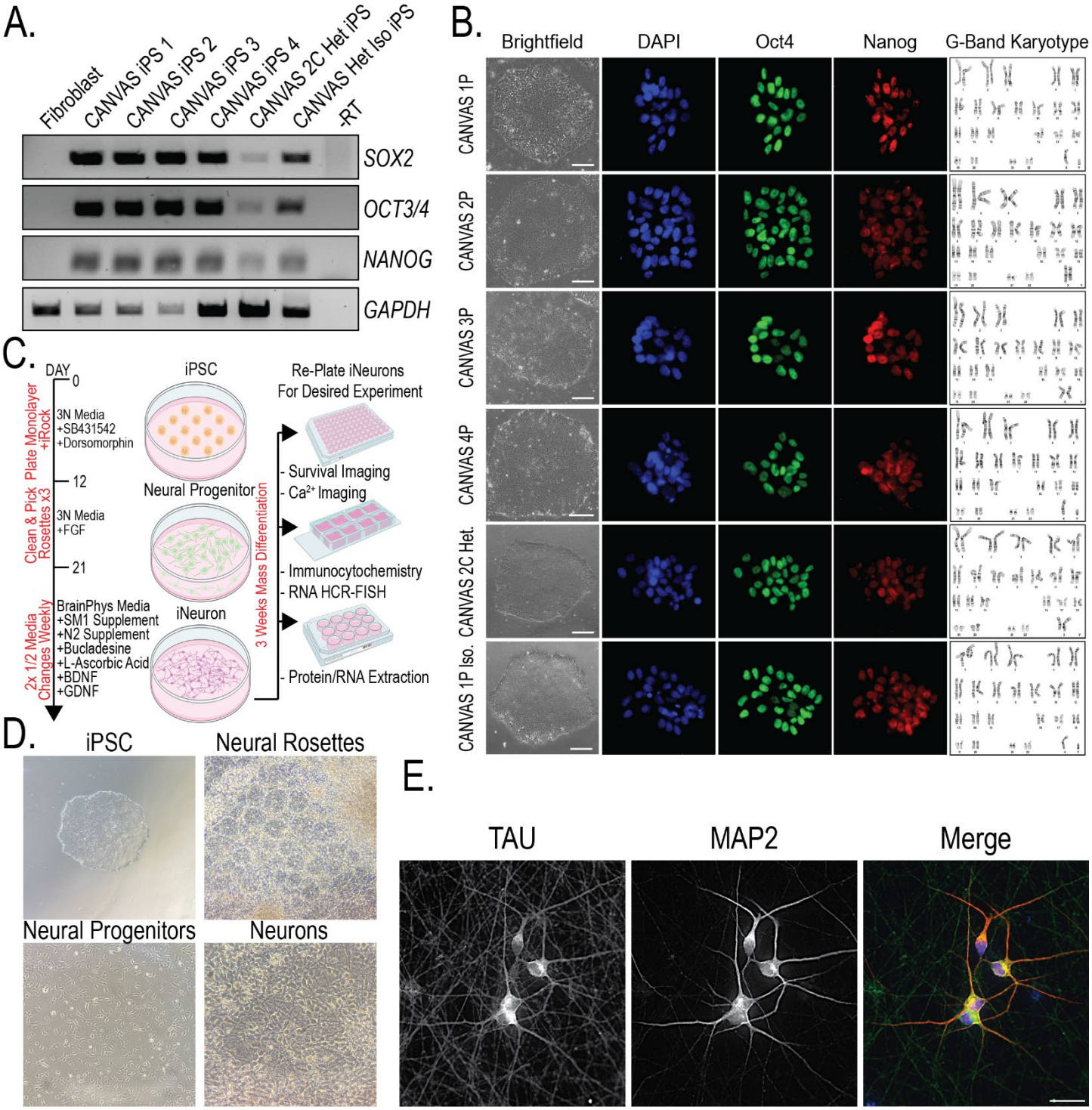
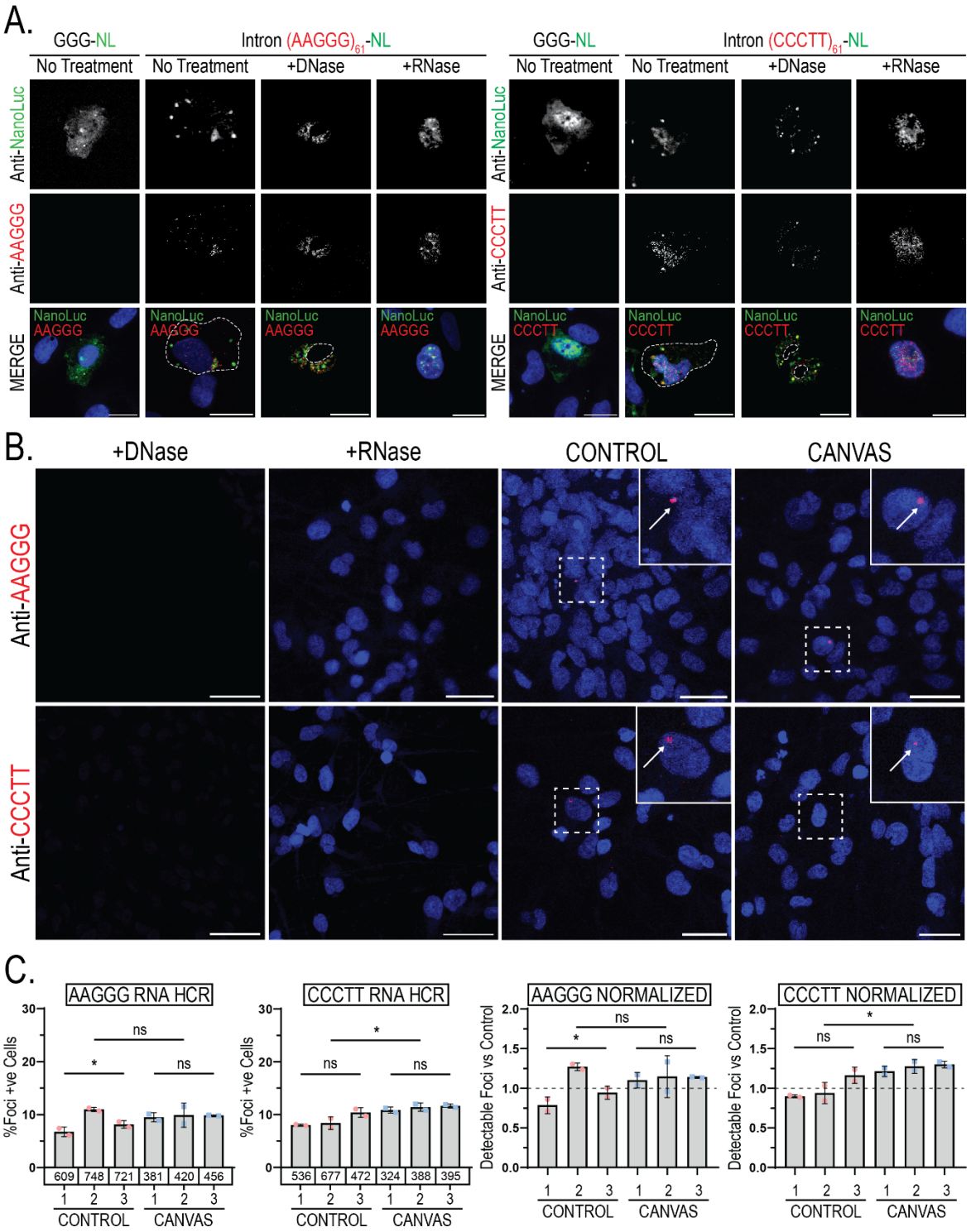
Supplementary Figures

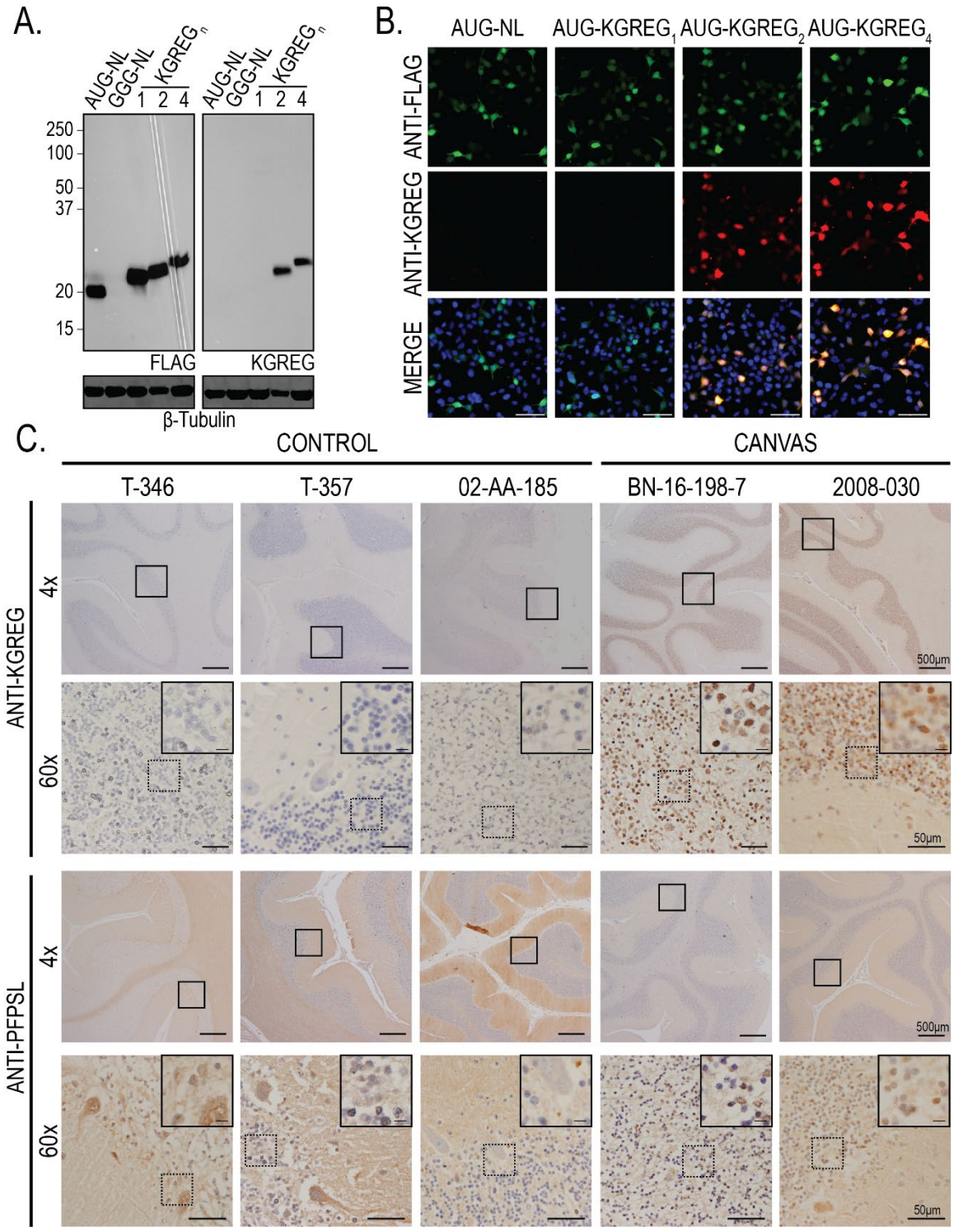


Supplementary Figure 1 –Generation, characterization, and differentiation of CANVAS patient iPSC-derived neurons. **A**) RT-PCR of CANVAS patient derived iPSCs for the pluripotency mRNA markers *SOX2*, *OCT3/4*, and *NANOG*. **B**) G-Band Karyotype analysis (WiCell) and Immunocytochemistry of CANVAS patient iPSCs for the pluripotency markers OCT4 and NANOG, scale = 25 µm. **C**) Schematic outlining the process of differentiating patient-derived iPSCs to neural progenitor cells and glutamatergic neurons by dual-SMAD inhibition for experimentation. **D**) Brightfield images of the stages of neuronal differentiation from patient-derived iPSCs, showing iPSCs, neural rosettes, neural progenitor cells, mass-differentiated neuronal cells and re-plated neuronal cells for experimentation. **E**) Immunocytochemistry of a representative patient iPSC-derived neurons stained for the neuronal markers Tau and MAP2. Scale = 25 µm.

