Identification and therapeutic rescue of autophagosome and glutamate receptor defects in *C9ORF72* and sporadic ALS neurons

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Supplemental Materials

Supplemental Table 2. ALS-related genes queried for rare or disease-causing variants in sporadic ALS lines.

Supplemental Table 3. Sequences of primers and antisense oligonucleotides used in this study.

Supplemental Table 4. Gene counts from RNA-seq data.

Supplemental Table 5. Description of RNA-seq samples.

Supplemental Materials:

Supplemental Table 1. Control and Patient iPSC information

NINDS/ Coriell Code	Sample name	Mutation	Disease	Age of one set	Age of Sampling	Gender
ND03231	control 1	N/A	N/A	N/A	56	M
ND05280	control 2	N/A	N/A	N/A	72	F
ND03719	control 3	N/A	N/A	N/A	33	M
ND06769	C9-ALS 1	C9ORF72	ALS/FTD	45	46	F
ND10689	C9-ALS 2	C9ORF72	ALS/FTD	49	51	F
ND12099	C9-ALS 3	C9ORF72	ALS/FTD	48	49	M
ND13454	sALS 1	sporadic	ALS	44	45	M
ND09711	sALS 2	sporadic	ALS	52	52	M
ND08705	sALS 3	sporadic	ALS	32	34	M
ND14185	sALS 4	sporadic	ALS	45	46	M
ND10739	sALS 5	sporadic	ALS	31	34	M
ND11813	sALS 6	sporadic	ALS	34	63	M

Supplemental Table 2. ALS-related genes queried for rare or disease-causing variants in sporadic ALS lines. Gene list derived from: https://ghr.nlm.nih.gov/condition/amyotrophic-lateral-sclerosis#genes.
* - repeat-primed PCR was used to screen this gene.

ALS2	*ANG*	**C9ORF72*	*CHCHD10*	*CHMP2B*	*DCTN1*
ERBB4	*FIG4*	*FUS*	*HNRNPA1*	*MATR3*	*NEFH*
OPTN	*PFN1*	*PRPH*	*SETX*	*SIGMAR1*	*SMN1*
SOD1	*SPG11*	*SQSTM1*	*TARDBP*	*TBK1*	*TRPM7*
TUBA4A	*UBQLN2*	*VAPB*	*VCP*		

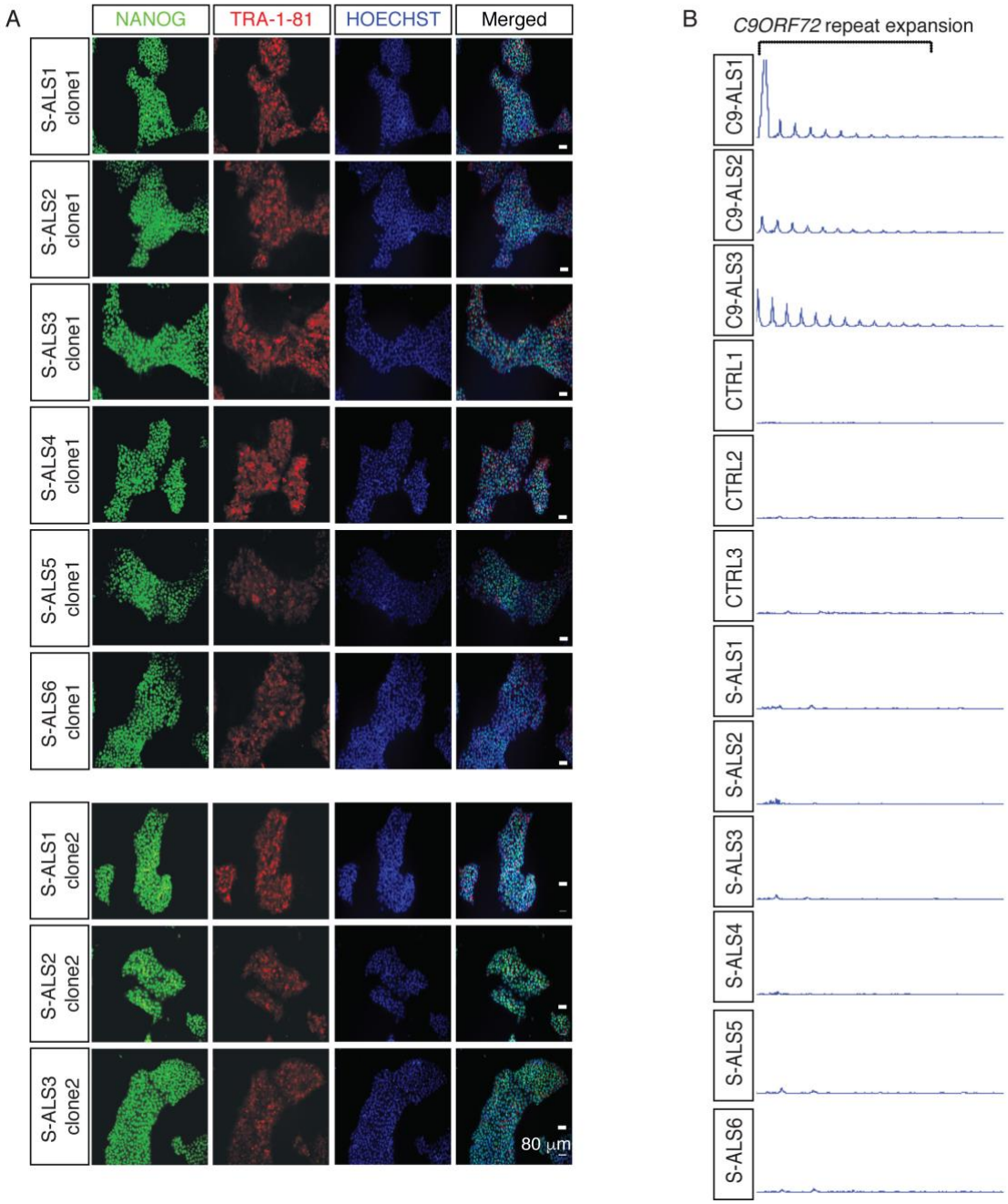
Supplemental Table 3. Sequences of primers and antisense oligonucleotides used in this study.

Primer	Sequence
qRT-PCR primer for *ATG5*, Forward	5'-AGAAGCTGTTTCGTCCTGTGG
qRT-PCR primer for *ATG5*, Reverse	5'-AGGTGTTTCCAACATTGGCTC
qRT-PCR primer for *ATG10*, Forward	5'-ATAAGATGCGACTGCTACAGGG
qRT-PCR primer for *ATG10*, Reverse	5'-CAATGCTCAGCCATGATGTGAT
qRT-PCR primer for *PAR1*, Forward	5'-CGGCAGTGATTGGCAGTTTG
qRT-PCR primer for *PAR1*, Reverse	5'-TGAGCAAGATAGAGGCGTACA
qRT-PCR primer for *PAR2*, Forward	5'-CTGTGGGTCTTTCTTTTCCGAA
qRT-PCR primer for *PAR2*, Reverse	5'-CAAGGGGAACCAGATGACAGA
qRT-PCR primer for *PAR3*, Forward	5'-GCAAAGCCAACCTTACCCATT
qRT-PCR primer for *PAR3*, Reverse	5'-GAGGTAGATGGCAGGTATCAGT
qRT-PCR primer for *GAPDH*, Forward	5'-CGAGATCCCTCCAAAATCAA
qRT-PCR primer for *GAPDH*, Reverse	5'-GTCTTCTGGGTGGCAGTGAT
qRT-PCR primer for *C9ORF72* variants 1 and 3, Forward	5'- GGGTCTAGCAAGAGCAGGTG
qRT-PCR primer for *C9ORF72* variants 1 and 3, Forward	5'- GTCTTGGCAACAGCTGGAGAT
For ASOs, m=2'-O-Methoxyethyl, *- phosphorothioate linkage	
PAR1 ASO	mU*mA*mC*mU*mU*G*C*C*A*T*C*A*A*C*T*mG*mC*mC*mC*mA
PAR2 ASO	mA*mA*mG*mA*mC*C*C*A*C*A*G*G*G*C*C*mA*mU*mG*mC*mC
PAR3 ASO	mG*mU*mG*mG*mC*T*C*C*T*G*T*C*C*A*G*mC*mC*mU*mU*mC
Scrambled ASO	mC*mC*mU*mU*mC*C*C*U*G*A*A*G*G*U*mU*mC*mC*mU*mC*mC*

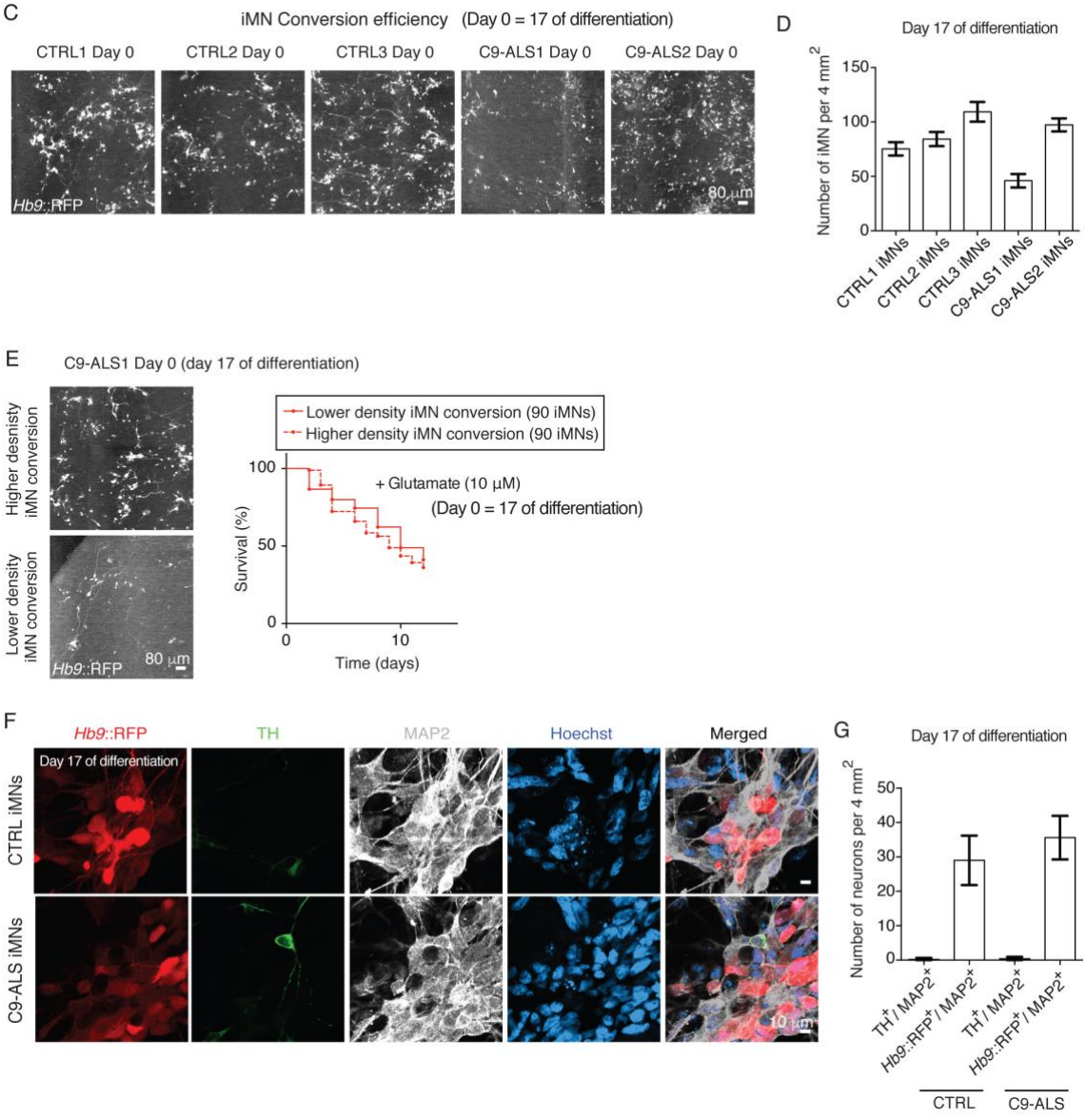
Supplemental Table 4. Gene counts from RNA-seq data.

Supplemental Table 5. Description of RNA-seq samples.

