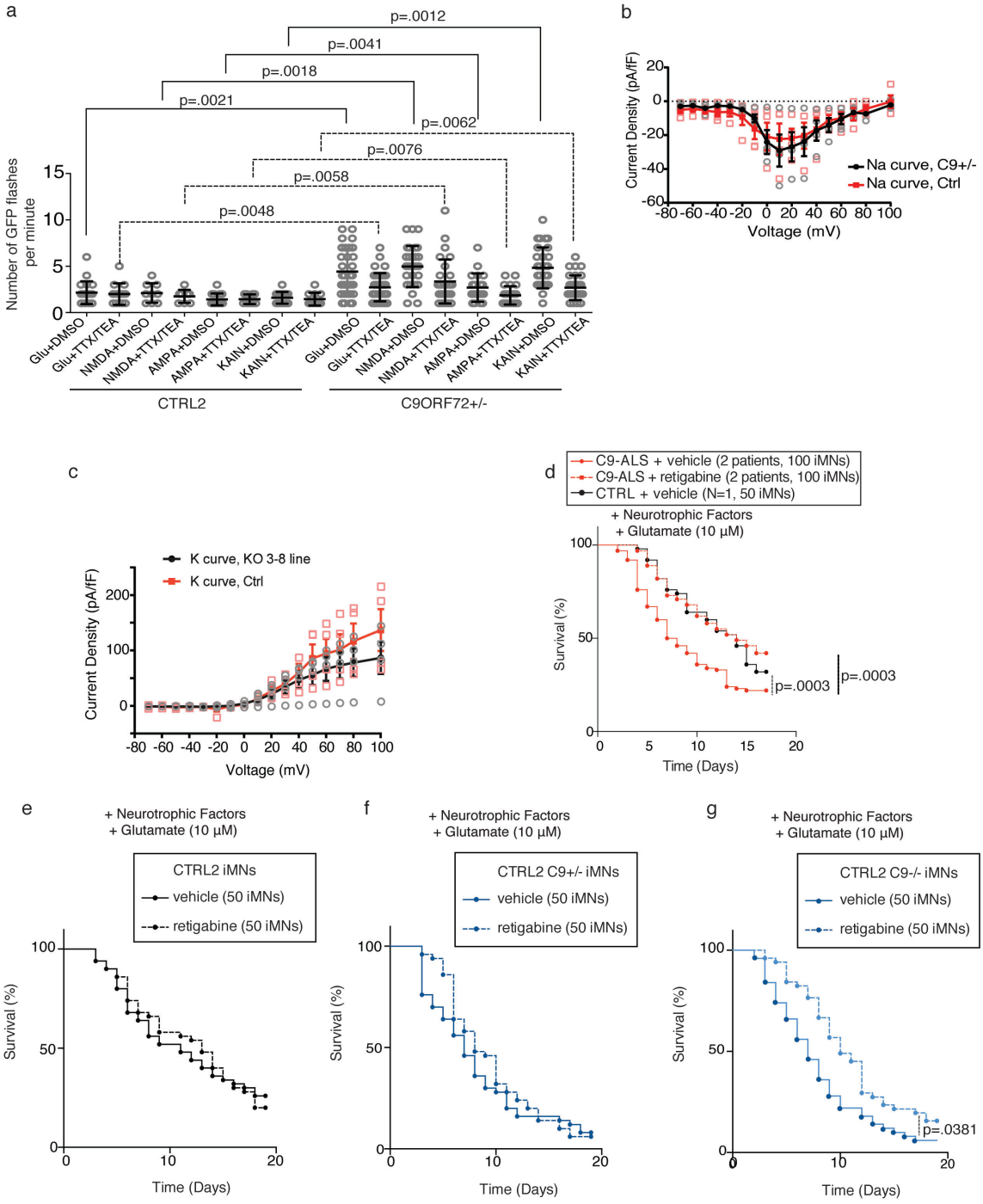
Supplementary Figure 11, related to Figure 4. Gene Expression Profile of Dox-NIL Motor Neurons. **a,** Immunofluorescence analysis of HB9 levels in Dox-NIL iMN cultures. Scale Bar: 100 μm. **b,** Immunofluorescence analysis of TUJ1, MAP2, and SMI-32 levels in Dox-NIL iMN cultures. Scale Bar: 40 μm. Experiments in (a-b) were repeated twice with similar results. **c,** Heatmap and dendrogram of iMNs, Dox-NIL iMNs, and iPSCs. The heatmap depicts the computed euclidean distance value (of the rlog transformed DESeq2 normalized gene counts) of each sample compared to each other. RNA sequencing experiments were performed and quantified using 2 biological replicates/individual differentiations per sample.

Supplementary Figure 12. Reduced C9ORF72 levels lead to increased motor neuron glutamate receptor levels *in vivo*

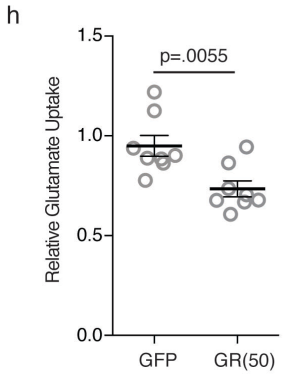


Supplementary Figure 12, related to Figure 4. Reduced C9ORF72 levels lead to increased glutamate receptor levels on motor neurons in vivo. **a-b,** Immunohistochemistry and quantification of NR1 (**a**) and GluR1 **(b**) in post-mortem lumbar sections of *Nestin-Cre+/− C9orf72*$^{loxP/loxP}$ mice. For (a), n=297 control and 146 *C9orf72*$^{-/-}$ motor neurons, and for (b), n=106 control and 174 *C9orf72*$^{-/-}$ motor neurons quantified from 10 ventral horn sections from 2 animals per genotype. Two-sided Mann-Whitney test, median +/- interquartile range. Data points represent a single cell. **c,** Western blot showing the purity of post-synaptic density-enriched (PSD) preparations. PSD-95 resides only in the post-synaptic density-enriched fraction and not in the total or triton-soluble fractions. NR1 shows a similar pattern, but as expected, p53 and Synapsin show the opposite pattern. This experiment was repeated twice with similar results. **d,** Representative immunohistochemistry and quantification of motor neuron marker levels in human post-mortem lumbar spinal cord neurons, using CHAT and SMI-32 antibodies to identify spinal motor neurons. Scale bars: 4 μm. Quantification derived from n=11 fields of view taken from 4 different spinal cord sections, covering 48 large neurons. Mean +/- s.e.m. **e,** Representative NR1 immunohistochemistry and quantification of control and C9-ALS post-mortem lumbar spinal cord. N=30 control and 32 C9-ALS motor neurons taken from 3 donors per genotype. Data points represent a single cell. Two-tailed t-test, t-value = 5.562, degrees of freedom = 60. Scale bars: 4 μm. Mean +/- s.e.m.

Supplementary Figure 13 (a-g). Reduced *C9ORF72* Expression Sensitizes iMNs to Glutamate