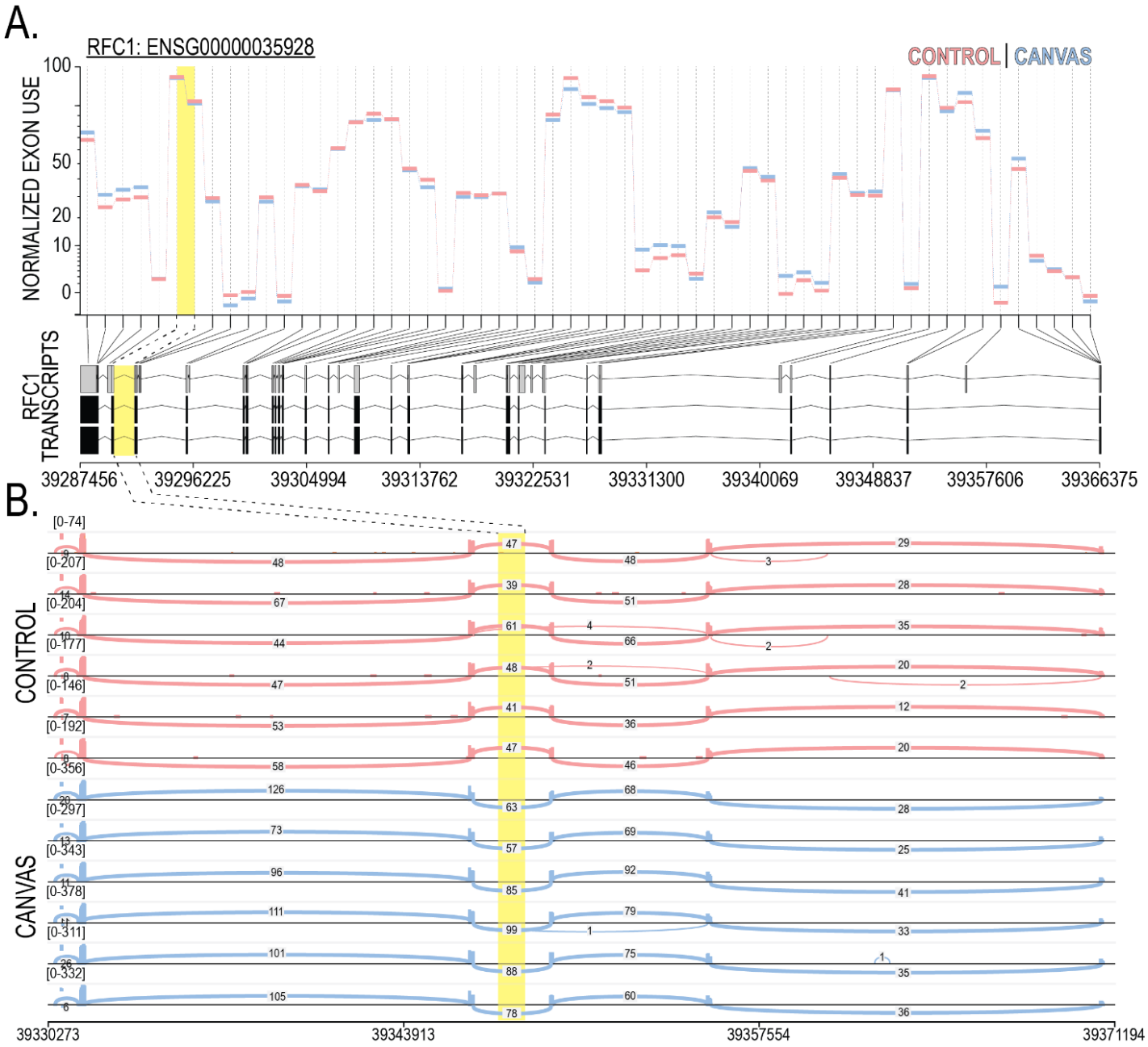


Supplementary Figure 2 – RNA HCR probe validation and analysis of RNA foci formation in CANVAS patient and control iPSC-derived neurons.

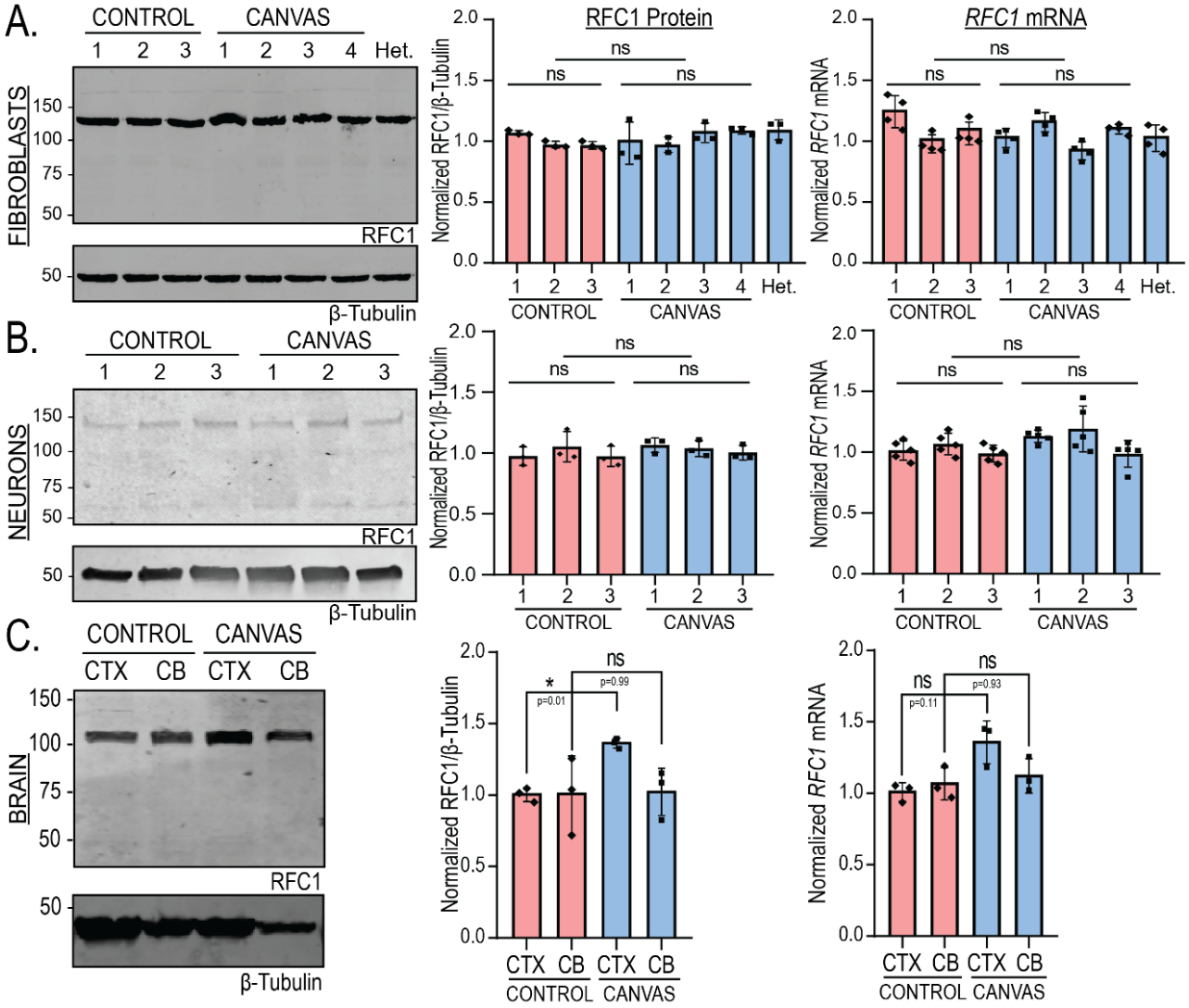
A) Confocal images of HEK293 cells transfected with control GGG-NL plasmid or plasmid expressing intronic AAGGG or CCCTT expansion followed by RNA HCR-FISH for AAGGG and CCCTT RNAs. Cells were fixed and treated with either anti-Nanoluc, anti-AAGGG, or anti-CCCTT fluorescent probes after no treatment, DNase, or RNase treatment to assess probe specificity. Scale = 10 μm. **B**) Confocal Images of representative CANVAS and control patient