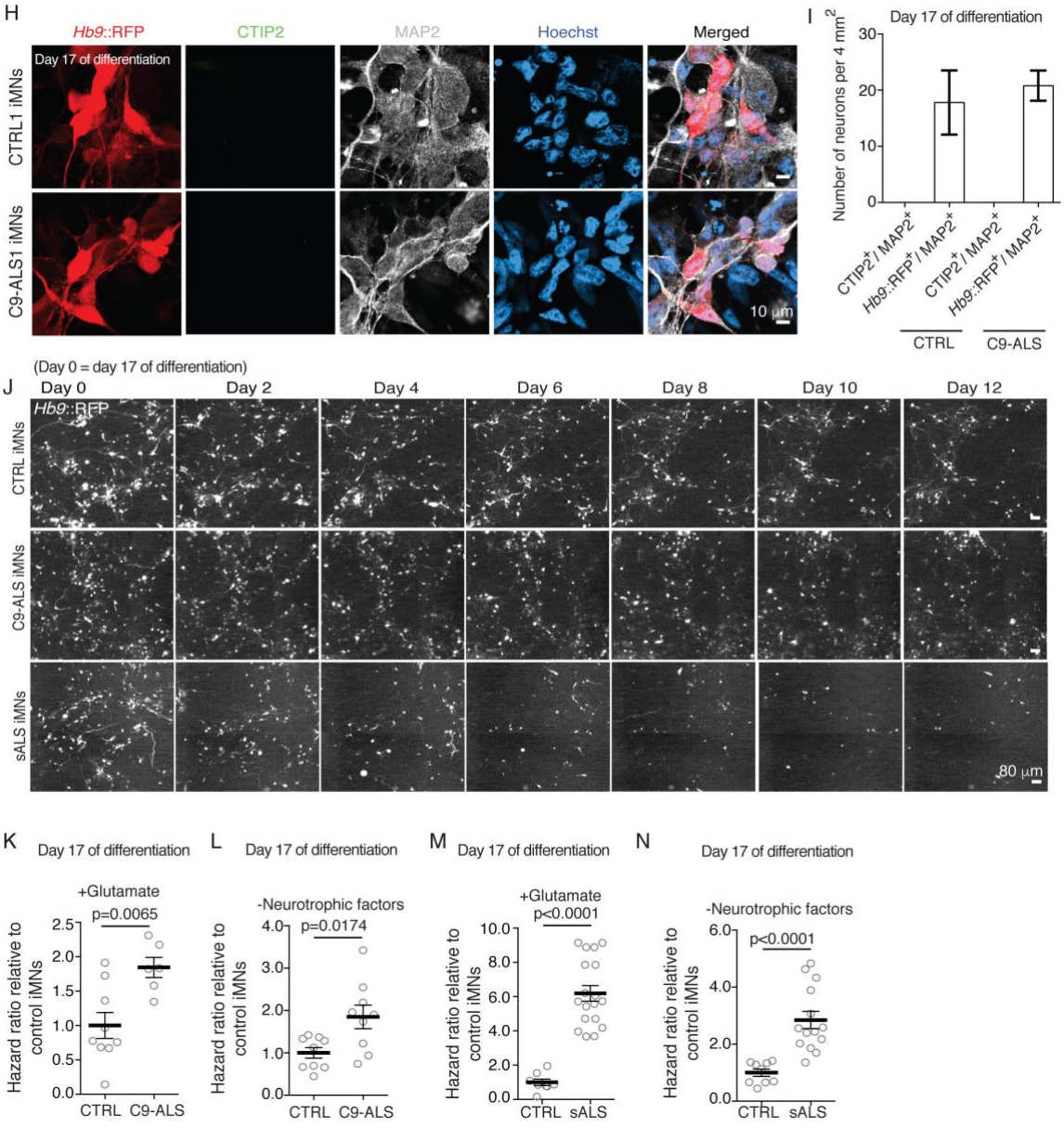
Supplemental Figure 1(A-B).
Identification of neurodegenerative phenotypes in sporadic ALS patient iMNs



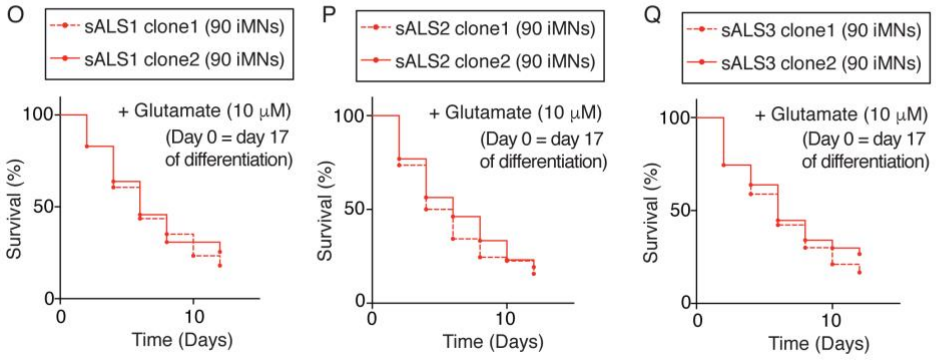
Supplemental Figure 1(C-G).
Identification of neurodegenerative phenotypes in sporadic ALS patient iMNs



Supplemental Figure 1(H-N).
Identification of neurodegenerative phenotypes in sporadic ALS patient iMNs



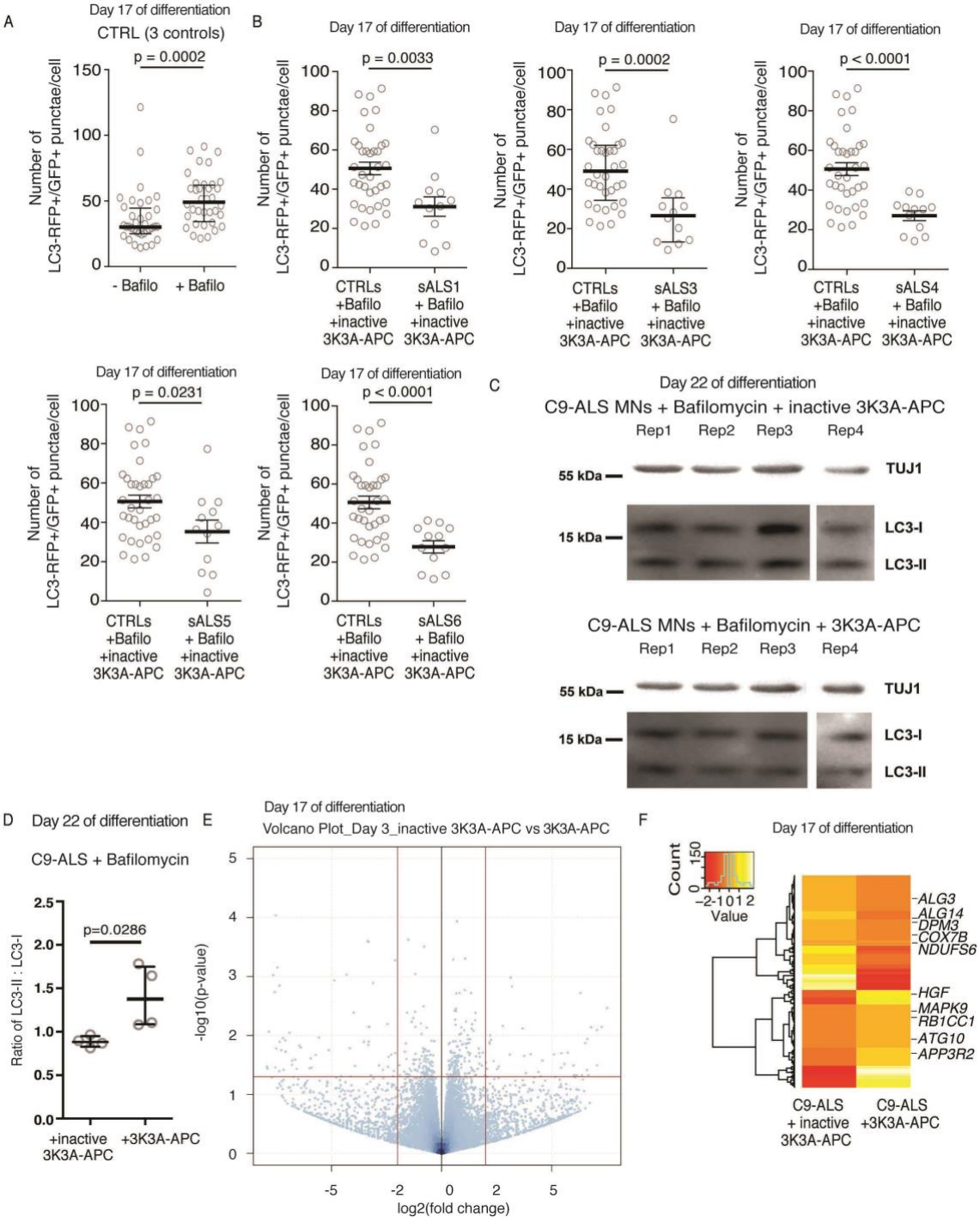
Supplemental Figure 1(O-Q).
Identification of neurodegenerative phenotypes in sporadic ALS patient iMNs



Supplemental Figure 1. Identification of neurodegenerative phenotypes in sporadic ALS patient iMNs. (A) Sporadic ALS iPSC lines expressing markers of pluripotency including NANOG (green) and TRA-1-81 (red). Nuclei (blue) are labeled with Hoechst. Scale bars: 80 μm. **(B)** Repeat-primed PCR data used to detect the presence of the *C9ORF72* repeat expansion. **(C-D)** Images (C) and quantification (D) of the density of iMNs generated from multiple iPSC lines at the start of the iMN survival assay. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.d. **(E)** Survival of iMNs from the same line at when cultured at high or low density. iMNs quantified from 3 biologically independent iMN conversions per line. Scale bars: 80 μm. Two-sided log-rank test using the entire survival time course. **(F-G)** Images (F) and quantification (G) of the number of TH^+ neurons in iMN cultures. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.d. Scale bars: 10 μm. **(H-I)** Images (H) and quantification (I) of the number of $CTIP2^+$ neurons in iMN cultures. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.d. Scale bars: 10 μm. **(J)** Representative images showing the number of controls, C9-ALS, or sporadic ALS iMNs over time during an iMN survival assay. Scale bars: 80 μm. **(K)** Hazard ratio (relative to control iMNs) of iMNs from three controls or two C9-ALS patients in excess glutamate. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test. **(L)** Hazard ratio (relative to control iMNs) of iMNs from three controls or three C9-ALS patients in the neurotrophic factor withdrawal condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test with Welch's correction. **(M)** Hazard ratio (relative to control iMNs) of iMNs from three controls or six sporadic ALS patients in excess glutamate. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test with Welch's correction. **(N)** Hazard ratio (relative to control iMNs) of iMNs from three controls or five sporadic ALS patients in the neurotrophic factor withdrawal condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test with Welch's correction. **(O-Q)** Survival of iMNs in excess glutamate from two different iPSC lines derived from sporadic ALS patient 1 (O), 2 (P), or 3 (Q).

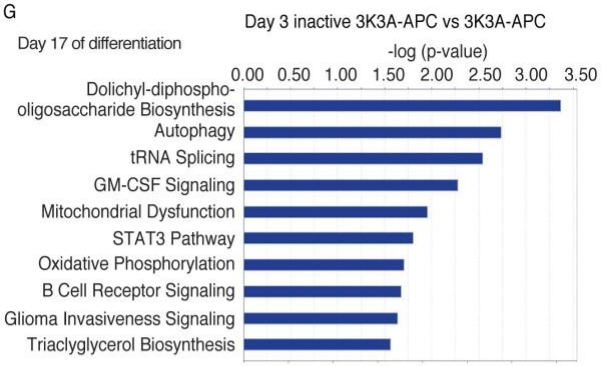
iMNs quantified from 3 biologically independent iMN conversions per line. Scale bars: 80 μm. Two-sided log-rank test using the entire survival time course. The day of differentiation stated on each panel indicates the day of differentiation on which the experimental treatment or time course was initiated.

Supplemental Figure 2(A-J).
C9ORF72 and sporadic ALS iMNs share autophagosome formation abnormalities that are rescued by 3K3A-APC



Supplemental Figure 2(G-K).
C9ORF72 and sporadic ALS iMNs share autophagosome formation abnormalities that are rescued by 3K3A-APC

G



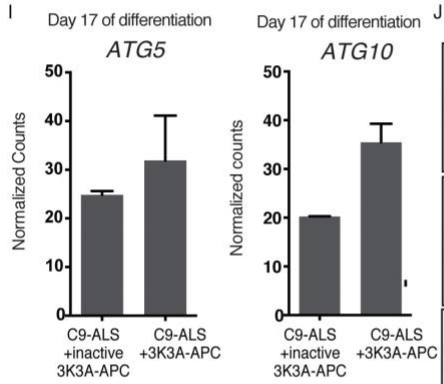
H

Day 3 of treatment (Day 17 of differentiation)

KEGG Pathway_Day 3 inactive 3K3A-APC vs 3K3A-APC

	KEGG Pathway	Combined Score
1	Regulation of Autophagy	14.56
2	Huntington's disease	14.55
3	Alzheimer's disease	8.64
4	Cardiac muscle contraction	7.76
5	ErbB signaling pathway	7.34
6	Neurotrophin signaling pathway	7.19
7	cAMP signaling pathway	6.95
8	Amphetamine addiction	6.09
9	Long-term potentiation	6.03
10	Oxidative phosphorylation	5.92