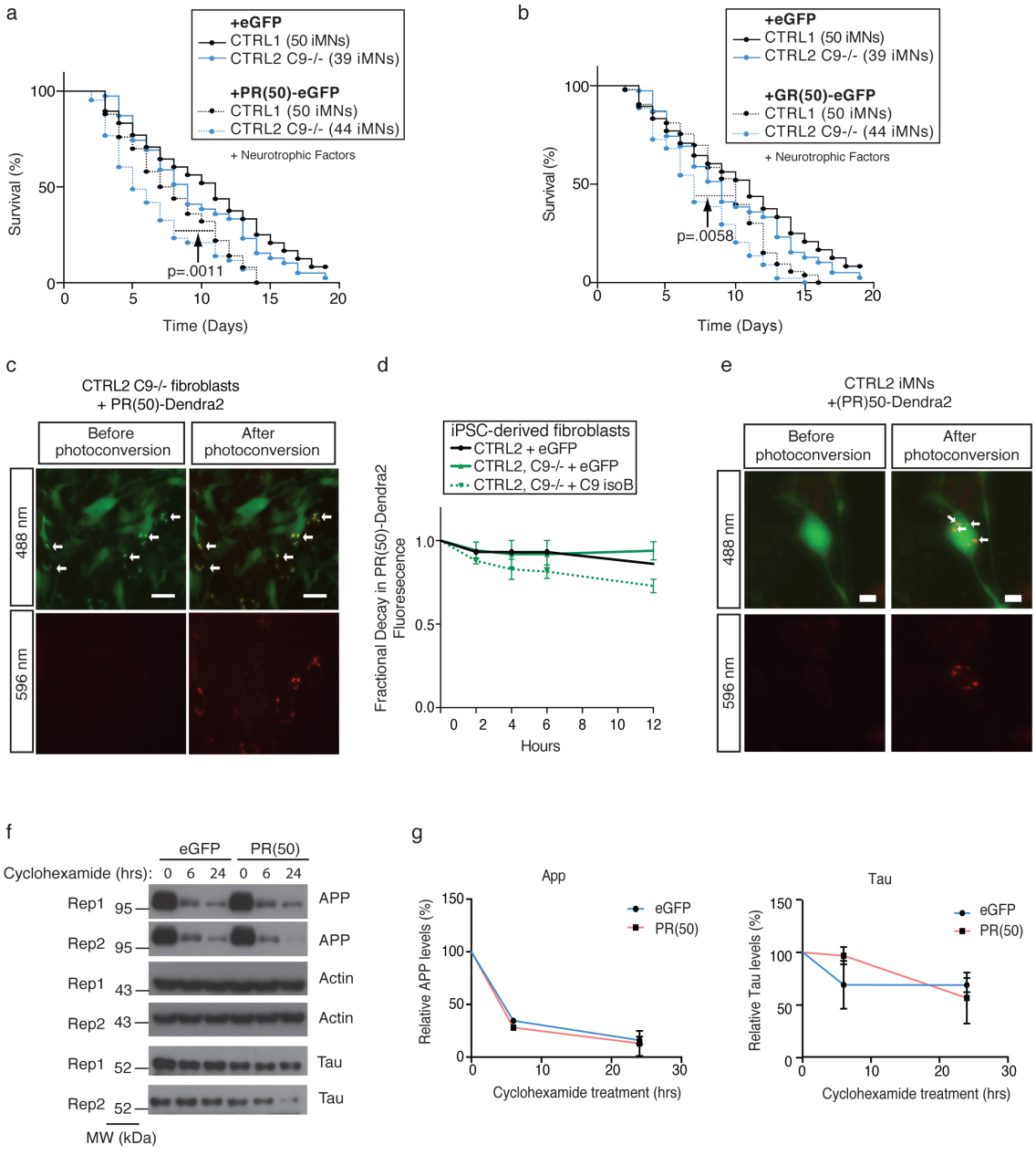


Supplementary Figure 13h. Reduced *C9ORF72* Expression Sensitizes iMNs to Glutamate



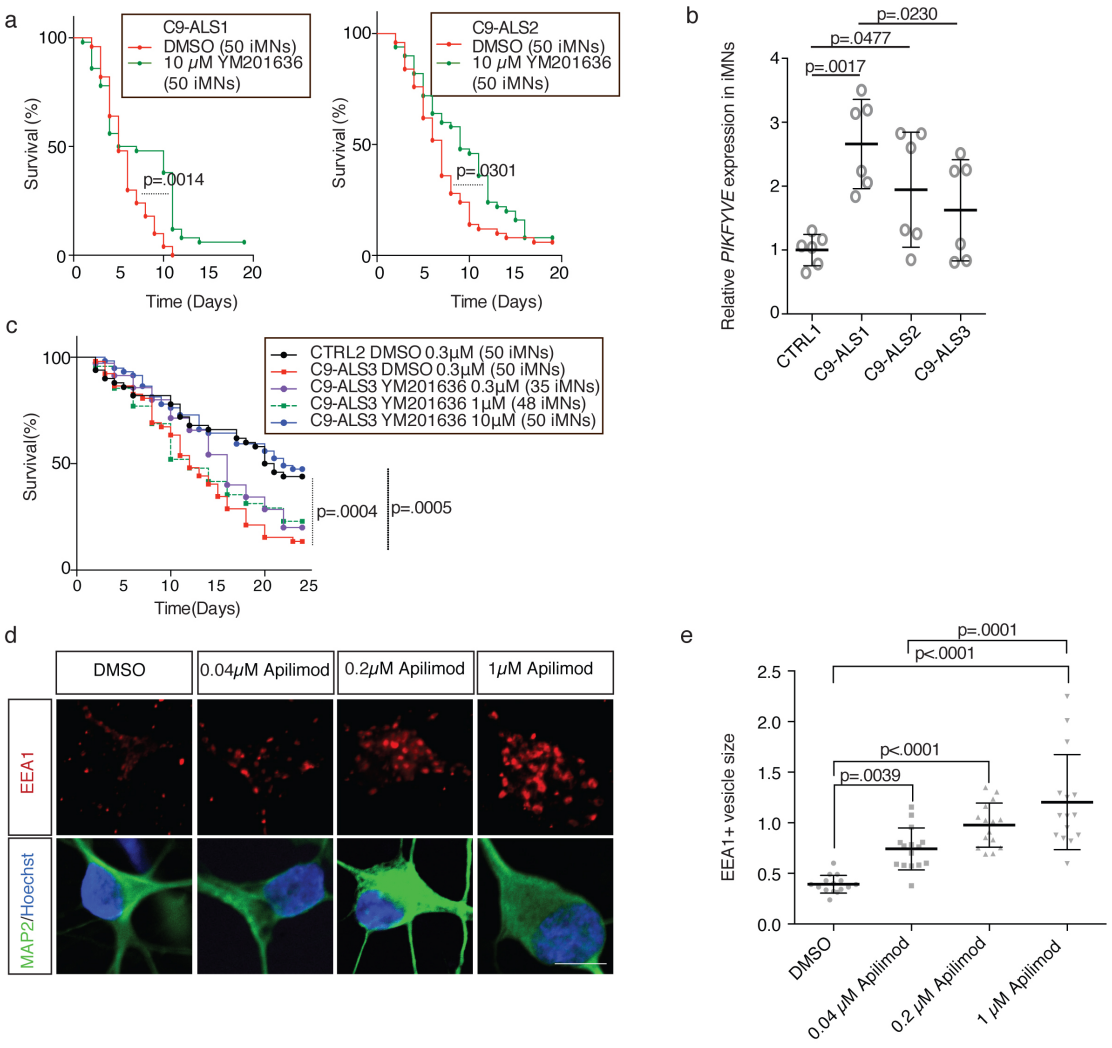
Supplementary Figure 13, related to Fig. 4. Reduced *C9ORF72* Expression Sensitizes iMNs to Glutamate. **a,** GCaMP6 assay to look at the relative contributions of different glutamate receptors to the hyperexcitability phenotype of C9ORF72 mutant iMNs. C9ORF72$^{+/-}$ iMNs have heightened responses to NMDA, AMPA, and kainite, as well as to glutamate (Glu) that activates all three receptor types. This is still true when calcium influx from action potentials is blocked by TTX/TEA treatment (blue bars)(mean ± s.e.m. n=20 (CTRL2 + Glutamate + DMSO), 13 (CTRL2 + Glutamate + TTX/TEA), 9 (CTRL2 + NMDA + DMSO), 8 (CTRL2 + NMDA + TTX/TEA), 14 (CTRL2 + AMPA + DMSO), 9 (CTRL2 + AMPA + TTX/TEA), 15 (CTRL2 + kainate + DMSO), 11 (CTRL2 + kainate + TTX/TEA), 35 (C9$^{+/-}$ + Glutamate + DMSO), 38 (C9$^{+/-}$ + Glutamate + TTX/TEA), 29 (C9$^{+/-}$ + NMDA + DMSO), 28 (C9$^{+/-}$ + NMDA + TTX/TEA), 20 (C9$^{+/-}$ + AMPA + DMSO), 22 (C9$^{+/-}$ + AMPA + TTX/TEA), 29 (C9$^{+/-}$ + kainate + DMSO), and 32 (C9$^{+/-}$ + kainate + TTX/TEA) iMNs quantified from 3 biologically independent iMN conversions. One-way ANOVA with Tukey correction for all comparisons. F-value (DFn, DFd): (15, 316) = 11.06. **b,** Sodium and **c,** potassium curves for control and *C9ORF72*+/- iMNs obtained by patch clamp electrophysiology. Curves are representative of for each genotype (n=4 curves per genotype). Two-tailed t-test at each voltage. Mean +/- s.e.m. **d-g,** The potassium channel agonist retigabine does not affect control (CTRL2) (**e)** or iMNs with heterozygous (**f**) mutations in *C9ORF72*, but enhances the survival of iMNs with homozygous (**g**) mutations in *C9ORF72* and C9-ALS iMNs (**d**). n=50 iMNs per condition quantified from 3 biologically independent iMN conversions. **h,** Glutamate uptake by human primary astrocytes expressing GFP or GR(50)-GFP. Astrocytes were incubated with 200 μM glutamate for 2 hours before uptake was measured. Mean +/- s.e.m. n=8 biologically independent astrocyte cultures per condition. Two-tailed t-test. t-value = 3.278, degrees of freedom = 14. All iMN survival statistical analysis was performed using a two-sided log-rank test and experiments were performed in a Molecular Devices ImageExpress. Electrophysiology experiments were performed and quantified using 2 biologically independent iMN conversions.