iPSC-derived neurons after RNA HCR-FISH utilizing either anti-AAGGG or anti-CCCTT fluorescent probes to assess sense or antisense RNA foci formation. Scale = 10 μm. **C**) Quantification of foci positive neurons for control (n=3) and CANVAS (n=3) patient iPSC-derived neurons with total n-numbers of neuronal cells analyzed per cell line indicated. AAGGG ($F(5, 12) = 3.619$, $P=0.074$), CCCTT ($F(5, 12) = 8.293$, $P=0.011$). Data were analyzed by one-way ANOVA with Sidak's post-hoc multiple comparison tests. Error = SD.



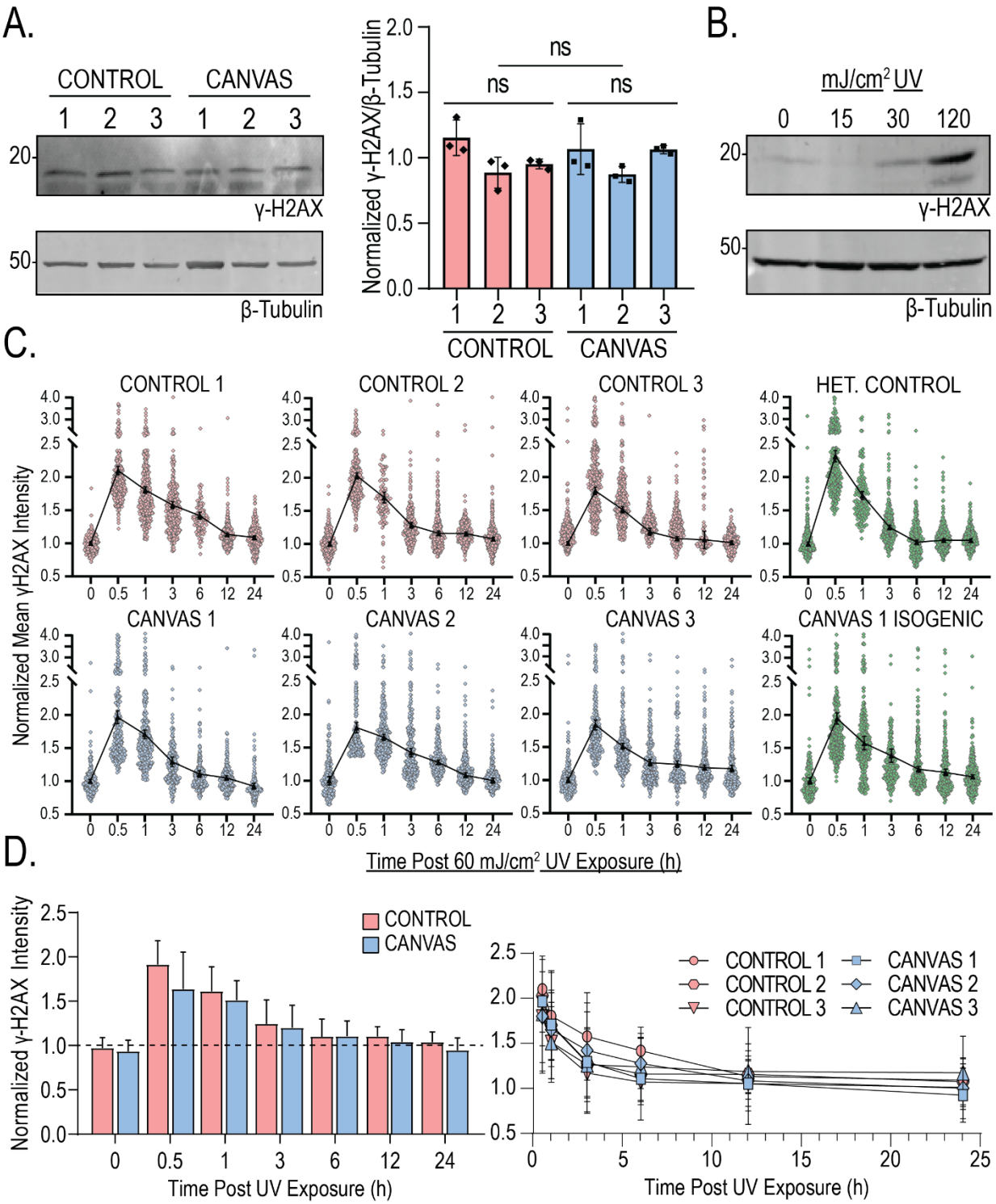
Supplementary Figure 3 – Analysis of repeat-derived peptides using KGREG & PFPSL antibodies.