I



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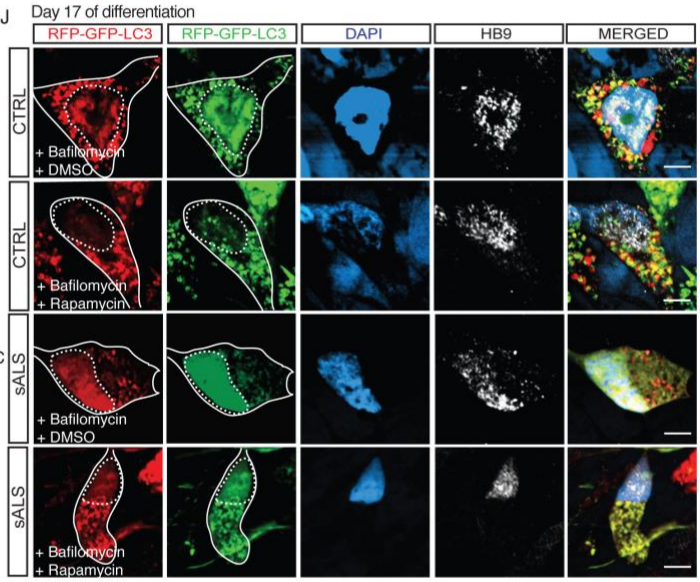
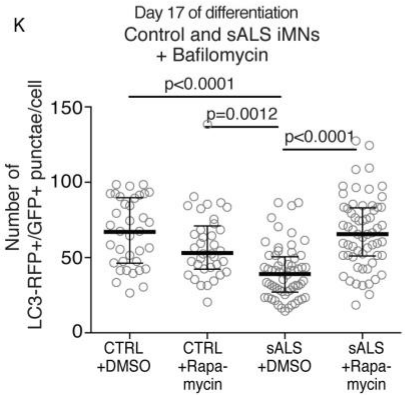
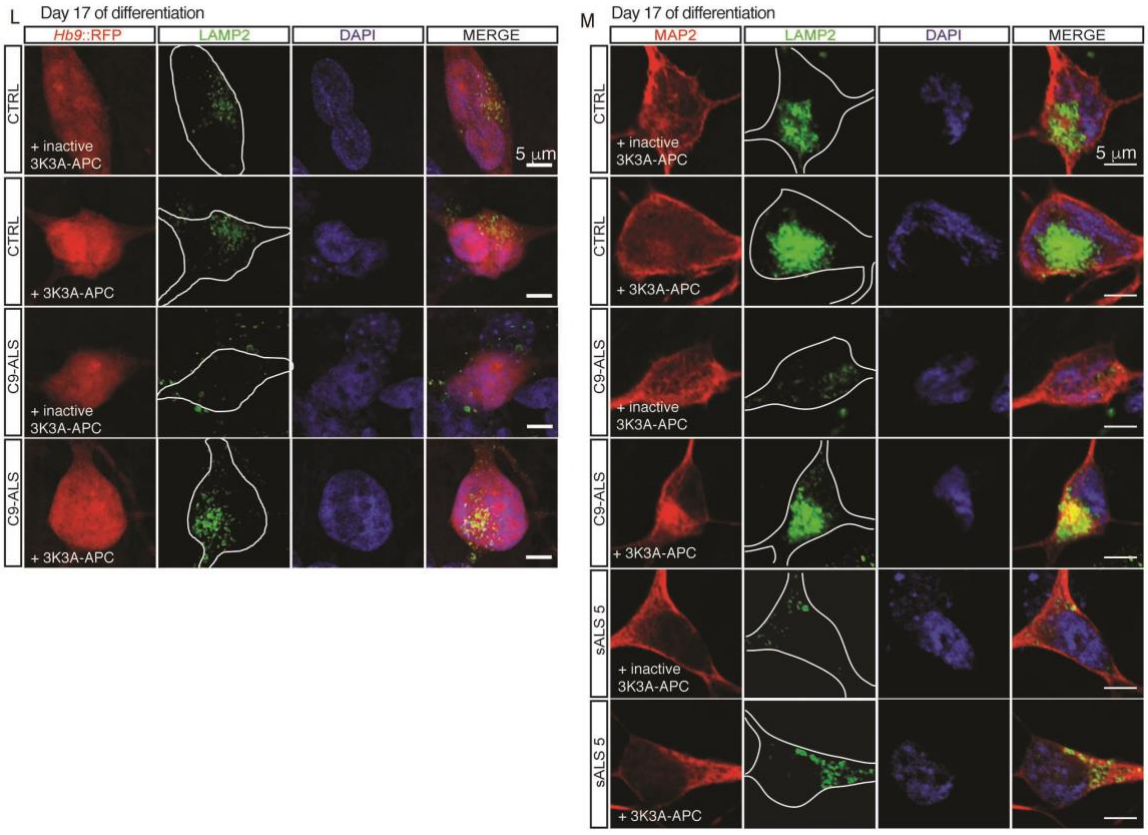


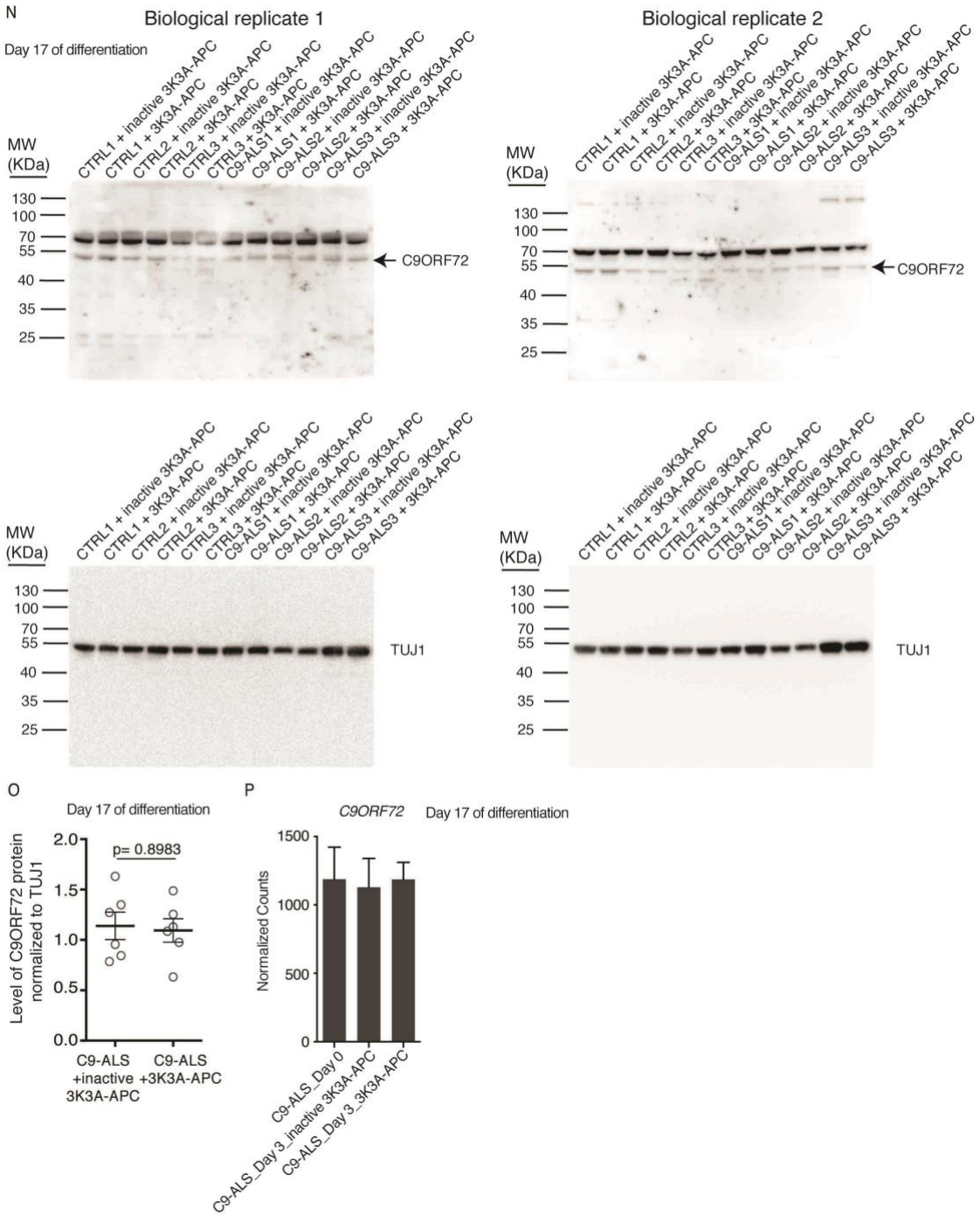
K



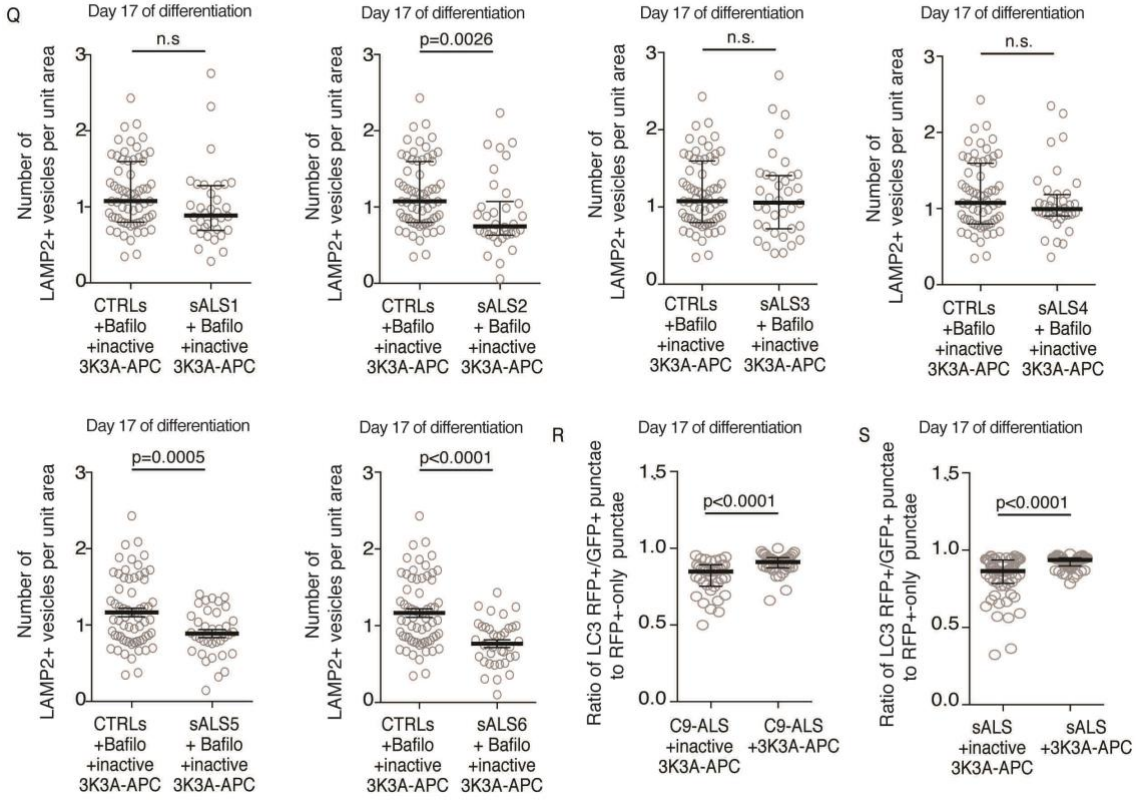
Supplemental Figure 2(L-M).
C9ORF72 and sporadic ALS iMNs share autophagosome formation abnormalities that are rescued by 3K3A-APC



Supplemental Figure 2(N-P).
C9ORF72 and sporadic ALS iMNs share autophagosome formation abnormalities that are rescued by 3K3A-APC



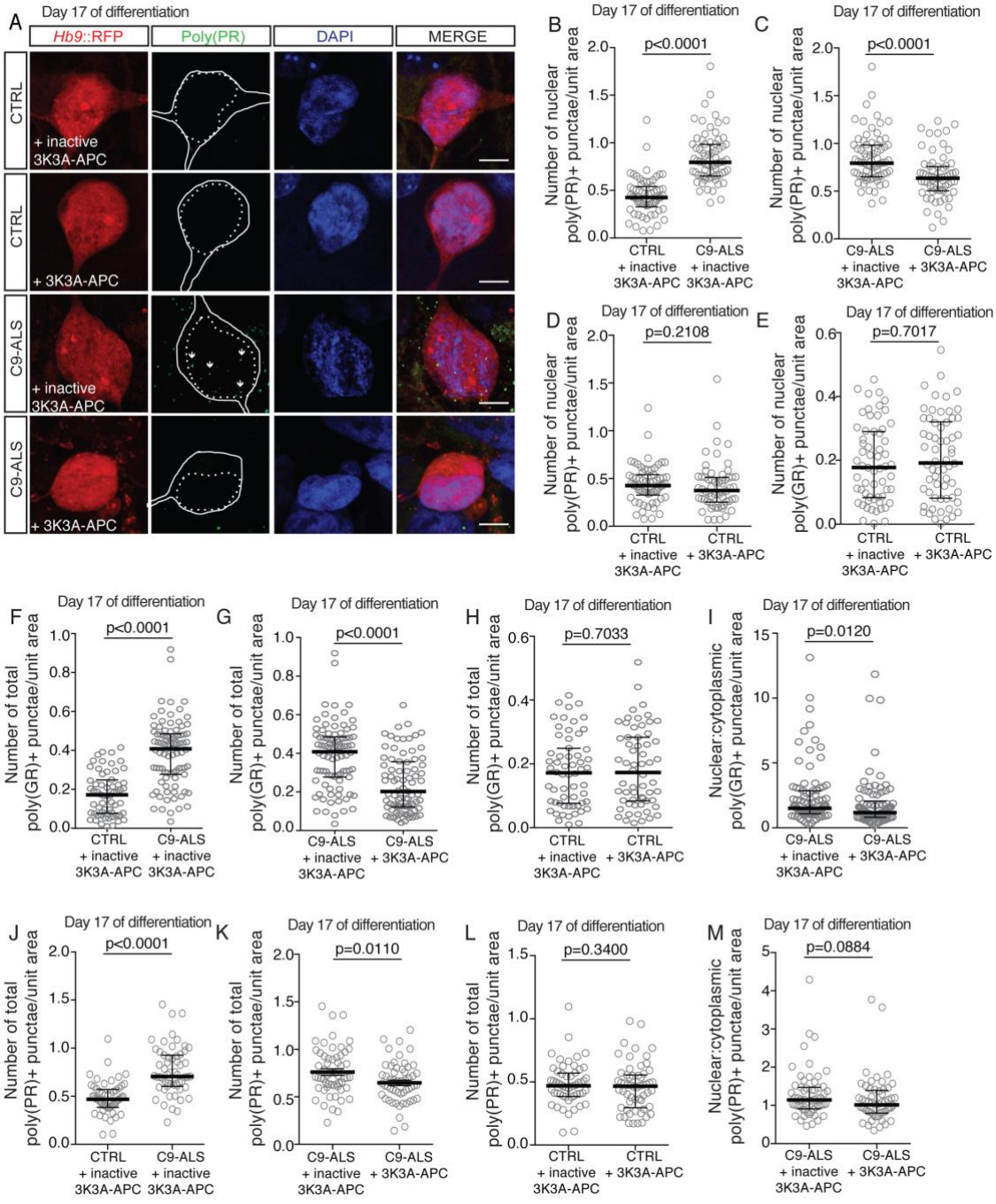
Supplemental Figure 2(Q-S).
C9ORF72 and sporadic ALS iMNs share autophagosome formation abnormalities that are rescued by 3K3A-APC