Supplementary Figure 14. C9ORF72 levels modulate DPR toxicity

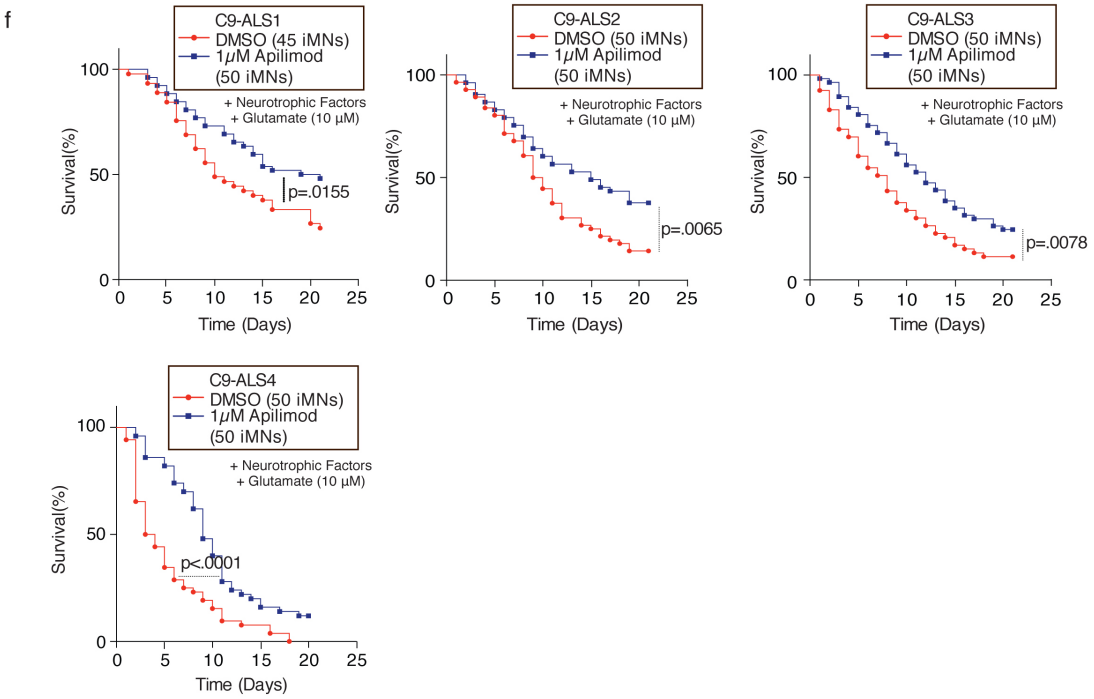


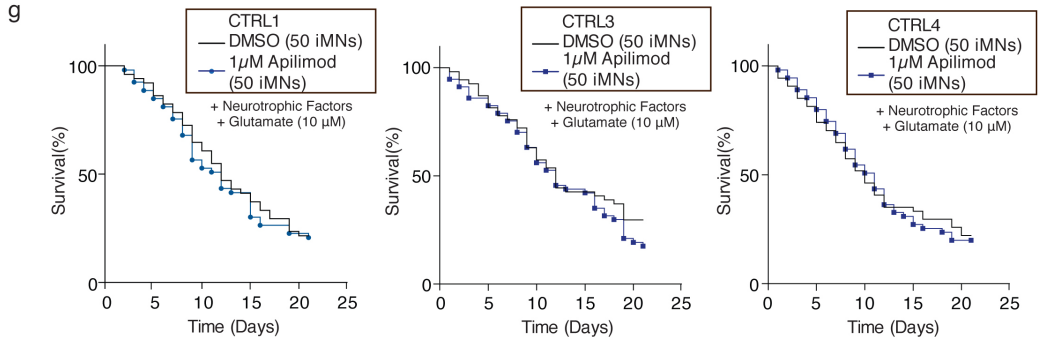
Supplementary Data Figure 14, related to Figure 5. C9ORF72 levels modulate DPR toxicity. **a-b,** Survival of control and CRISPR-mutant *C9ORF72*$^{-/-}$ iMNs in excess glutamate with overexpression of eGFP or PR(50)-eGFP (**a**) or GR(50)-eGFP (**b**). n=50 (CTRL + GFP or + DPR), 39 (*C9ORF72*$^{-/-}$ + GFP), or 44 (*C9ORF72*$^{-/-}$ + DPR) iMNs quantified from 3 biologically independent iMN conversions. **c,** PR(50)-Dendra2-expressing CTRL2 or *C9ORF72*$^{-/-}$ fibroblasts before and after Dendra2 Photoconversion (scale bar: 15 μm). **d,** Relative decay in Dendra2 fluorescence over 12 hours with overexpression of eGFP or C9ORF72 isoform B-T2A-eGFP in iPSC-derived fibroblasts. Mean +/- s.d. n= 2 biologically independent cell cultures per condition, of 10 cells quantified per culture. **e,** PR(50)-Dendra2-expressing CTRL2 iMNs before and after photoconversion (scale bar: 10 μm). **f,** Western blots and **g,** quantification of the decay of APP, ACTIN, and Tau over 6 and 24 hours in iMNs after application of continuous cyclohexamide treatment to inhibit protein synthesis. Protein decay in iMNs expressing GFP or PR(50)-GFP is shown. Mean protein level +/- s.d. of two biologically independent motor neuron differentiations. Experiments in (c, e, f) were repeated twice with similar results. All neuronal survival assays were performed and quantified using 3 biological replicates/individual differentiations.

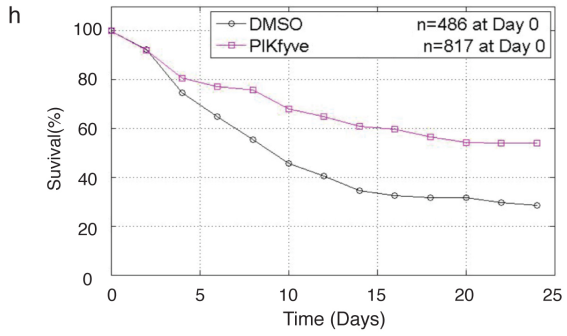
Supplementary Figure 15 (a-e). Chemical and genetic modulators of vesicle trafficking rescue *C9ORF72* patient iMN survival