A) Analysis of lysates from HEK293 cells transfected with control plasmid or plasmids expressing 1x, 2x, and 4x KGREG FLAG-tagged plasmids using anti-FLAG M2 (1:1000) and anti-KGREG (1:100) antibodies. **B**) ICC of HEK293 cells transfected with control plasmid or plasmids expressing 1x, 2x, and 4x KGREG FLAG-tagged plasmids using anti-FLAG M2 (1:100) and anti-KGREG (1:100) antibodies, scale = 25 µm. **C**) Immunohistochemistry of control (n=3) and RFC1 expansion CANVAS (n=2) patient post-mortem cerebellar vermis tissue stained with sense anti-KGREG or antisense anti-PFPSL antibodies (1:100, acid AR). Scale = 500 µm (4x), 50 µm (60x) and 20 µm (inset).



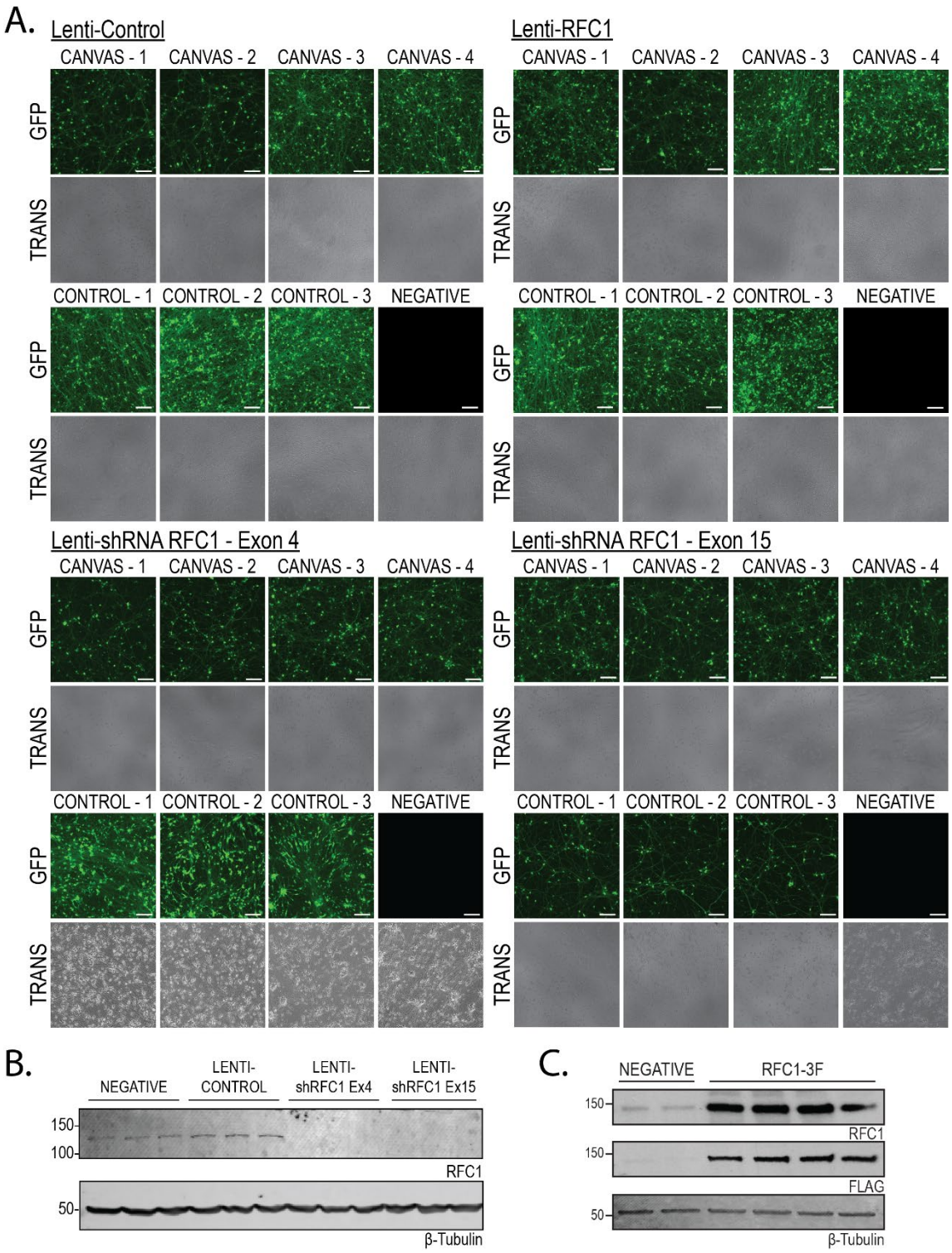
Supplementary Figure 4 – Analysis of *RFC1* isoform and alternative exon usage RNA short-read sequencing.
A) Normalized exon usage for *RFC1* mRNA transcripts in CANVAS patient iPSC-derived neurons, analyzed by DEXSeq of paired-end RNASeq reads. **B**) Sashimi plot of *RFC1* exons 1-5 in CANVAS patient iPSC-derived neurons indicating no skipping of exons flanking the intron 2 repeat expansion region (highlighted yellow).