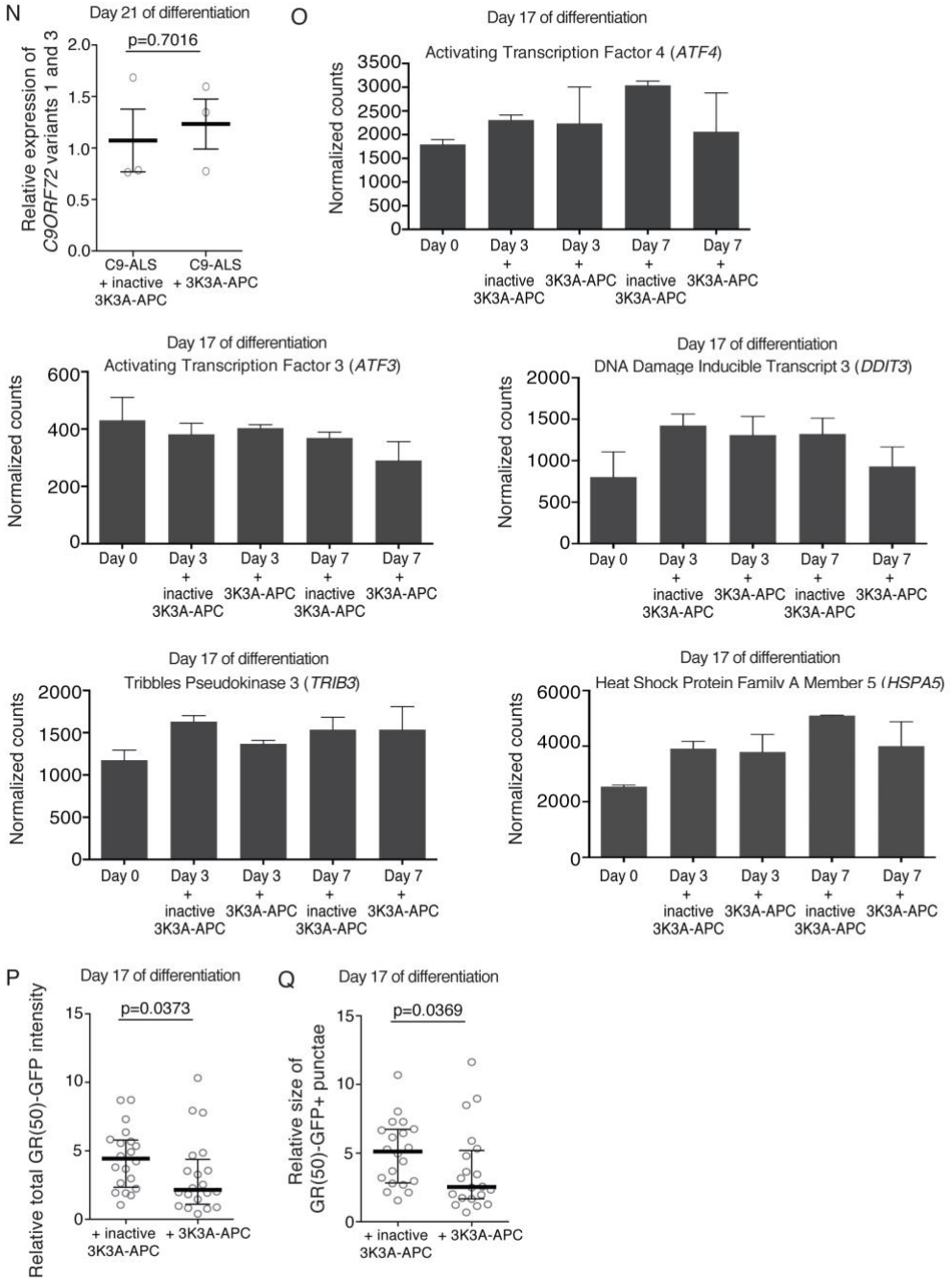
Supplemental Figure 2. C9ORF72 and sporadic ALS iMNs share autophagosome formation abnormalities that are rescued by 3K3A-APC. (A) Number of RFP^+/GFP^+ vesicles per iMN in C9-ALS iMNs treated with DMSO or 50 nM Bafilomycin for 24 hours. iMNs from 3 controls and 3 C9-ALS patients were quantified. n=30 iMNs per line per condition across 2 independent iMN conversions were quantified. Median +/- interquartile range. Two-tailed Mann-Whitney test. Bafilo = Bafilomycin. Grey circles represent individual iMNs. **(B)** Number of RFP^+/GFP^+ vesicles per iMN in sporadic ALS iMNs treated with 50 nM Bafilomycin and inactive 3K3A-APC or 3K3A-APC for 24 hours. iMNs from 5 sporadic ALS lines were quantified. n=30 iMNs (controls) or 12 iMNs (sporadic ALS patients) per line per condition across 2 independent iMN conversions were quantified. For control (CTRL) vs sALS1, 4, 5, 6, mean +/- s.e.m. and unpaired t-test. For CTRL vs sALS 3, median +/- interquartile range and two-tailed Mann-Whitney test. Bafilo = Bafilomycin. Grey circles represent individual iMNs. **(C, D)** Immunoblot analysis (C) and quantification (D) of LC3-I and LC3-II in C9-ALS iPSC-derived motor neurons + 50 nM Bafilomycin + 10 nM inactive 3K3A-APC or 3K3A-APC for 48 hours. Samples from four independent motor neuron treatments are shown for each treatment condition. Mean +/- interquartile range. Two-tailed Mann-Whitney test. Grey circles represent individual samples. Samples for replicate 4 were run on the same gel as the other samples but were noncontiguous. The full unedited gels for (C) are shown. **(E)** The difference in gene expression between inactive 3K3A-APC- and

3K3A-APC-treated C9-ALS iMNs at day 3 of treatment. Genes are plotted based on their log2 fold change (x-axis) from InAPC- to APC- treated cells and the log10 of the p-value for the significance of this change as determined by DESeq2. RNA-seq data from two samples per condition were used for this analysis. **(F)** Heatmap showing log2 fold change of differentially expressed genes in 10 nM inactive 3K3A-APC- and 3K3A-APC-treated C9-ALS iMNs relative to the average of both at 3 days. RNA-seq data from two independent iMN conversions and treatments were analyzed. **(G)** Ingenuity Pathway Analysis of RNA-seq data of C9-ALS iMNs treated with 10 nM inactive or active 3K3A-APC for 3 days. **(H)** KEGG pathway analysis of RNA-seq data from C9-ALS iMNs treated with 10 nM inactive or active 3K3A-APC for 3 days. Two samples included per condition. **(I)** mRNA levels of *ATG5* or *ATG10* in C9-ALS iMNs treated with 10 nM inactive or active 3K3A-APC for 3 days. Data were derived from RNA-seq analyses. **(J)** mRFP-GFP-LC3 fluorescence in control or sporadic ALS iMNs treated with 50 nM Bafilomycin and 10 μM DMSO or rapamycin. Bafilo = Bafilomycin. Scale bars = 5 μm. sALS = sporadic ALS. Solid and dotted lines outline the cell body and nucleus, respectively. **(K)** Number of RFP^+/GFP^+ vesicles per iMN in control or sporadic ALS iMNs treated with 50 nM Bafilomycin and 10 μM DMSO or rapamycin for 24 hours. iMNs from 3 controls and 5 sporadic ALS patients were quantified. n=12 iMNs per line per condition across 2 independent iMN conversions were quantified. Each grey circle represents a single iMN. Median +/- interquartile range. Non-parametric Kruskal-Wallis testing. **(L)** Images showing the number of $LAMP2^+$ lysosomes in control or C9-ALS iMNs treated with inactive 3K3A-APC or 3K3A-APC. White solid lines outline iMN cell bodies. Scale bars = 5 μm. **(M)** Images showing the number of $LAMP2^+$ lysosomes in control, C9-ALS, or sporadic ALS $MAP2^+$ iMNs treated with inactive 3K3A-APC or 3K3A-APC. White solid lines outline iMN cell bodies. Scale bars = 5 μm. **(N-O)** Western blots (N) and quantification (O) showing levels of C9ORF72 in C9-ALS iMNs treated with inactive or active 3K3A-APC for 3 days. iMNs from two independent conversions were tested. Grey circles represent individual samples. Mean +/- s.e.m. Two-tailed t-test. The full unedited gels for (N) are shown. **(P)** mRNA levels of *C9ORF72* in Hb9::RFP^+ flow-sorted C9-ALS patient iMNs. n=3 biological replicates of iMNs from one C9-ALS patient line per condition. Mean +/- s.d. One-way ANOVA. **(Q)** Number of $LAMP2^+$ vesicles in sporadic ALS iMNs treated with 50 nM Bafilomycin and 10 nM inactive 3K3A-APC for 24 hours. iMNs from 3 controls and 6 sporadic ALS patients were quantified. n=33 iMNs per line per condition across 2 independent iMN conversions were quantified. Median +/- interquartile range and non-parametric Mann-Whitney testing for sALS 1-4 and mean +/- s.e.m. and unpaired t-test for sALS 5, 6. **(R, S)** Ratio of RFP^+/GFP^+ vesicles to RFP^+-only vesicles in control, C9-ALS (R), or sporadic ALS (S) iMNs treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 24 hours. iMNs from 3 controls, 3 C9-ALS, and 6 sporadic ALS patients were quantified. n=30 (controls and C9-ALS) or 12 (sporadic ALS) iMNs per line per condition across 2 independent iMN conversions were quantified. Each grey circle represents a single iMN. Mean +/- s.e.m. Two-tailed unpaired t-test. The day of differentiation stated on each panel indicates the day of differentiation on which the experimental treatment or time course was initiated.

Supplemental Figure 3(A-M). Rescue of autophagosome formation abnormalities by 3K3A-APC improves proteostasis



Supplemental Figure 3(N-Q). Rescue of autophagosome formation abnormalities by 3K3A-APC improves proteostasis



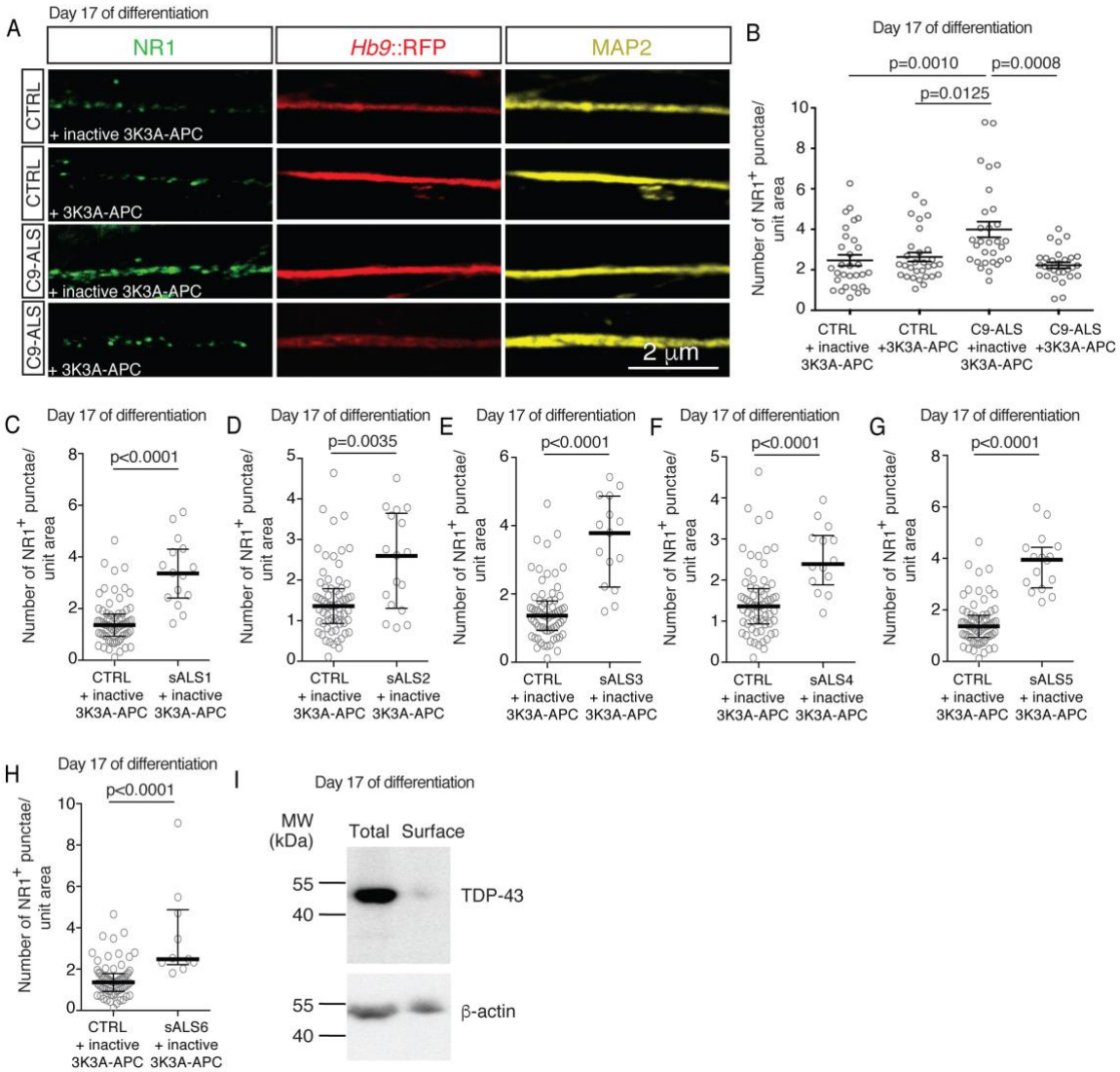
Supplemental Figure 3. Rescue of autophagosome formation abnormalities by 3K3A-APC improves proteostasis. **(A-D)** Immunostaining (A) and quantification (B-D) to determine endogenous poly(PR)$^{+}$ punctae in control or C9-ALS iMNs with 10 nM inactive 3K3A-APC or 3K3A-APC treatment for 6 days. Quantified values represent the number of nuclear poly(PR)$^{+}$ punctae in n=30 iMNs per line per condition from two