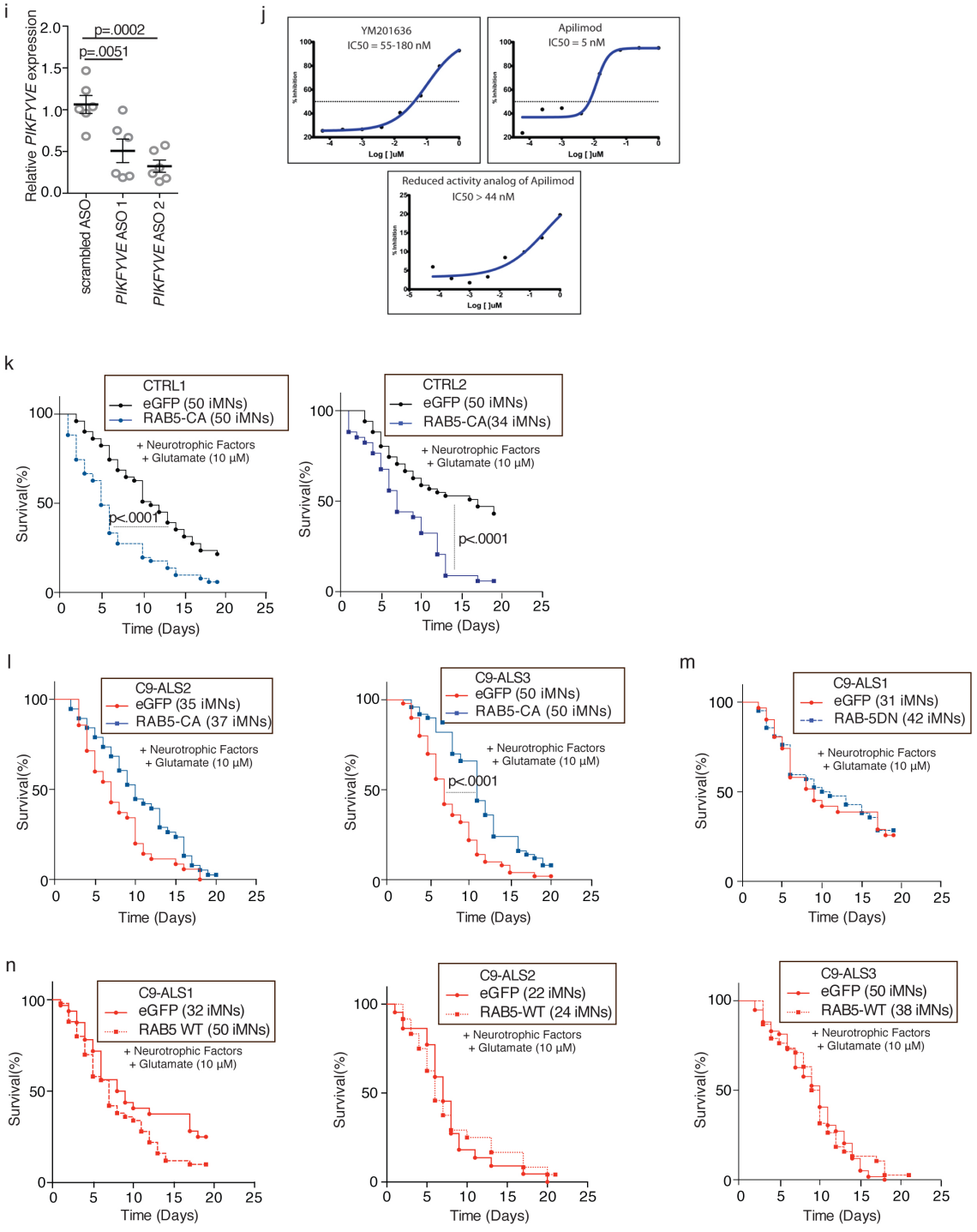
Supplementary Figure 15(f-h). Chemical and genetic modulators of vesicle trafficking rescue *C9ORF72* patient iMN survival



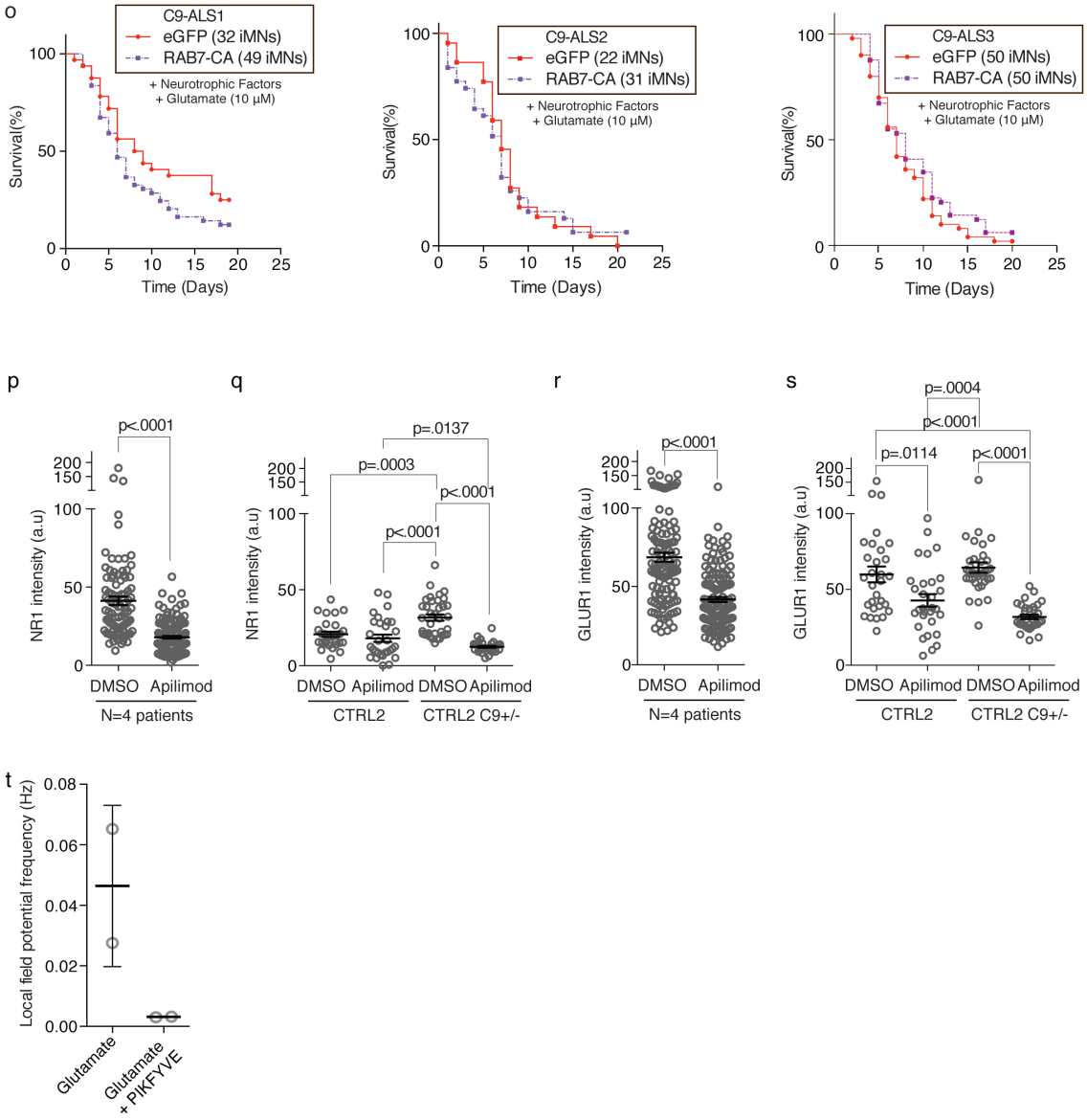




Supplementary Figure15(i-n). Chemical and genetic modulators of vesicle trafficking rescue *C9ORF72* patient iMN survival