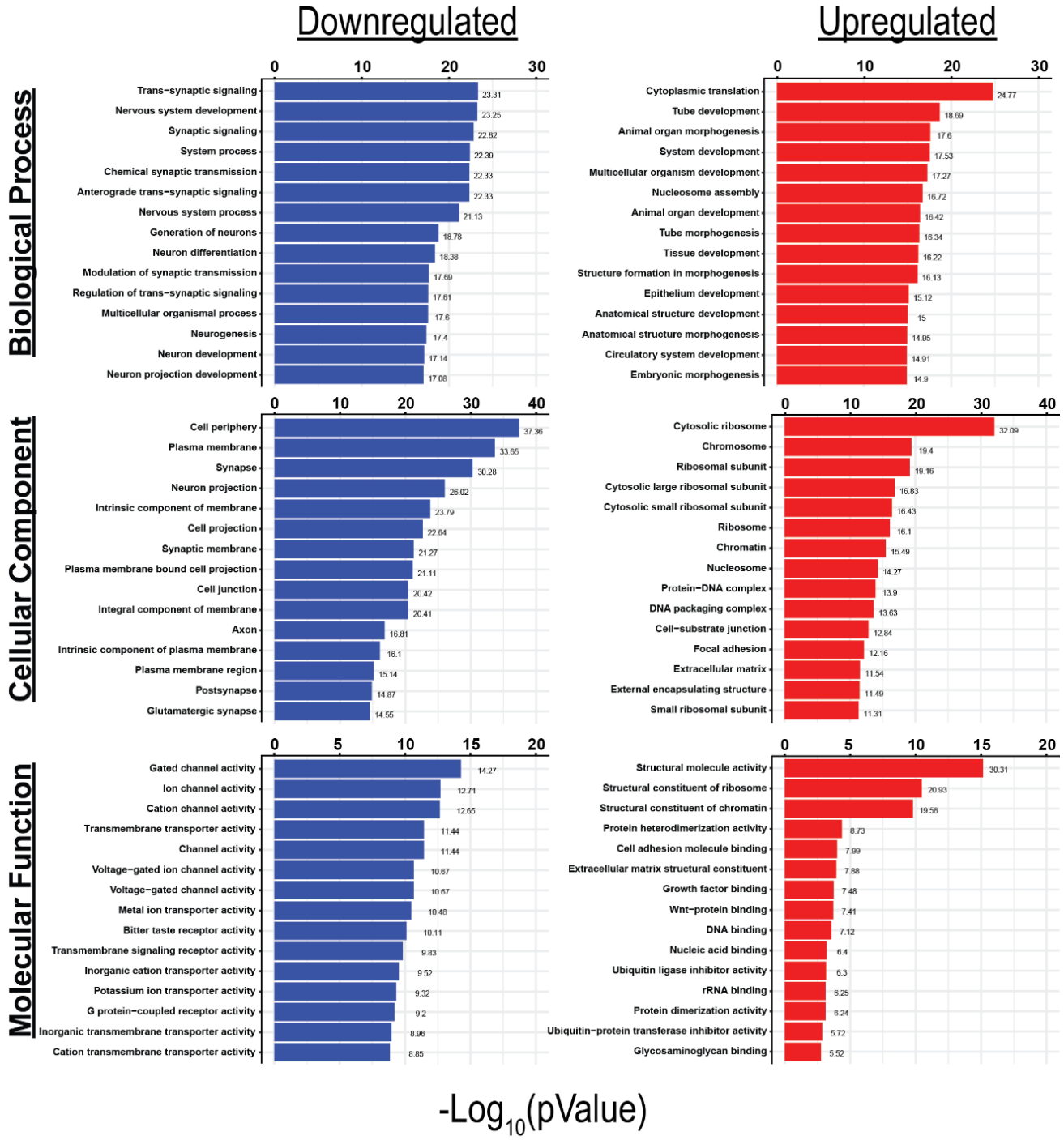
Supplementary Figure 5 – RFC1 protein and mRNA expression is maintained in AAGGG expansion contexts.
A) Analysis of RFC1 expression (**left**), quantification of normalized RFC1 expression (**center**, F(7,24) = 1.592, P=0.208), and quantification of normalized RFC1 mRNA expression (**right**, F(7,24) = 4.944, P=0.0014) from CANVAS (n=4) and control (n=3) patient-derived fibroblasts. **B**) Analysis of RFC1 expression (**left**), quantification of normalized RFC1 expression (**center**, F(5,18) = 0.707, P=0.629)), and quantification of normalized RFC1 mRNA expression (**right**, F(5,24) = 3.029, P=0.029) from CANVAS and control (n=3) patient iPSC-derived neurons. **C**) Analysis of RFC1 protein (**left**), quantification of normalized RFC1 protein expression (**center**, F(3,8) = 2.010, P=0.191) from CANVAS (n=1) and control (n=1) patient post-mortem brain tissue, and quantification of normalized RFC1 mRNA expression (**right**, F(3,8) = 5.171, P=0.0281). N = 3 biological replicates. Data were analyzed by one-way ANOVA with Sidak's post-hoc multiple comparison tests. Error = SD.



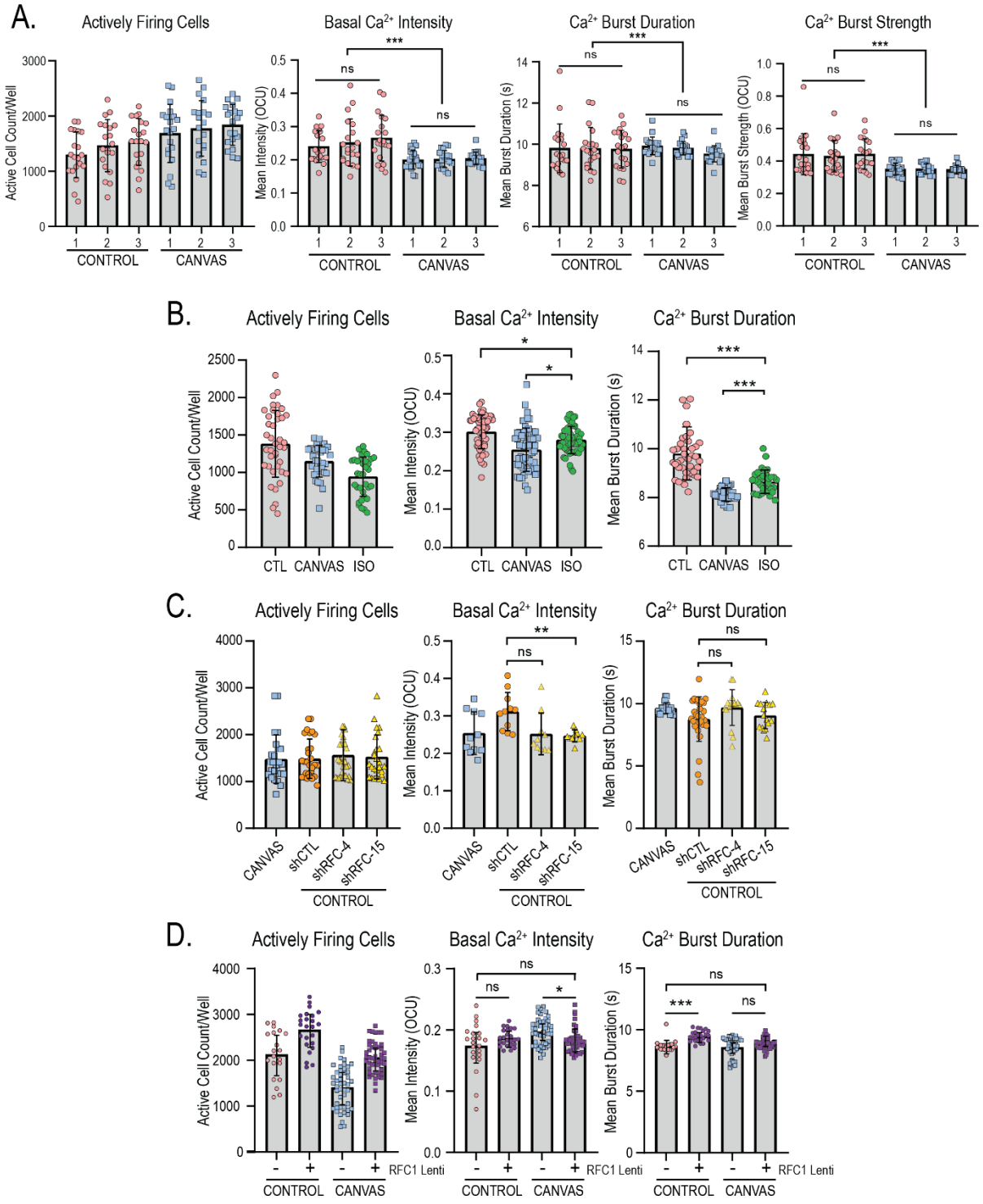
Supplementary Figure 6 – Analysis of DNA damage accumulation and recovery in patient iPSC-derived neurons. **A**) Analysis of γ-H2AX expression (**left**), and quantification of normalized γ-H2AX levels (**right**, $F(5, 