control or two C9-ALS patient lines. For each line, iMNs were quantified from two independent iMN conversions per line per condition. Median +/- interquartile range. Each grey circle represents the number of poly(PR)$^+$ punctae/unit area in a single iMN. Non-parametric Mann-Whitney testing. Solid and dotted lines in (A) outline the cell body and nucleus, respectively. Scale bar = 5 μm. **(E)** Number of nuclear poly(GR)$^+$ punctae in control iMNs treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Quantified values represent the number of nuclear poly(GR)$^+$ punctae in n=30 iMNs per line per condition from two control lines. For each line, iMNs were quantified from two independent iMN conversions per line per condition. Median +/- interquartile range. Each grey circle represents the number of poly(GR)$^+$ punctae/unit area in a single iMN. Non-parametric Mann-Whitney testing. **(F-H)** Number of total (nuclear and cytoplasmic) poly(GR)$^+$ punctae in control or C9-ALS iMNs treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Quantified values represent the number of total poly(GR)$^+$ punctae in n=30 iMNs (controls) or 41-44 iMNs (C9-ALS) per line per condition from two control or C9-ALS lines. For each line, iMNs were quantified from two independent iMN conversions per line per condition. Median +/- interquartile range. Each grey circle represents the number of poly(GR)$^+$ punctae/unit area in a single iMN. Non-parametric Mann-Whitney testing. **(I)** Ratio of nuclear:cytoplasmic poly(GR)$^+$ punctae/unit area in C9-ALS iMNs treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Quantified values represent the number of nuclear:cytoplasmic poly(GR)$^+$ punctae in n=30 iMNs (controls) or 41-44 iMNs (C9-ALS) per line per condition from two control or C9-ALS lines. For each line, iMNs were quantified from two independent iMN conversions per line per condition. Median +/- interquartile range. Each grey circle represents the ratio of nuclear:cytoplasmic poly(GR)$^+$ punctae/unit area in a single iMN. Non-parametric Mann-Whitney testing. **(J-L)** Number of total (nuclear and cytoplasmic) poly(PR)$^+$ punctae in control or C9-ALS iMNs treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Quantified values represent the number of total poly(GR)$^+$ punctae in n=30 iMNs per line per condition from two control or C9-ALS lines. For each line, iMNs were quantified from two independent iMN conversions per line per condition. Median +/- interquartile range except for (K), which is mean +/- s.e.m. Each grey circle represents the number of poly(PR)$^+$ punctae/unit area in a single iMN. Non-parametric Mann-Whitney testing except for (K), which was tested by unpaired t-test. **(M)** Ratio of nuclear:cytoplasmic poly(PR)$^+$ punctae/unit area in C9-ALS iMNs treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Quantified values represent the number of nuclear:cytoplasmic poly(GR)$^+$ punctae in n=30 iMNs per line per condition from two control or C9-ALS lines. For each line, iMNs were quantified from two independent iMN conversions per line per condition. Median +/- interquartile range. Each grey circle represents the ratio of nuclear:cytoplasmic poly(PR)$^+$ punctae/unit area in a single iMN. Non-parametric Mann-Whitney testing. **(N)** qRT-PCR analysis of *C9ORF72* variants 1 and 3 (repeat-expansion-containing transcripts) mRNA in C9-ALS patient motor neurons treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 3 days. n=3 biological replicates of iMNs from one C9-ALS patient line per condition. Mean +/- s.e.m. Unpaired t-test. **(O)** RNA-seq data showing expression levels of genes associated with the integrated stress response in *Hb9*::RFP$^+$ C9-ALS iMNs treated with 10 nM inactive 3K3A-APC or 3K3A-APC for the durations shown. n=3 biological replicates of iMNs from one C9-ALS patient line per condition. Mean +/- s.e.m. One-way ANOVA.

(P) Relative total GFP intensity of GR(50)-GFP$^+$ punctae in iMNs 6 days before degeneration when treated with 10 nM inactive 3K3A-APC or 3K3A-APC. n=20 iMNs quantified for each condition from 3 independent conversions. Each grey circle represents the total GFP intensity of GR(50)-GFP$^+$ punctae in one iMN. Median +/- interquartile range. Mann-Whitney test. **(Q)** Relative size of GR(50)-GFP$^+$ punctae in iMNs 6 days before degeneration when treated with 10 nM inactive 3K3A-APC or 3K3A-APC. n=20 iMNs quantified for each condition from 3 independent conversions. Each grey circle represents the size of GR(50)-GFP$^+$ punctae in one iMN. Median +/- interquartile range. Mann-Whitney test. The day of differentiation stated on each panel indicates the day of differentiation on which the experimental treatment or time course was initiated.

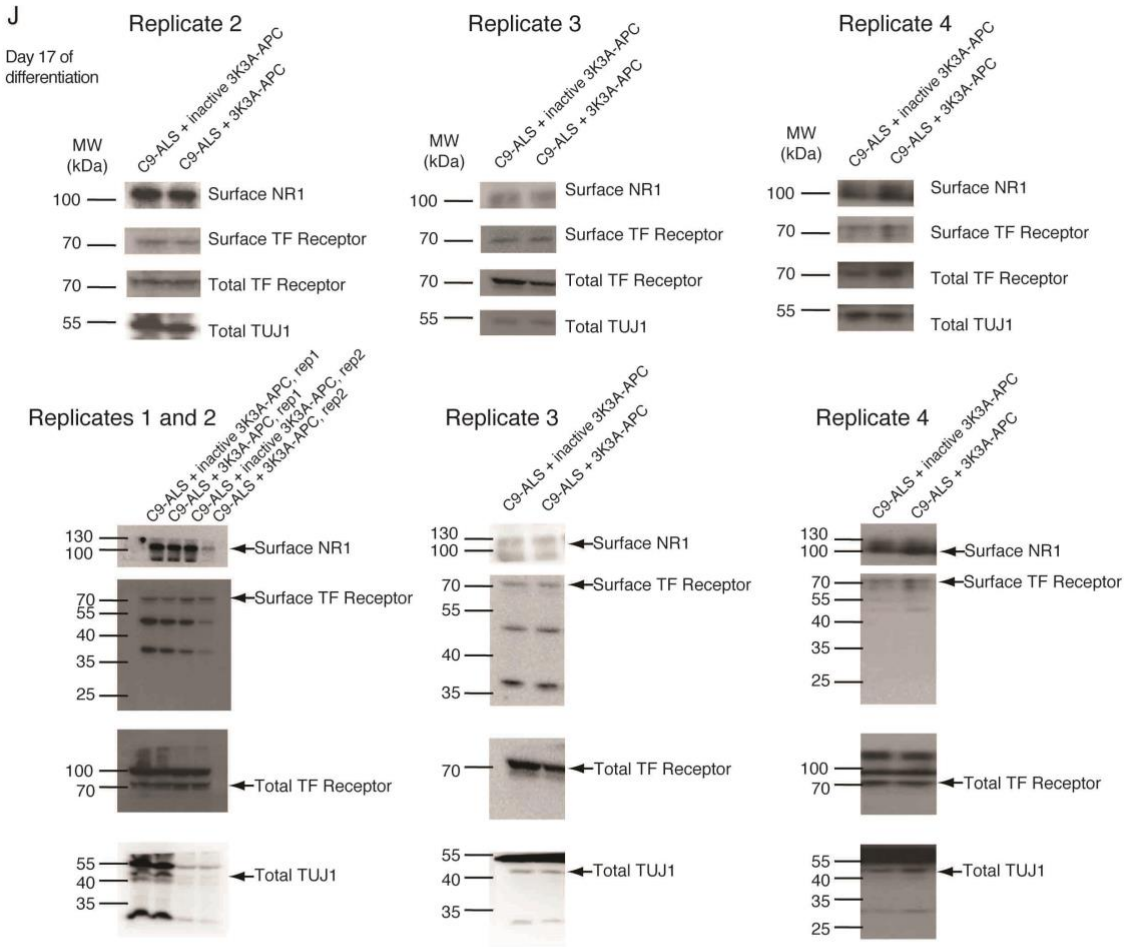
Supplemental Figure 4(A-I).

C9ORF72 and sporadic ALS iMNs have elevated glutamate receptor levels that are normalized by 3K3A-APC

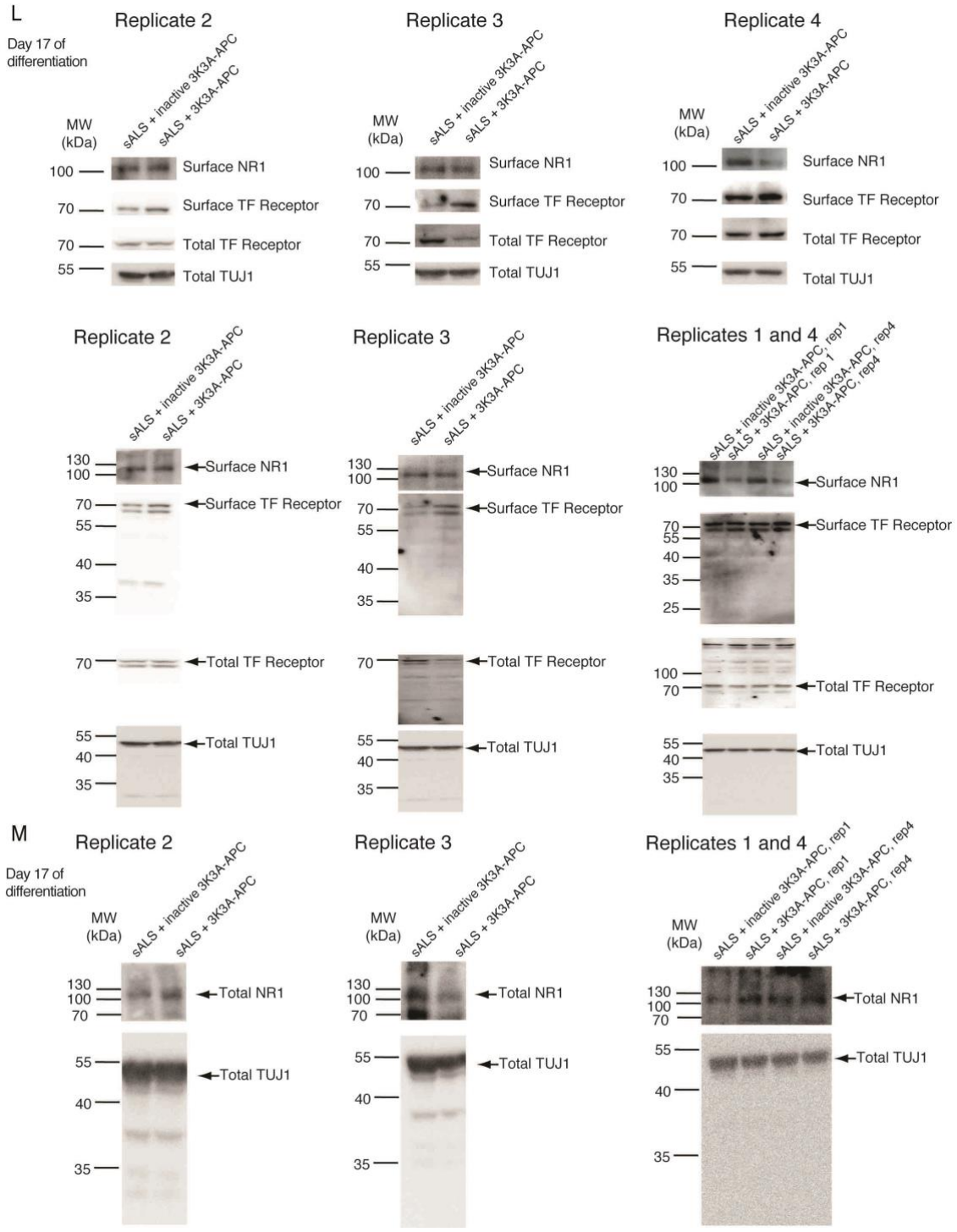


Supplemental Figure 4(J, K).