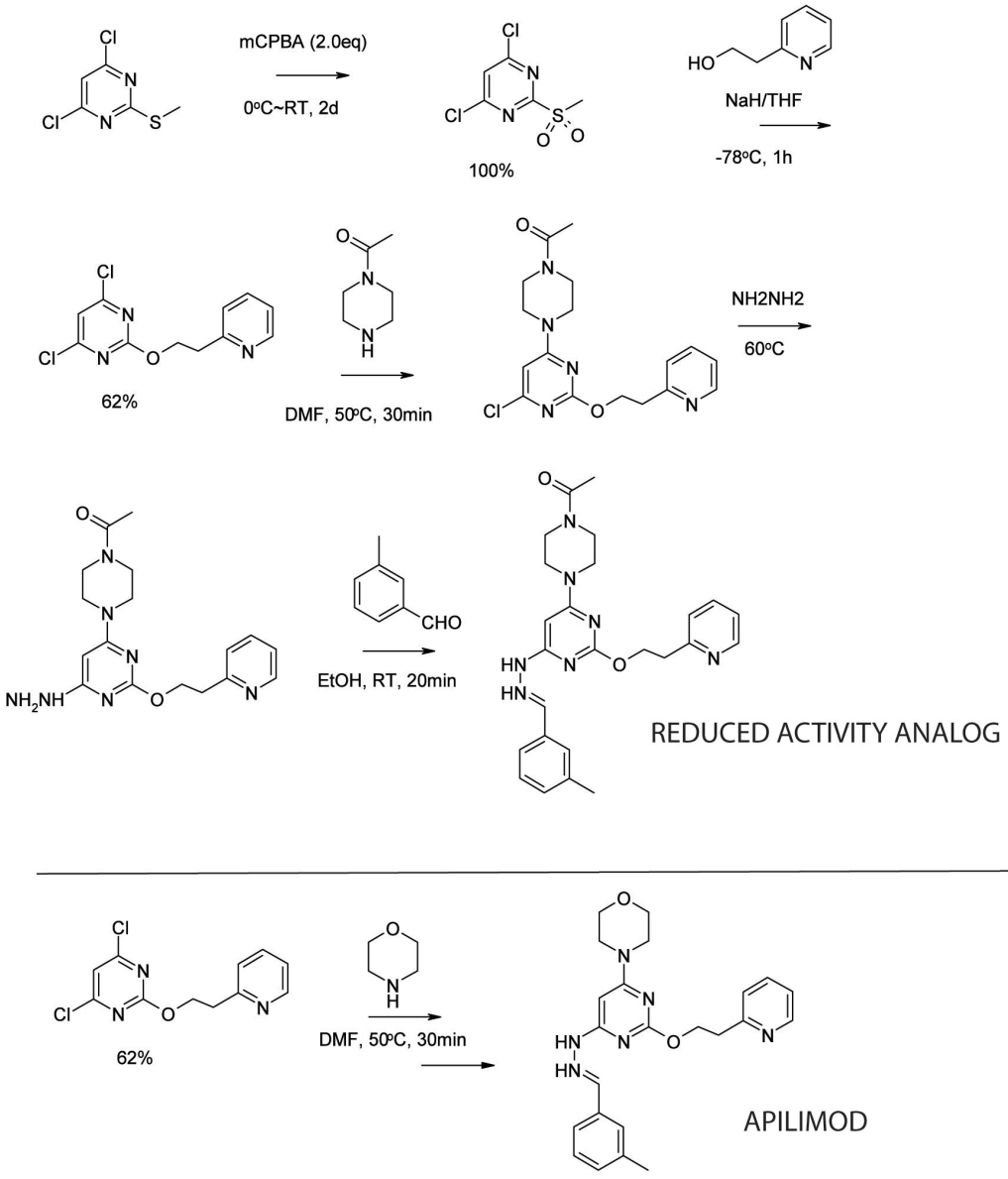
Supplementary Figure15(o-t). Chemical and genetic modulators of vesicle trafficking rescue *C9ORF72* patient iMN survival



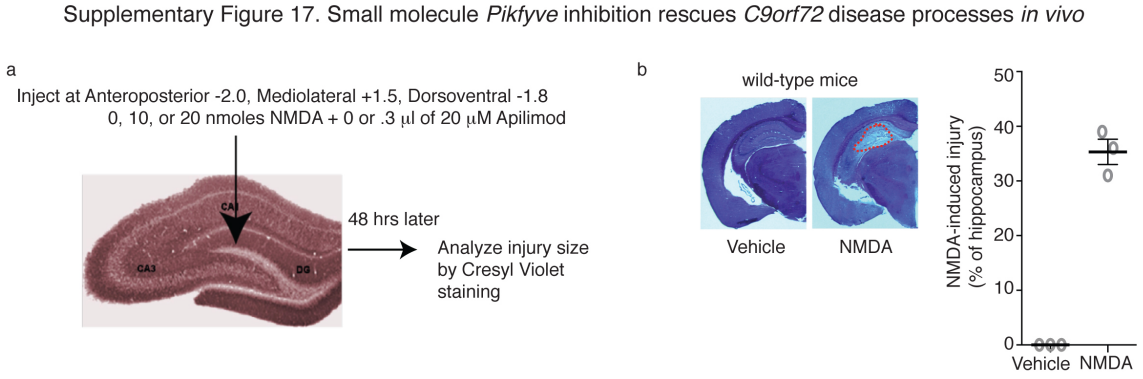
Supplementary Data Figure 15, related to Figure 6. Chemical and genetic modulators of vesicle trafficking rescue C9ORF72 patient iMN survival. **a,** Survival of iMNs in excess glutamate from two independent *C9ORF72* patient lines with DMSO or YM201636 treatment. **b,** PIKFYVE mRNA expression in control and C9-ALS iMNs. n=6 biologically independent iMN conversions per line. One-way ANOVA with Tukey correction. F-value (DFn, DFd): (3, 20) = 5.775. Mean +/- s.d. **c,** Survival of *C9ORF72* patient iMNs with a dose titration of YM201636. **d,** Immunostaining of $EEA1^+$ vesicles in *C9ORF72* patient motor neurons to measure their size after a 3 hour treatment of Apilimod at the indicated doses. Scale bar = 10 μm. **e,** Quantification of $EEA1^+$ vesicle size in *C9ORF72* patient motor neurons after a 3 hour treatment of Apilimod. n=15 $EEA1^+$ vesicles per condition quantified from two biologically independent motor neuron conversions for each condition. One-way ANOVA with Tukey correction. F-value (DFn, DFd): (3, 56) = 22.64. Mean +/- s.d. **f,** Survival of iMNs in excess glutamate from four independent *C9ORF72* patient lines with DMSO or Apilimod treatment. **g,** Survival of iMNs in excess glutamate from two independent control lines with DMSO or Apilimod treatment. **h,** Automated quantification of *C9ORF72* patient 1 iMN survival with DMSO or Apilimod treatment using SVCell 4.0 (DRVision Technologies, LLC). Automated survival analysis replicates manual counting results. n=486 (DMSO) or 817 (Apilimod) C9-ALS iMNs quantified from 3 biologically independent iMN conversions per condition. **i,** qRT-PCR of *PIKFYVE* expression in iMN cultures treated with 3 μM scrambled or *PIFKVYE*-targeting ASOs for 72 hrs. One way-ANOVA with Tukey correction across all comparisons, F-value (DFn, DFd): (2, 15) = 12.07. n= 6 biologically independent iMN conversions in each condition. Mean +/- s.d. **j,** Rates of PIKFYVE inhibition and IC_{50} measurements for YM201636, Apilimod, and the reduced-activity analog of Apilimod using an *in vitro* biochemical assay for PIKFYVE activity (Signalchem). **k,** Survival of iMNs in excess glutamate from two independent control lines lines with GFP or constitutively-active RAB5 (RAB5CA-T2A-GFP) overexpression. **l,** Survival of iMNs in excess glutamate from two independent *C9ORF72* patient with GFP or RAB5CA-T2A-GFP overexpression. **m,** Survival of *C9ORF72* patient iMNs in excess glutamate with GFP or dominant-negative RAB5 (RAB5DN-T2A-GFP) overexpression. **n,** Survival of iMNs in excess glutamate from three independent *C9ORF72* patient lines with GFP or wild-type RAB5 (RAB5-T2A-GFP) overexpression. **o,** Survival of iMNs in excess glutamate from three independent *C9ORF72* patient lines with GFP or constitutively-active RAB7 (RAB7-T2A-GFP) overexpression. **p-q,** Quantification of immunostaining of NR1 levels in *C9ORF72* patient and $C9ORF72^{+/-}$ iMNs with or without Apilimod treatment. For (p), n=100 iMNs for DMSO and 140 iMNs for Apilimod from 4 different donor lines per genotype, two biologically independent iMN conversions per line, two-tailed t-test with Welch's correction, t-value = 8.139, degrees of freedom = 116.9. For (q), n=29 iMNs for CTRL + DMSO, 30 iMNs for CTRL + Apilimod, 36 iMNs for $C9ORF72^{+/-}$ + DMSO, and 32 iMNs for $C9ORF72^{+/-}$ + Apilimod, each from two biologically independent iMN conversions, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 123) = 20.55. Each grey open circle represents the number of NR1+ puncta per unit area on a single neurite (one neurite quantified per iMN). Mean +/- s.e.m. **r-s,** Quantification of immunostaining of GLUR1 levels in *C9ORF72* patient and $C9ORF72^{+/-}$iMNs with or without Apilimod treatment. For (r), n=100 iMNs for DMSO and 140 iMNs for Apilimod