18) = 2.962$, $P=0.057$) from CANVAS (n=3) and control (n=3) patient iPSC-derived neurons. Data were analyzed by one-way ANOVA with Sidak's post-hoc multiple comparison tests. N = 3 biological replicates. **B**) Analysis of γ-H2AX levels in control iPSC-derived neurons exposed to 0, 15, 30, and 120 mJ/cm^2 UV irradiation. **C**) Quantification of mean γ-H2AX staining in control (n=3), CANVAS (n=3), heterozygous *RFC1* expansion (n=1), and CANVAS heterozygous isogenic corrected (n=1) patient iPSC-derived NeuN+ neuronal nuclei over a 24h period after 60 mJ/cm^2 UV exposure. **D**) Comparison of control (n=3) and CANVAS (n=3) γ-H2AX levels after 60 mJ/cm^2 UV exposure (left) and comparison of first derivative rates of γ-H2AX decline (right, $F(5, 30) = 0.033$, $P=0.999$). Error = SD.



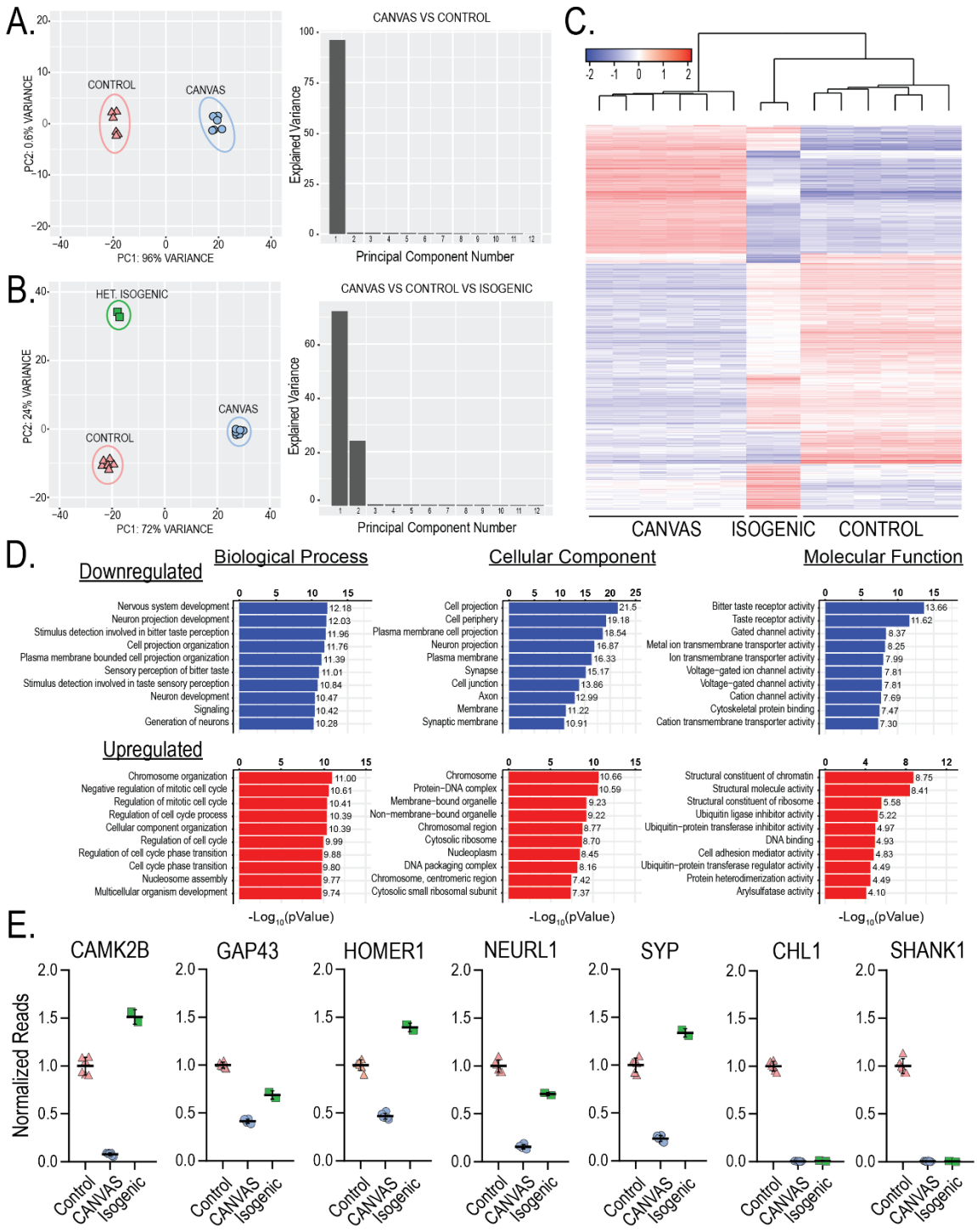
Supplementary Figure 7 – Efficiency of lentiviral transduction in CANVAS patient and control iPSC-derived neurons. **A**) Brightfield and GFP fluorescence images of CANVAS (n=4) and control (n=3) patient iPSC-derived neurons transduced with control lentivirus, RFC1 overexpression lentivirus, or lentiviruses encoding shRNAs to knockdown RFC1 at exon4 or exon15. Scale = 50 µm. **B-C**) Analysis of RFC1 expression (GTX129291 1:1000) upon shRNA RFC1 lentivirus treatment (**B**) or RFC1 overexpression lentivirus treatment (**C**) to assess for lentivirus efficiency.