C9ORF72 and sporadic ALS iMNs have elevated glutamate receptor levels that are normalized by 3K3A-APC

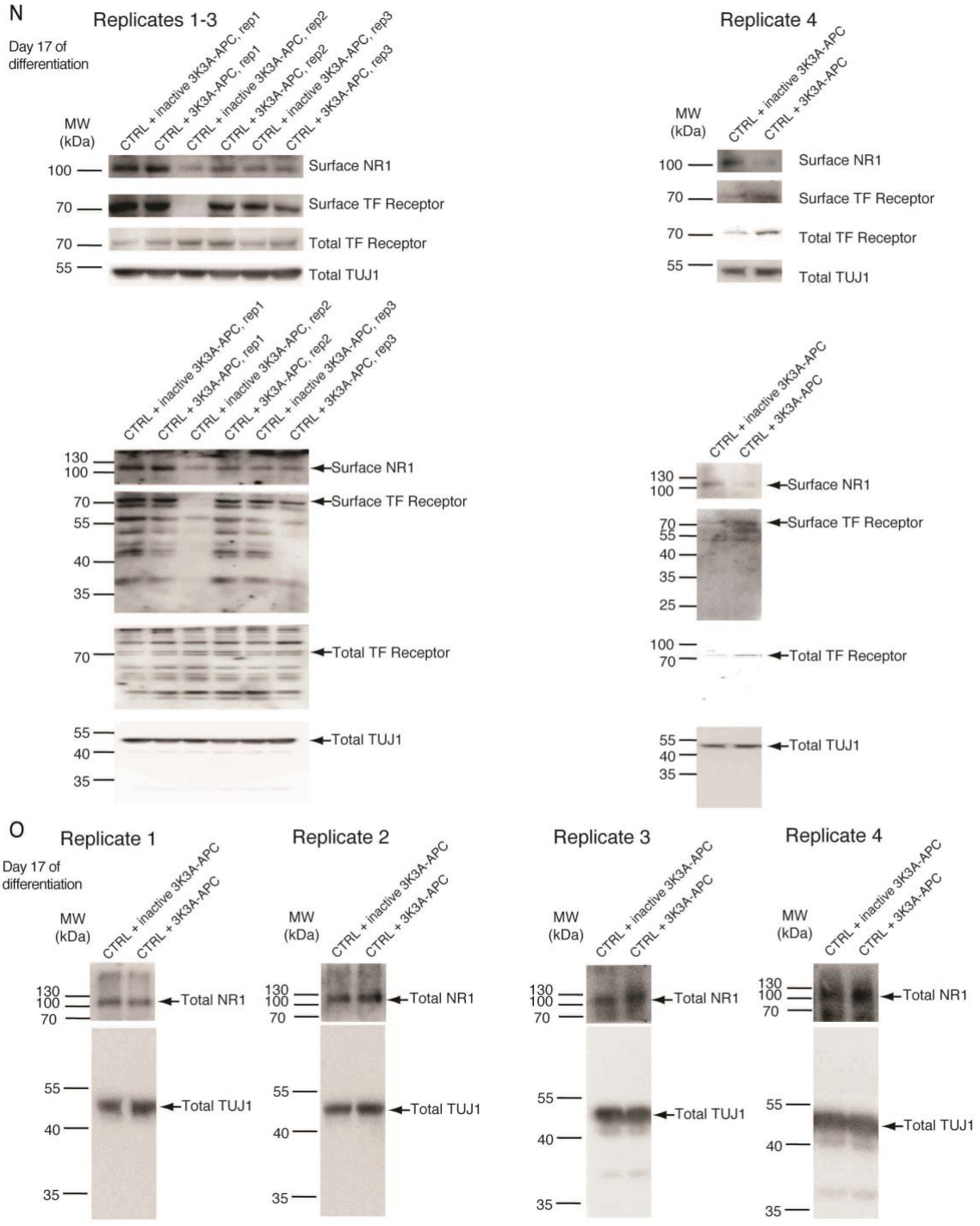


Supplemental Figure 4(L, M).
C9ORF72 and sporadic ALS iMNs have elevated glutamate receptor levels that are normalized by 3K3A-APC

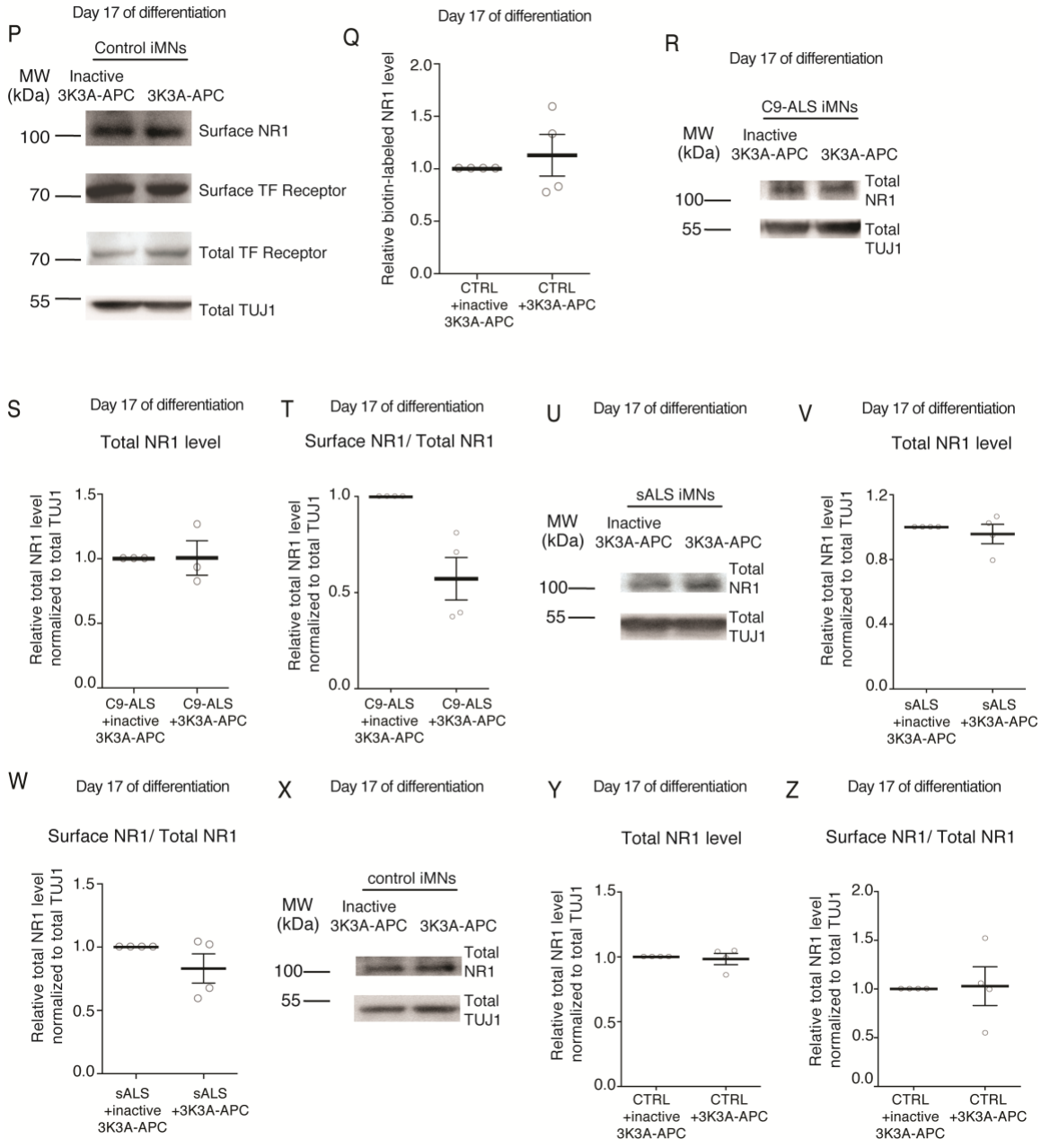


Supplemental Figure 4(N, O).

C9ORF72 and sporadic ALS iMNs have elevated glutamate receptor levels that are normalized by 3K3A-APC



Supplemental Figure 4(P-Z).
C9ORF72 and sporadic ALS iMNs have elevated glutamate receptor levels that are normalized by 3K3A-APC