from 4 different donor lines per genotype, two biologically independent iMN conversions per line, two-tailed t-test with Welch's correction, t-value = 8.176, degrees of freedom = 177.8. For (s), n=29 iMNs for CTRL + DMSO, 30 iMNs for CTRL + Apilimod, 36 iMNs for *C9ORF72*$^{+/-}$ + DMSO, and 32 iMNs for *C9ORF72*$^{+/-}$ + Apilimod, each from two biologically independent iMN conversions, one-way ANOVA with Tukey correction, F-value (DFn, DFd): (3, 124) = 16.74. Each grey open circle represents the number of GLUR1+ puncta per unit area on a single neurite (one neurite quantified per iMN). Mean +/- s.e.m. **t,** The effect of Apilimod on iPSC-derived motor neuron local field potentials (LFP) as measured by micro-electrode array (i)(mean ± s.d. of 2 biological replicates, two-tailed t-test). Statistical analysis was performed using a two-tailed Student's t-test for pairwise comparisons, or one-way ANOVA for multiple comparisons. For all iMN experiments, n=50 iMNs per condition quantified from 3 biologically independent iMN conversions, except in (c) in which n=35 and 48 for the .3 μM and 1 μM condition, respectively, and where indicated in (k-o). All iMN survival experiments were analyzed by a two-sided log-rank test, and statistical significance was calculated using the entire survival time course. iMN survival experiment in (c) was performed in a Nikon Biostation CT, and all others were performed in a Molecular Devices ImageExpress. For iMN survival experiments, if iMNs from more than one iPSC line were combined into one curve to improve clarity, the number of iPSC lines is indicated in the legend (each iPSC line is from a different donor).

Supplementary Figure 16. Chemical synthesis methods for Apilimod and the reduced-activity analog



Supplementary Data Figure 16, related to Figure 6. Chemical synthesis methods for Apilimod and the reduced-activity analog.



Supplementary Data Figure 17, related to Figure 6. Small molecule Pikfyve inhibition rescues C9orf72 disease processes *in vivo*. **a,** Overview of experimental procedure for inducing NMDA-injury in the hippocampus and testing the ability of Apilimod to mitigate this injury. The same injection procedure without NMDA was used to test the effect of Apilimod on dipeptide repeat protein levels in C9-BAC mice. **b,** NMDA-induced injury in the mouse hippocampus. The dosage of NMDA used was 20 nmoles. Dotted line outlines the injury site. Mean +/- s.e.m., n=3 animals per condition.

Supplementary Data Table 1. Locations of GGGGCC x 3 in the human motor neuron transcriptome. Human motor neuron transcriptome was defined as the genes with detectable signal in three motor neuron samples from (Kiskinis, E., et al. Pathways Disrupted in Human ALS Motor Neurons Identified through Genetic Correction of Mutant SOD1. Cell Stem Cell 14, 781-795 (2014)).
Samples used were GSM1314597, GSM1314598, GSM1314599 in the GSE54409 dataset in the Gene Expression Omnibus (GEO).

Number	Ensembl Gene/ Transcript ID	Gene/Transcript	Chromosome
1	ENSG00000175130.6	MARCKSL1	1:32333832-32336379
2	ENSG00000198035.9	AGAP9	1:48189612:48237508
3	ENSG00000036828.9	CASR	3:122183683-122286503
4	ENSG00000138685.8	FGF2	4:122826708-122898236
5	ENST00000264498.3	FGF2-001	4:122826708-122898236
6	ENST00000608478.1	FGF2-002	4:122826831-122895464
7	ENST00000517260.1	AC021205.1	4:122827014-122827090
8	ENSG00000138756.13	BMP2K	4:78776342-78916372
9	ENST00000389010.3	BMP2K-002	4:78776342-78879435
10	ENST00000502871.1	BMP2K-001	4:78776378-78879969
11	ENST00000335016.5	BMP2K-201	4:78776378-78912185
12	ENSG00000145214.9	DGKQ	4:958885-986895
13	ENST00000273814.3	DGKQ-001	4:958887-973556
14	ENSG00000156427.7	FGF18	5:171419656-171457623
15	ENSG00000096433.6	ITPR3	6:33620365-33696574
16	ENST00000244496.5	RRP36	6:43021645-43034156
17	ENSG00000013374.11	NUB1	7:151341699-151378449
18	ENSG00000104490.13	NCALD	8:101686543-102124907
19	ENST00000517531.1	NCALD-012	8:101719315-102123950
20	ENSG00000181790.6	BAI1	8:142449430-142545009
21	ENSG00000158856.13	DMTN	8:22048995-22082527
22	ENST00000522148.1	DMTN-012	8:22048995-22069056
23	ENSG00000029534.15	ANK1	8:41653220-41896762
24	ENSG00000251349.3	MSANTD3-TMEFF1	9:100442271-100577636
25	ENSG00000241697.3	TMEFF1	9:100473113-100577636
26	ENSG00000130723.13	PRRC2B	9:131394093-131500197
27	ENSG00000160360.7	GPSM1	9:136327476-136359605
28	ENSG00000148408.8	CACNA1B	9:137877789-138124624
29	ENSG00000147894.10	C9orf72	9:27546545-27573866
30	ENSG00000107816.13	LZTS2	10:100996618-101007836
31	ENST00000370220.1	LZTS2	10:100996618-101007836
32	ENST00000454422.1	LZTS2-006	10:100999853-101003760
33	ENSG00000242288.6	RP11-464F9.1	10:73674290-73730466
34	ENSG00000183020.9	AP2A2	11:924894-1012245

35	ENSG00000198176.8	TFDP1	13:113584721-113641470
36	ENSG00000259993.1	RP11-261B23.1	15:30223017-30225564
37	ENSG00000260211.2	RP13-395E19.2	15:32313126-32315654
38	ENST00000348261.5	CACNA1H-001	16:1153241-1221768
39	ENST00000358590.4	CACNA1H-201	16:1153241-1221771
40	ENSG00000196557.6	CACNA1H	16:1153241-1221771
41	ENSG00000059145.14	UNKL	16:1363205-1414751
42	ENSG00000177548.8	RABEP2	16:28904421-28936526
43	ENST00000358201.4	RABEP2-001	16:28904421-28925752
44	ENST00000562590.1	RABEP2-005	16:28910287-28925684
45	ENSG00000007384.11	RHBDF1	16:58059-76355
46	ENSG00000225485.3	ARHGAP23 (by BLAST)	17:38428418-38512392
47	ENSG00000267131.1	RP11-332H18.5	17:61361668-61400243
48	ENSG00000267280.1	TBX2-AS1	17:61393456-61411555
49	ENSG00000007314.7	SCN4A	17:63938554-63972918
50	ENSG00000007237.14	GAS7	17:9910609-10198551
51	ENSG00000172466.11	ZNF24	18:35332212-35345482
52	ENST00000589539.1	ZNF24-005	18:35340345-35343932
53	ENSG00000134046.7	MBD2	18:54151601-54224788
54	ENST00000256429.3	MBD2-001	18:54151601-54224788
55	ENST00000398398.2	MBD2-005	18:54202680-54224647
56	ENST00000583046.1	MBD2-002	18:54202681-54224617
57	ENSG00000101493.6	ZNF516	18:76358190-76495190
58	ENST00000353265.3	PARD6G-001	18:80157232-80247546
59	ENSG00000178184.11	PARD6G	18:80157232-80247546
60	ENSG00000267662.1	AC007796.1	19:31348881-31417794
61	ENSG00000104936.13	DMPK	19:45769717-45782552
62	ENSG00000196562.10	SULF2	20:47656348-47786616
63	ENST00000359930.4	SULF2-001	20:47656912-47786586
64	ENST00000463221.2	SULF2-008	20:47689548-47786054
65	ENSG00000198355.4	PIM3	22:49960513-49964080
66	ENST00000467480.1	PIM3-002	22:49960768-49961646
67	ENST00000375135.3	FGD1	X:54445454-54496166
68	ENSG00000102302.7	FGD1	X:54445454-54496166

Supplementary Data Table 2. Locations of GGGGCC x 4 in the human motor neuron transcriptome. Human motor neuron transcriptome was defined as the genes with detectable signal in three motor neuron samples (GSM1314597, GSM1314598, GSM1314599) in the GSE54409 dataset in the Gene Expression Omnibus (GEO).

	Ensembl Gene/ Transcript ID	Gene/Transcript	Chromosome
1	ENSG00000235016.1	SEMA3F-AS1	3:50116022-50156085
2	ENSG00000109794.9	FAM149A	4:186104419-186172667
3	ENSG00000169220.13	RGS14	5:177357837-177372601
4	ENST00000509289.1	RGS14-011	5:177371066-177371582
5	ENST00000244496.5	RRP36-001	6:43021645-43029597
6	ENSG00000124541.6	RRP36	6:43021645-43034156
7	ENSG00000179526.12	SHARPIN	8:144098633-144108124
8	ENSG00000167157.9	PRRX2	9:129665641-129722674
9	ENSG00000204172.7	AGAP10	10:47501854-47549750
10	ENSG00000254929.2	RP11-144G6.12	10:47517816-47553514
11	ENSG00000198035.9	AGAP9	10:48189612-48237508
12	ENSG00000204164.6	BMS1P5	10:48901102-48950972
13	ENSG00000255032.1	RP11-45A12.2	11:44719392-44736692
14	ENSG00000248265.1	FLJ12825	12:54058254-54122234
15	ENSG00000249388.1	RP11-834C11.6	12:54082118-54102693
16	ENSG00000140265.8	ZSCAN29	15:43358172-43371025
17	ENST00000561661.1	ZSCAN29-006	15:43369851-43371025
18	ENSG00000007545.11	CRAMP1L	16:1612325-1677908
19	ENSG00000099822.2	HCN2	19:589893-617159
20	ENSG00000175221.10	MED16	19:867630-893218

Supplementary Data Table 3. Control and Patient sample information. The C9-ALS/FTD lines used in the study possessed repeat expansions of ~1000-4000 base pairs.

NINDS/ Coriell Code	Sample name	Mutation	Disease	Age of Onset	Age at sampling	Gender	iPSC karyo-type
ND12133	control 1	control	N/A	N/A	43	F	46,XX,t(5;12)(q31.1;q22)[20]
ND03231	control 2	control	N/A	N/A	56	M	normal
ND11976	control 3	control	N/A	N/A	40	M	normal
ND03719	control 4	control	N/A	N/A	33	M	normal
ND00184	control 5	control	N/A	N/A	65	F	normal
ND05280	control 6	control	N/A	N/A	72	F	normal
ND06769	patient 1	C9ORF72	ALS/FTD	45	46	F	normal
ND10689	patient 2	C9ORF72	ALS/FTD	49	51	F	normal
ND12099	patient 3	C9ORF72	ALS/FTD	48	49	M	normal
ND14954	patient 4	C9ORF72	ALS/FTD	73	74	M	46,XY,der(18)t(12;18)(p11.2;q21.1)[20]
ND08957	patient 5	C9ORF72	ALS/FTD	46	48	M	normal
ND12100	patient 6	C9ORF72	ALS/FTD	55	56	M	normal
ND14587	SOD1A4V	SOD1A4V	ALS	46	46	F	normal

Supplementary Data Table 4. Primers, probes and single guide RNAs (sgRNAs) used in this study.

RP-PCR primer, Forward	5'- 6FAM-TGTAAAACGACGGCCAGTCAAGGA GGGAAACAACCGCAGCC
RP-PCR primer, Reverse-1	5'- CAGGAAACAGCTATGACC
RP-PCR primer, Reverse-2	5'-CAGGAAACAGCTATGACCGGGCCCGCC CCGACCACGCCCCGGCCCCGGCCCCGG
Primer for generating Southern probe, Forward	5'- AGAACAGGACAAGTTGCC
Primer for generating Southern probe, Reverse	5'- AACACACACCTCCTAAACC
Genotyping primers for *C9ORF72* exon 2, Forward	5'- GGATTCCACATCTTTGACTTAAGAGG
Genotyping primers for *C9ORF72* exon 2, Reverse	5'- ATGTGGAGCTACCATTTCGTACCT
qRT-PCR primer for *C9ORF72*, transcriptional variant 2 Forward	5'- GTGGCGAGTGGATATCTCCGGA
qRT-PCR primer for *C9ORF72*, transcriptional variants 2 Reverse	5'- TGGAGCCCAAATGTGCCTTACTC
qRT-PCR primer for *GAPDH*, Forward	5'- CGAGATCCCTCCAAAATCAA
qRT-PCR primer for *GAPDH*, Reverse	5'- GTCTTCTGGGTGGCAGTGAT
qRT-PCR primer for *GRIN1*, Forward	5'- CCAGCGTGTGGTTTGAGATG
qRT-PCR primer for *GRIN1*, Reverse	5'- TTCTCTGCCTTGGACTCACG
qRT-PCR primer for *GRIA1*, Forward	5'- ATTCCAAGGACAAGACAAGCG
qRT-PCR primer for *GRIA1*, Reverse	5'- CCGCTTGGATTCACTACGGG
qRT-PCR primer for *PIKFYVE*, Forward	5'- CGTCCCCAACACTGGACTCTGC
qRT-PCR primer for *PIKFYVE*, Reverse	5'- CCCTGGCCTCCTTCTGCTCTCTC
sgRNA-3 targeting *C9ORF72* exon 2	5'- UUAACACAUAUAAUCCGGAA

sgRNA-4 targeting *C9ORF72* exon 2	5’- CACCACUCUCUGCAUUUCGA
sgRNA targeting *AAVS1* locus	5’- GGGGCCACTAGGGACAGGATTGG
FOR ASOs, m = 2’OMe, *- phosphorothioate linkage	
C9ORF72 ASO 1	mG*mC*mC*mA*mT*G*A*T*T*T*C*T*T*G*T*mC*mT*mG*mG*mG
C9ORF72 ASO 2	mG*mG*mG*mA*mC*A*C*U*A*C*A*A*G*G*U*mA*mG*mU*mA*mU
PIKFYVE ASO 1	mU*mG*mG*mC*mC*T*C*C*T*T*C*T*G*C*T*mC*mU*mC*mU*mC
PIKFYVE ASO 2	mG*mC*mU*mG*mG*T*C*C*A*A*C*T*T*C*C*mA*mC*mU*mC*mA

Supplementary Data Table 5. Numbers of iMNs counted for each line for survival experiments.

	Main Figures	
Figures	**Line**	**Number of Neurons Tracked**
Figure 1b	CTRL1	50
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50
	C9-ALS3	50
Figure 1c	CTRL1	50
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50
	C9-ALS3	50
Figure 1f	CTRL1	50
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50
	C9-ALS3	50
Figure 1g	CTRL1	50
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50
	C9-ALS3	50
Figure 1h	CTRL1	50
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50
Figure 2b	CTRL1+eGFP	50
	CTRL2+eGFP	50
	C9-ALS1+eGFP	50
	C9-ALS1+isoA	50
	C9-ALS1+isoB	50
	C9-ALS2+eGFP	50
	C9-ALS2+isoA	50
	C9-ALS2+isoB	50
	C9-ALS3+eGFP	50
	C9-ALS3+isoA	50
	C9-ALS3+isoB	50
Figure 2c	SOD1-ALS+eGFP	50
	SOD1-ALS+isoA	50
	SOD1-ALS+isoB	50
Figure 2d	CTRL1+eGFP	50
	CTRL1+isoA	50
	CTRL1+isoB	50