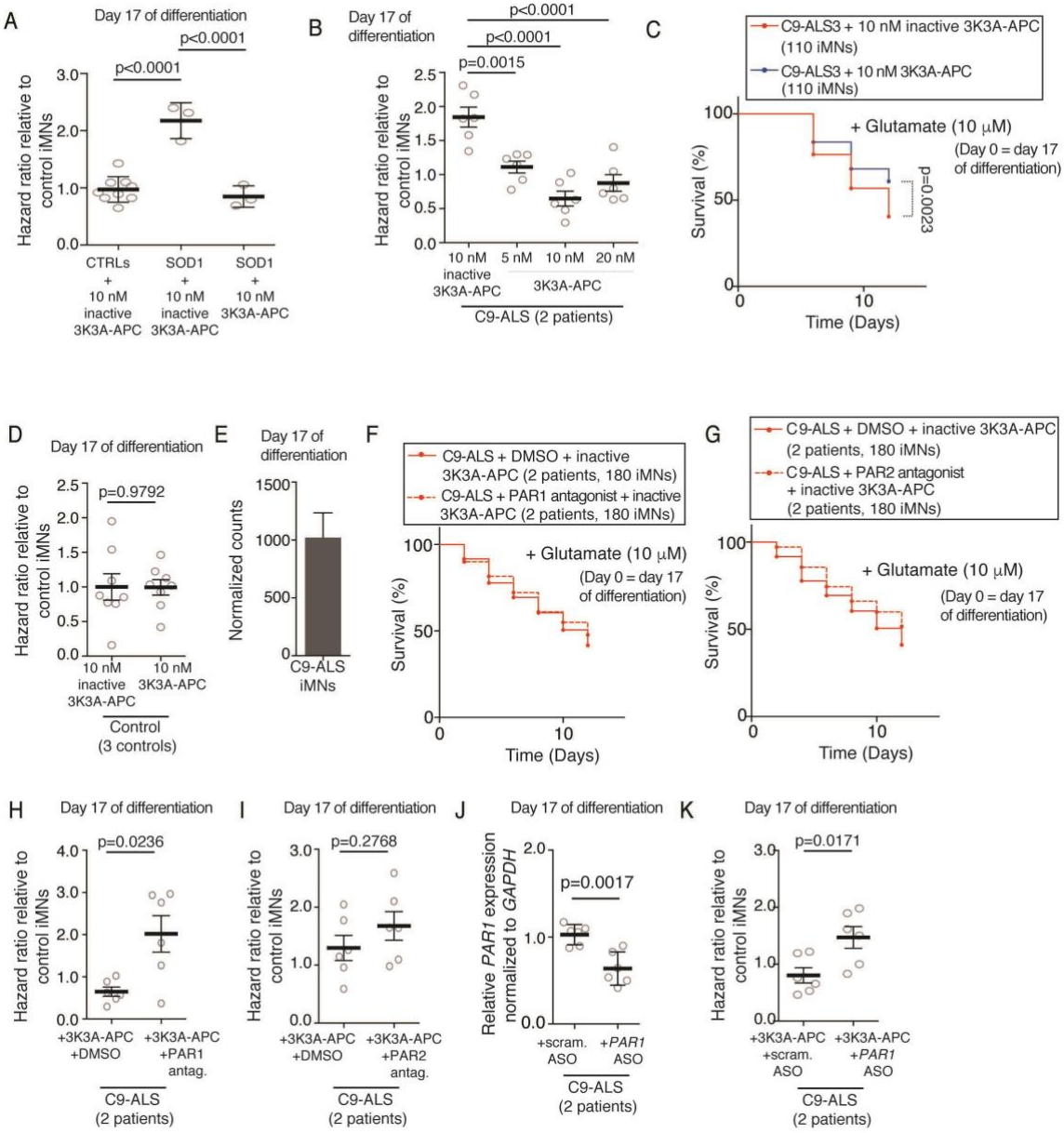


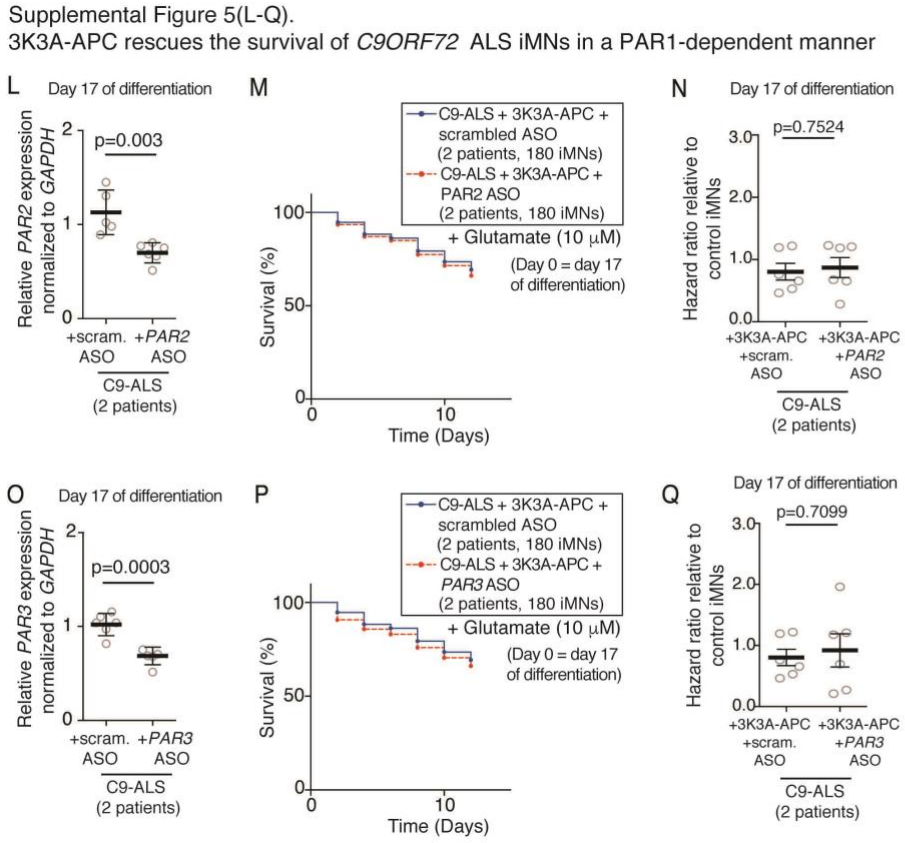
Supplemental Figure 4. Rescue of autophagosome abnormalities by 3K3A-APC improves proteostasis. (A) Confocal microscopy images of immunofluorescence shows NR1+ puncta on MAP2+ dendrites of iMNs treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Scale bar: 2 µm. This experiment was repeated 3 times with similar results. **(B)** Number of $NR1^+$ punctae per unit area in control or C9-ALS iMNs. Each grey circle represents the number of $NR1^+$ punctae per area unit on a single dendrite (one dendrite quantified per iMN). n=15 iMNs quantified per line per condition from two biologically independent iMN conversions from two control and two C9-ALS lines.

Median +/- interquartile range. Non-parametric Kruskal-Wallis testing. Each grey circle represents a single iMN. **(C-H)** Number of $NR1^+$ punctae per unit area in control or sporadic ALS iMNs. Each grey circle represents the number of $NR1^+$ punctae per area unit on a single neurite. n=15 (controls) or 15-17 (sporadic ALS) iMNs quantified per line per condition from two biologically independent iMN conversions from two control or six individual sporadic ALS lines. Median +/- interquartile range. Non-parametric Kruskal-Wallis testing. Each grey circle represents a single iMN. **(I)** Western blot of the surface-bound and total fractions of iMNs to determine the amount of intracellular protein (TDP-43) contaminating the surface-bound protein fraction. (I) is the full unedited gel. **(J)** Western blot analysis of surface NR1 after surface protein biotinylation in C9-ALS iMNs generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. TF = Transferrin. Samples from four independent iMN conversions and treatments were used. **(K)** Western blot analysis of total NR1 in C9-ALS iMNs generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Samples from three independent iMN conversions and treatments were used. **(L)** Western blot analysis of surface NR1 after surface protein biotinylation in sporadic ALS iMNs generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. TF = Transferrin. Samples from four independent iMN conversions and treatments were used. **(M)** Western blot analysis of total NR1 in sporadic ALS iMNs generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Samples from four independent iMN conversions and treatments were used. **(N)** Western blot analysis of surface NR1 after surface protein biotinylation in control iMNs generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. TF = Transferrin. Samples from four independent iMN conversions and treatments were used. **(O)** Western blot analysis of total NR1 in control iMNs generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. Samples from four independent iMN conversions and treatments were used. **(P)** Immunoblotting analysis of surface NR1 after surface protein biotinylation in control iMNs (one control) generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. TF = Transferrin. **(Q)** Quantification of NR1 immunoblotting from (P). n=4 biologically independent iMN conversions. Each grey circle represents an individual sample. Mean +/- s.e.m. Unpaired t-test. The ratio of surface to total Transferrin Receptor was used to normalize for the membrane protein extraction efficiency and TUJ1 was used to normalize for neuron number. **(R-T)** Immunoblotting analysis (R) and quantification (S, T) of total NR1 (S) and surface:total NR1 (T) in C9-ALS iMNs (one patient) generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. TF = Transferrin. Mean +/- s.e.m. Unpaired t-test. **(U-W)** Immunoblotting analysis (U) and quantification (V, W) of total NR1 (V) and surface:total NR1 (W) in sporadic ALS iMNs (one patient) generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-APC for 6 days. TF = Transferrin. Mean +/- s.e.m. Unpaired t-test. **(X-Z)** Immunoblotting analysis (X) and quantification (Y, Z) of total NR1 (Y) and surface:total NR1 (Z) in control iMNs (one control) generated with 3 factors (*NGN2*, *ISL1*, and *LHX3*) and treated with 10 nM inactive 3K3A-APC or 3K3A-

APC for 6 days. TF = Transferrin. Mean +/- s.e.m. Unpaired t-test. The day of differentiation stated on each panel indicates the day of differentiation on which the experimental treatment or time course was initiated. (J-O) are the full unedited gels for Fig. 4F, H and Supplemental Fig. 4P, R, U, X.

Supplemental Figure 5(A-K).
3K3A-APC rescues the survival of *C9ORF72* ALS iMNs in a PAR1-dependent manner

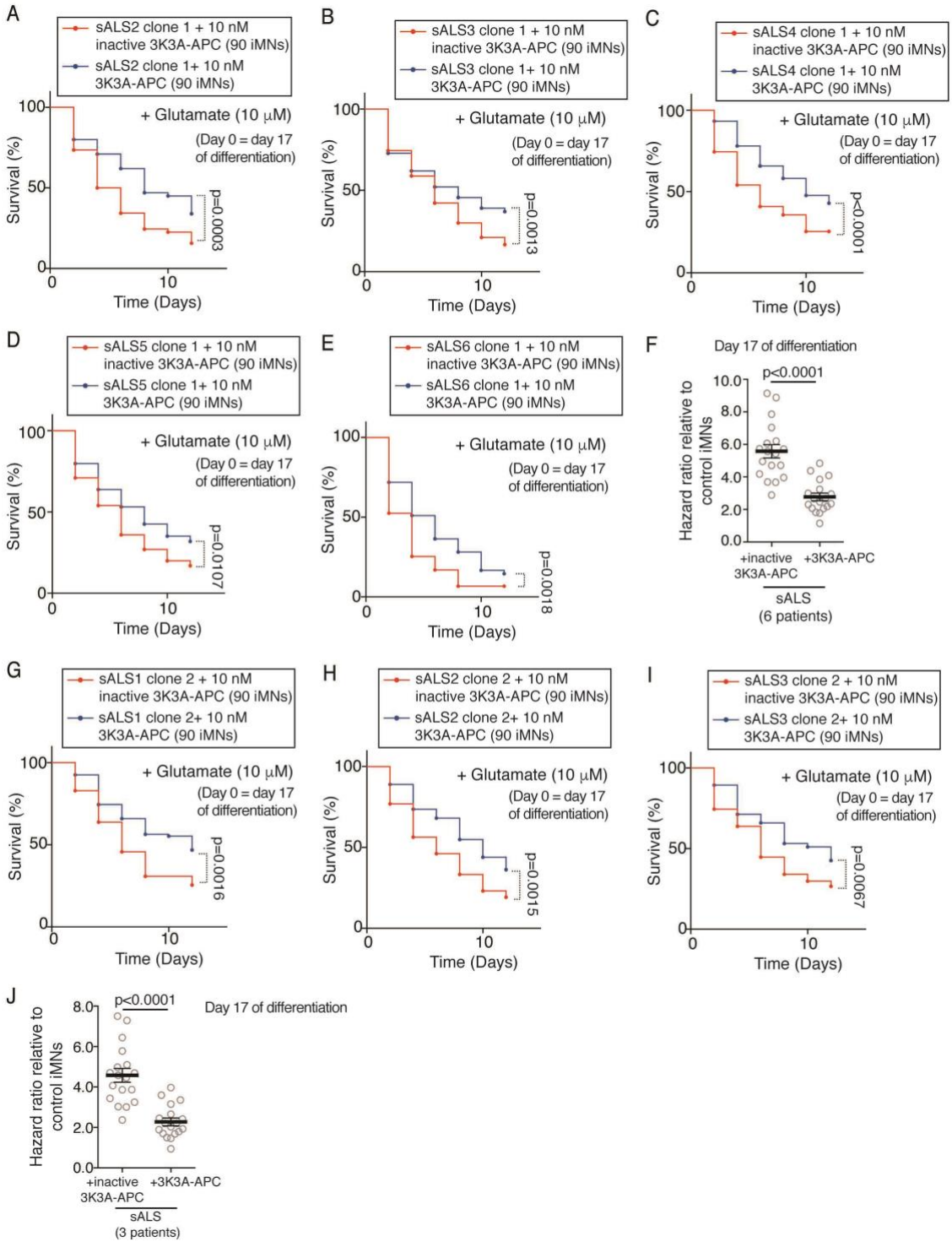




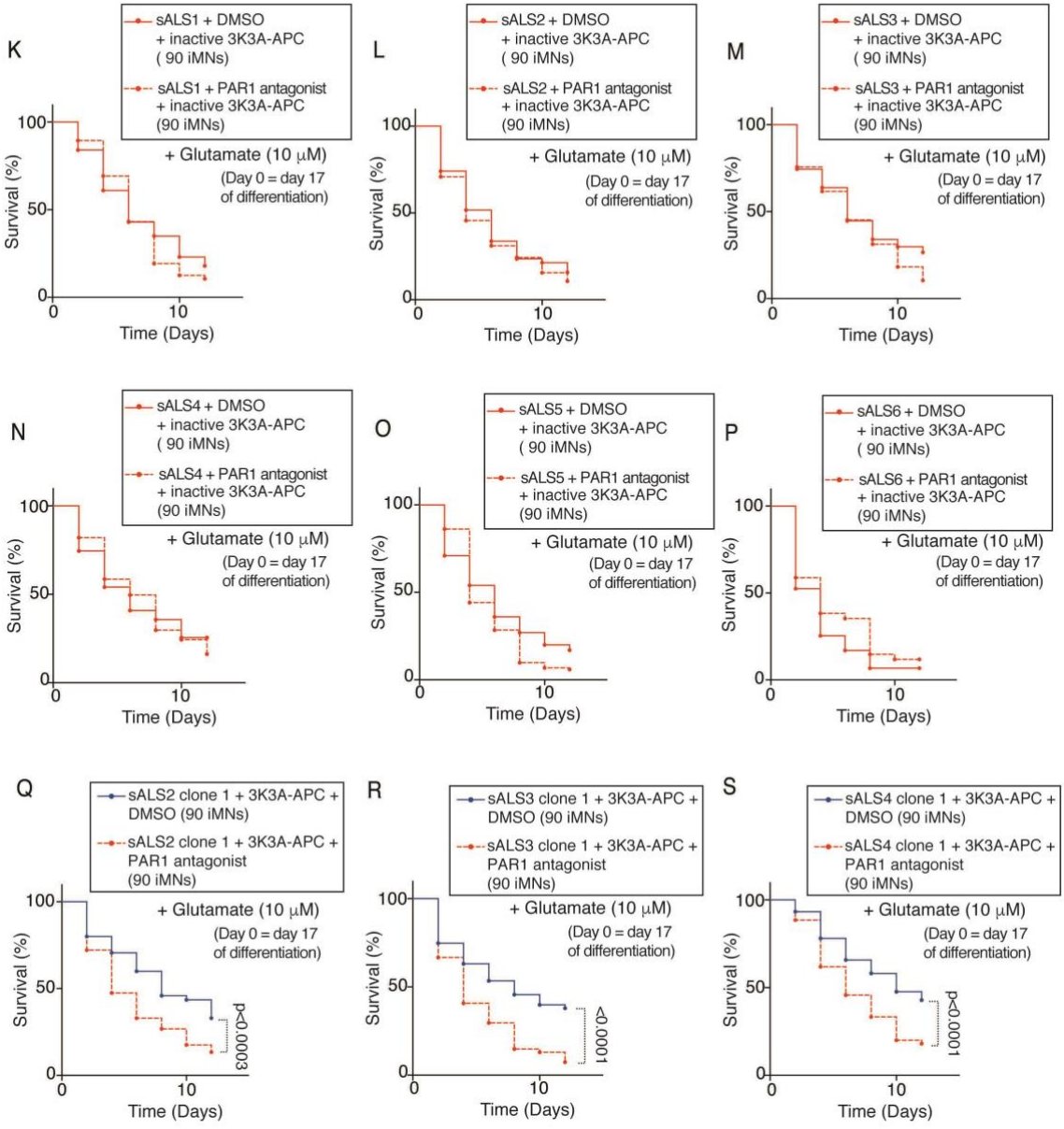
Supplemental Figure 5. 3K3A-APC rescues the survival of C9ORF72 ALS iMNs in a PAR1-dependent manner. (A) Hazard ratio (relative to control iMNs) of control (three controls) or *SOD1*A4V ALS (one patient) iMNs treated with inactive 3K3A-APC or 3K3A-APC in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. One-way ANOVA. **(B)** Hazard ratio (relative to control iMNs) of C9-ALS iMNs from two patients treated with inactive or different concentrations of 3K3A-APC in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. One-way ANOVA. **(C)** Survival of C9-ALS3 iMNs with excess glutamate with inactive 3K3A-APC or 3K3A-APC. iMNs quantified from 3 biologically independent iMN conversions per line. Two-sided log-rank test using the entire survival time course. **(D)** Hazard ratio (relative to control iMNs treated with inactive 3K3A-APC) of control iMNs from three controls treated with inactive 3K3A-APC or 3K3A-APC in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test. **(E)** RNA-seq data showing the number of gene counts for the *F2R* gene in flow-purified, *Hb9*::RFP^{+} C9-ALS iMNs. **(F, G)** Survival of C9-ALS iMNs in excess glutamate with or without 3 μM PAR1 antagonist treatment (F) or PAR2 antagonist treatment (G). n=90 iMNs per line per condition, iMNs from both lines shown in aggregate for clarity. iMNs quantified from 3 biologically independent iMN conversions per line. **(H)** Hazard ratio (relative to control iMNs) of C9-ALS iMNs from two patients treated with 3K3A-APC and DMSO or a PAR1 antagonist in the

excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test. Antag. = antagonist. **(I)** Hazard ratio (relative to control iMNs) of C9-ALS iMNs from two patients treated with 3K3A-APC and DMSO or a PAR2 antagonist in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test. **(J)** qRT-PCR measuring *PAR1* expression in C9-ALS iMNs from two patients treated with a scrambled ASO or a *PAR1* ASO. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.d. Unpaired t-test. Scram. = scrambled. **(K)** Hazard ratio (relative to control iMNs) of C9-ALS iMNs from two patients treated with 3K3A-APC and a scrambled ASO or a *PAR1* ASO in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test. Scram. = scrambled. **(L)** qRT-PCR measuring *PAR2* expression in C9-ALS iMNs from two patients treated with a scrambled ASO or a *PAR2* ASO. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.d. Unpaired t-test. Scram. = scrambled. **(M)** Survival of iMNs from 2 C9-ALS lines in excess glutamate with 3K3-APC with or without 9 μM *PAR2* ASO treatment. n=90 iMNs per line per condition, iMNs from both lines shown in aggregate for clarity. iMNs quantified from 3 biologically independent iMN conversions per line. Each trace includes neurons from at 2 donors with the specified genotype. Two-sided log-rank test using the entire survival time course. **(N)** Hazard ratio (relative to control iMNs) of C9-ALS iMNs from two patients treated with 3K3A-APC and a scrambled ASO or a *PAR2* ASO in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test. Scram. = scrambled. **(O)** qRT-PCR measuring *PAR3* expression in C9-ALS iMNs from two patients treated with a scrambled ASO or a *PAR3* ASO. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.d. Unpaired t-test. Scram. = scrambled. **(P)** Survival of iMNs from 2 C9-ALS lines in excess glutamate with 3K3-APC with or without 9 μM *PAR3* ASO treatment. n=90 iMNs per line per condition, iMNs from both lines shown in aggregate for clarity. iMNs quantified from 3 biologically independent iMN conversions per line. Each trace includes neurons from at 2 donors with the specified genotype. Two-sided log-rank test using the entire survival time course. **(Q)** Hazard ratio (relative to control iMNs) of C9-ALS iMNs from two patients treated with 3K3A-APC and a scrambled ASO or a *PAR3* ASO in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test. Scram. = scrambled. For A, B, D, H-K, L, N, O, and Q, grey circles represent individual samples. The day of differentiation stated on each panel indicates the day of differentiation on which the experimental treatment or time course was initiated.

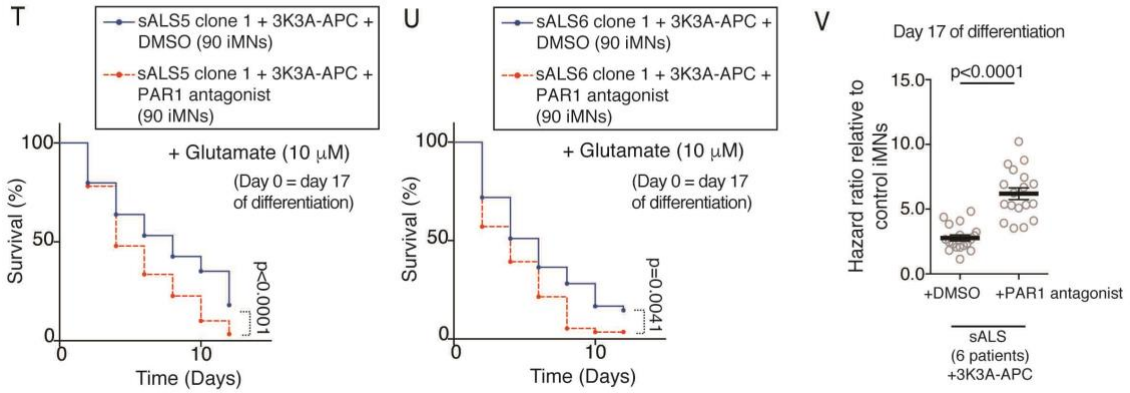
Supplemental Figure 6(A-J).
3K3A-APC rescues the survival of sporadic ALS iMNs in a PAR1-dependent manner



Supplemental Figure 6(K-S).
3K3A-APC rescues the survival of sporadic ALS iMNs in a PAR1-dependent manner



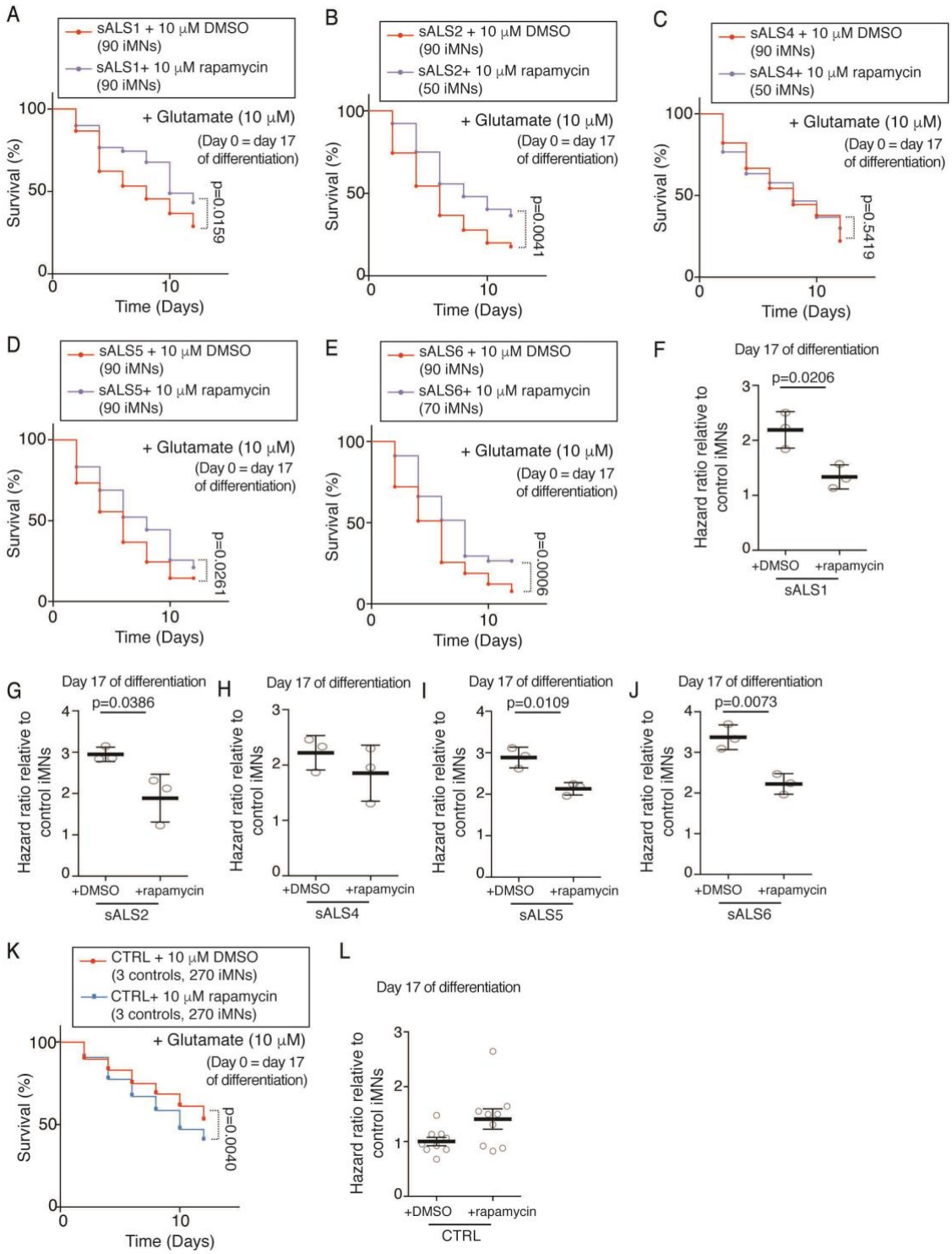
Supplemental Figure 6(T-V).
3K3A-APC rescues the survival of sporadic ALS iMNs in a PAR1-dependent manner



Supplemental Figure 6. 3K3A-APC rescues the survival of sporadic ALS iMNs in a PAR1-dependent manner. (A-E) Survival of sALS2 (A), sALS3 (B), sALS4 (C), sALS5 (D), or sALS6 (E) sporadic ALS iMNs with excess glutamate with inactive 3K3A-APC or 3K3A-APC. iMNs quantified from 3 biologically independent iMN conversions per line. Two-sided log-rank test using the entire survival time course. **(F)** Hazard ratio (relative to control iMNs treated with inactive 3K3A-APC) of sporadic ALS iMNs from six sporadic ALS patients treated with inactive 3K3A-APC or 3K3A-APC in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test with Welch's correction. Grey circles represent individual samples. **(G-I)** Survival of iMNs from a second clone of sALS1 (G), sALS2 (H), and sALS3 (I) in excess glutamate with inactive 3K3A-APC or 3K3A-APC. iMNs quantified from 3 biologically independent iMN conversions per line. Two-sided log-rank test using the entire survival time course. **(J)** Hazard ratio (relative to control iMNs treated with inactive 3K3A-APC) of iMNs from clone 2 of three sporadic ALS patients treated with inactive 3K3A-APC or 3K3A-APC in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test. Grey circles represent individual samples. **(K-P)** Survival of sALS1 (K), sALS2 (L), sALS3 (M), sALS4 (N), sALS5 (O), or sALS6 (P) sporadic ALS iMNs with excess glutamate with inactive 3K3A-APC and DMSO or a PAR1 antagonist. iMNs quantified from 3 biologically independent iMN conversions per line. Two-sided log-rank test using the entire survival time course. **(Q-U)** Survival of sALS2 (Q), sALS3 (R), sALS4 (S), sALS5 (T), or sALS6 (U) sporadic ALS iMNs with excess glutamate with 3K3A-APC and DMSO or a PAR1 antagonist. iMNs quantified from 3 biologically independent iMN conversions per line. Two-sided log-rank test using the entire survival time course. **(V)** Hazard ratio (relative to control iMNs) of iMNs from six sporadic ALS patients treated with 3K3A-APC and DMSO or a PAR1 antagonist in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.e.m. Unpaired t-test with Welch's correction. Grey circles represent individual samples. The day of differentiation stated on each panel

indicates the day of differentiation on which the experimental treatment or time course was initiated.

Supplemental Figure 7(A-L).
Rapamycin rescues the survival of iMNs from some sporadic ALS lines



Supplemental Figure 7. Rapamycin rescues the survival of iMNs from some sporadic ALS lines. (A-E) Survival of sALS1 (A), sALS2 (B), sALS4 (C), sALS5 (D),

or sALS6 (E) sporadic ALS iMNs with excess glutamate with 10 μM DMSO or rapamycin. iMNs quantified from 3 biologically independent iMN conversions per line. Two-sided log-rank test using the entire survival time course. **(F-J)** Hazard ratio (relative to control iMNs treated with DMSO) of sporadic ALS iMNs from five sporadic ALS patients treated with DMSO or rapamycin in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line. Mean +/- s.d. Unpaired t-test. Grey circles represent individual samples. **(G-J)** Survival of iMNs from a second clone of sALS1 (G), sALS2 (H), and sALS3 (I) in excess glutamate with inactive 3K3A-APC or 3K3A-APC. iMNs quantified from 3 biologically independent iMN conversions per line. Two-sided log-rank test using the entire survival time course. **(K)** Survival of control iMNs (3 controls) with excess glutamate with 10 μM DMSO or rapamycin. iMNs quantified from 3 biologically independent iMN conversions per line. Two-sided log-rank test using the entire survival time course. n=90 iMNs per line. **(L)** Hazard ratio (relative to control iMNs treated with DMSO) of control iMNs (three controls) treated with DMSO or rapamycin in the excess glutamate condition. iMNs quantified from 3 biologically independent iMN conversions per line per condition. Mean +/- s.e.m. Unpaired t-test. Grey circles represent individual samples. The day of differentiation stated on each panel indicates the day of differentiation on which the experimental treatment or time course was initiated.