	CTRL2+eGFP	50
	CTRL2+isoA	50
	CTRL2+isoB	50
Figure 2f	CTRL2+eGFP	50
	CTRL2 C9+/-+eGFP	50
	CTRL2 C9 -/-+eGFP	50
	C9-ALS1+eGFP	50
	C9-ALS2+eGFP	50
Figure 2g	CTRL1+eGFP	50
	CTRL1+isoA	50
	CTRL1+isoB	50
	CTRL2+eGFP	50
	CTRL2+isoA	50
	CTRL2+isoB	50
Figure 5a	CTRL1+eGFP	50
	CTRL1+PR	50
	CTRL2 C9+/- +eGFP	49
	CTRL2 C9+/- +PR	47
Figure 5b	CTRL1+eGFP	50
	CTRL1+GR	50
	CTRL2 C9+/- +eGFP	49
	CTRL2 C9+/- +GR	40
Figure 5c	CTRL1+eGFP	50
	CTRL1+PR	50
	C9-ALS1+eGFP	50
	C9-ALS1+PR	42
	C9-ALS2+eGFP	50
	C9-ALS2+PR	40
Figure 5d	CTRL1+eGFP	50
	CTRL1+GR	50
	C9-ALS1+eGFP	50
	C9-ALS1+GR	48
	C9-ALS2+eGFP	50
	C9-ALS2+GR	45
Figure 6d	C9-ALS2+DMSO	50
	C9-ALS2+Apilimod	50
	C9-ALS2+reduced-activity analog	50
Figure 6e	Scrambled ASO	50
	PIKFYVE ASO 1	50
	PIKFYVE ASO 2	50
	Supplementary Figures	
Supp Fig3a	CTRL1	34
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50

	C9-ALS3	50
Supp Fig3b	CTRL1	50
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50
Supp Fig3c	C9-ALS3, Clone1	50
	C9-ALS3, Clone2	50
Supp Fig3d	CTRL1	50
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50
	C9-ALS3	50
Supp Fig3e	CTRL1	50
	CTRL2	50
	CTRL3	50
	CTRL4	50
	C9-ALS4	50
	C9-ALS5	31
	C9-ALS6	40
Supp Fig3f	CTRL1	50
	CTRL2	50
	C9-ALS1	50
	C9-ALS2	50
	C9-ALS3	50
Supp Fig3i	CTRL1	1075
	CTRL2	1075
	CTRL3	1075
	CTRL4	1075
	C9-ALS1	1075
	C9-ALS2	1075
	C9-ALS3	1075
	C9-ALS4	1075
Supp Fig3j	CTRL2	100
	CTRL2 C9+/-	100
	CTRL2 C9-/-	100
Supp Fig3k	CTRL1	1075
	CTRL2	1075
	CTRL3	1075
	CTRL4	1075
	C9-ALS1	1075
	C9-ALS2	1075
	C9-ALS3	1075
	C9-ALS4	1075
Supp Fig3l	CTRL2	100
	CTRL2 C9+/-	100
	CTRL2 C9-/-	100

Supp Fig3o	CTRL1-iDA	50
	CTRL2-iDA	50
	C9-ALS2-iDA	50
	C9-ALS3-iDA	50
Supp Fig4c	C9-ALS1+eGFP	50
	C9-ALS1+isoA	50
	C9-ALS1+isoB	50
	C9-ALS2+eGFP	50
	C9-ALS2+isoA	50
	C9-ALS2+isoB	50
	C9-ALS3+eGFP	50
	C9-ALS3+isoA	50
	C9-ALS3+isoB	50
Supp Fig4d	CTRL1+eGFP	50
	CTRL1+isoA	50
	CTRL1+isoB	50
	CTRL2+eGFP	50
	CTRL2+isoA	50
	CTRL2+isoB	50
Supp Fig4k	CTRL2+eGFP	50
	CTRL2 C9+/- +eGFP	50
	CTRL2 C9+/- +isoA	50
	CTRL2 C9+/- +isoB	50
Supp Fig4l	CTRL2+eGFP	50
	CTRL2 C9-/- +eGFP	50
	CTRL2 C9-/- +isoA	50
	CTRL2 C9-/- +isoB	50
Supp Fig13e	CTRL2+DMSO	50
	CTRL2+Retigabine	50
Supp fig13f	CTRL2 C9+/- +DMSO	50
	CTRL2 C9+/- +Retigabine	50
Supp fig13g	CTRL2 C9-/- +DMSO	50
	CTRL2 C9-/- +Retigabine	50
Supp fig14a	CTRL1+eGFP	50
	CTRL1+PR(50)-eGFP	50
	CTRL2 C9-/- +eGFP	50
	CTRL2 C9-/- + PR(50)-eGFP	44
Supp fig14b	CTRL1+eGFP	50
	CTRL1+GR(50)-eGFP	50
	CTRL2 C9-/- +eGFP	39
	CTRL2 C9-/- + GR(50)-eGFP	44
Supp fig15a	C9-ALS1+DMSO	50
	C9-ALS1-YM201636	50
	C9-ALS2+DMSO	50
	C9-ALS2-YM201636	50
Supp fig15c	CTRL2+0.3μM DMSO	50

	C9-ALS2+0.3μM DMSO	50
	C9-ALS2+0.3μM YM201636	35
	C9-ALS2+1μM YM201636	48
	C9-ALS2+10μM YM201636	50
Supp fig15f	C9-ALS1+DMSO	45
	C9-ALS1+1μM Apilimod	50
	C9-ALS2+DMSO	50
	C9-ALS2-1μM Apilimod	50
	C9-ALS3+DMSO	50
	C9-ALS3+1μM Apilimod	50
	C9-ALS4+DMSO	50
	C9-ALS4+1μM Apilimod	50
Supp fig15g	CTRL1+DMSO	50
	CTRL1+1μM Apilimod	50
	CTRL3+DMSO	50
	CTRL3+1μM Apilimod	50
	CTRL4+DMSO	50
	CTRL4+1μM Apilimod	50
Supp fig15h	C9-ALS2+DMSO	486
	C9-ALS2+1μM Apilimod	817
Supp fig15k	CTRL1+eGFP	50
	CTRL1+RAB5-CA	50
	CTRL2+eGFP	50
	CTRL2+RAB5-CA	34
Supp fig15l	C9-ALS2+eGFP	35
	C9-ALS2+RAB5-CA	37
	C9-ALS3+eGFP	50
	C9-ALS3+RAB5-CA	50
Supp fig15m	C9-ALS1+eGFP	31
	C9-ALS1+RAB5-DN	42
Supp fig15n	C9-ALS1+eGFP	32
	C9-ALS1+RAB5-WT	50
	C9-ALS2+eGFP	22
	C9-ALS2+RAB5-WT	24
	C9-ALS3+eGFP	50
	C9-ALS3+RAB5-WT	38
	C9-ALS1+eGFP	32
Supp fig15o	C9-ALS1+RAB7-CA	49
	C9-ALS2+eGFP	22
	C9-ALS2+RAB7-CA	31
	C9-ALS3+eGFP	50
	C9-ALS3+RAB7-CA	50

Supplementary Data Table 6. Live imager used for each iMN survival experiment.

Figure No	Machine Used for Live Imaging
1b	BS (Nikon Biostation CT)
1c	BS
1d	BS
1f	BS
1g	BS
1h	MD (Molecular Devices ImageExpress)
2b	BS
2c	MD
2d	BS
2f	MD
2g	BS
5a	MD
5b	MD
5c	MD
5d	MD
6d	MD
6e	MD
Supp Fig3a	BS
Supp Fig3b	MD
Supp Fig3c	MD
Supp Fig3d	BS
Supp Fig3e	BS
Supp Fig3f	BS
Supp Fig3i	MD
Supp Fig3j	MD
Supp Fig3k	MD
Supp Fig3l	MD
Supp Fig3o	MD
Supp Fig4c	BS
Supp Fig4d	BS
Supp Fig4k	MD
Supp Fig4l	MD
Supp Fig13e	MD
Supp Fig13f	MD
Supp Fig13g	MD
Supp Fig14a	MD
Supp Fig14b	MD
Supp Fig15a	MD
Supp Fig15c	BS
Supp Fig15f	BS for C9-ALS1,2 MD for C9-

ALS3,4

Supp Fig15g	MD
Supp Fig15k	MD
Supp Fig15l	MD
Supp Fig15m	MD
Supp Fig15n	MD
Supp Fig15o	MD