Supplementary Figure 8 – Gene ontology (GO) analysis of dysregulated transcripts detected in CANVAS patient vs control iPSC-derived neurons. Full Gene Ontology (GO) pathway analysis of the top up and downregulated Biological Process, Cellular Component, and Molecular Functions in CANVAS patient vs control iPSC-derived neurons. N = 6 biological replicates from 3 individual CANVAS and control patients.

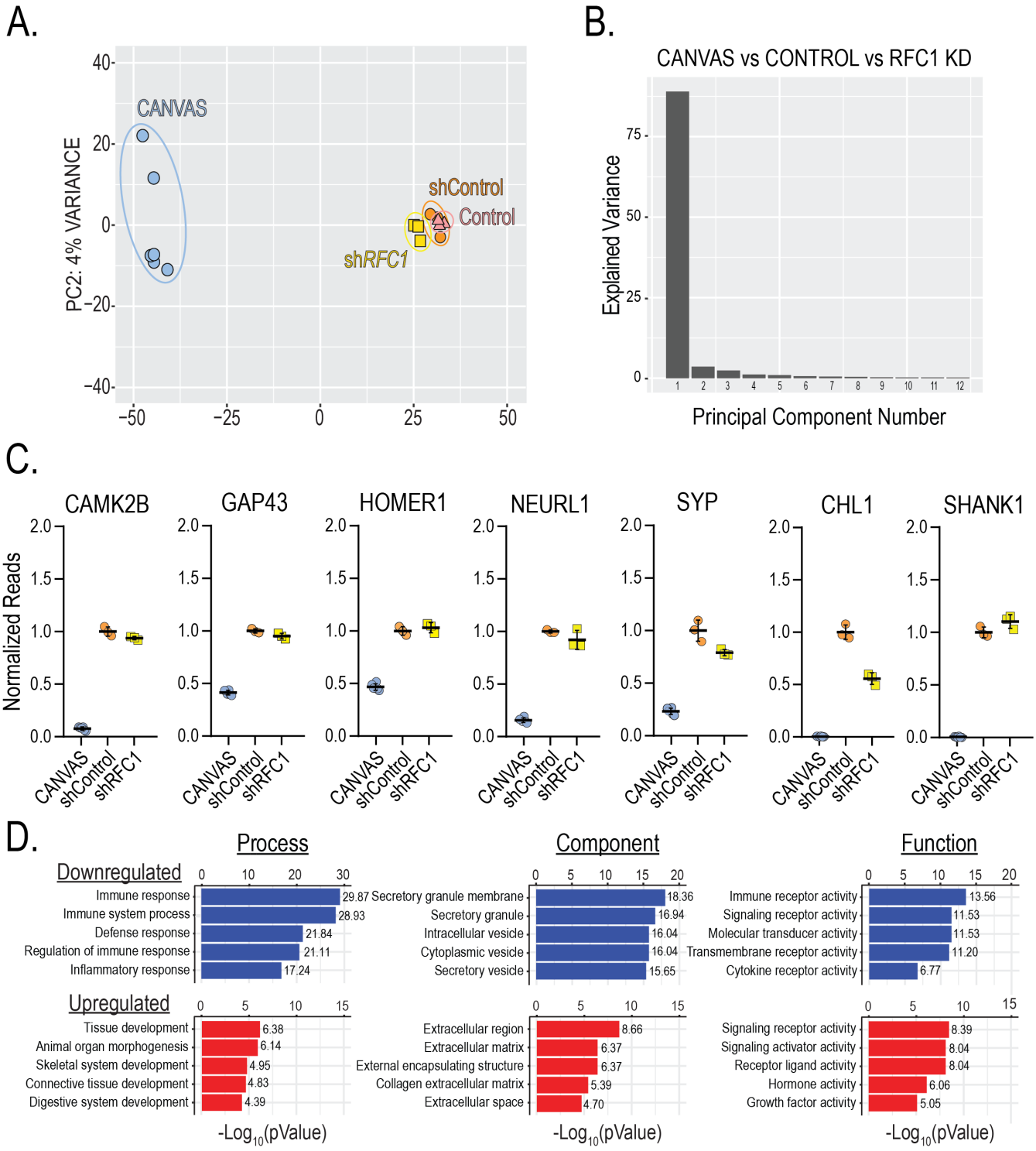


Supplementary Figure 9 – Calcium imaging metrics from CANVAS patient and control iPSC-derived neurons. **A)** Analysis of Ca^{2+} imaging metrics for control (n=3) and CANVAS (n=3) patient iPSC-derived neurons. Basal Intensity ($F(5, 114) = 7.075$, $P<0.0001$), Burst Duration ($F(5, 114) = 0.5371$, $P=0.745$), Burst Strength ($F(5, 114) = 7.573$, $P<0.0001$). **B**) Analysis of Ca^{2+} imaging metrics for control (n=3), CANVAS (n=3), and Heterozygous Isogenic (n=1) patient iPSC-derived neurons. Basal Intensity ($F(2, 165) = 14.31$, $P<0.0001$), Burst Duration ($F(2, 101) = 48.79$, $P<0.0001$). **C**) Analysis of Ca^{2+} imaging metrics for CANVAS (n=3) and control (n=3) patient iPSC-derived neurons treated with shControl or shRFC1 exon4/exon15 lentiviruses. Basal Intensity ($F(3, 78) = 4.279$, $P=0.011$), Burst Duration ($F(3, 78) = 2.520$, $P=0.0641$). **D**) Analysis of Ca^{2+} imaging metrics for control (n=3) and CANVAS (n=3) patient iPSC-derived neurons treated with control or RFC1-overexpression lentivirus. Basal Intensity ($F(3, 135) = 5.621$, $P=0.0012$), Burst Duration ($F(3, 135) = 14.42$, $P<0.0001$). Each data point represents the mean of ~1000-3000 active cells per well. Data were analyzed by one-way ANOVA with Sidak's post-hoc multiple comparison tests. N= 3 biological replicates, error = SD.

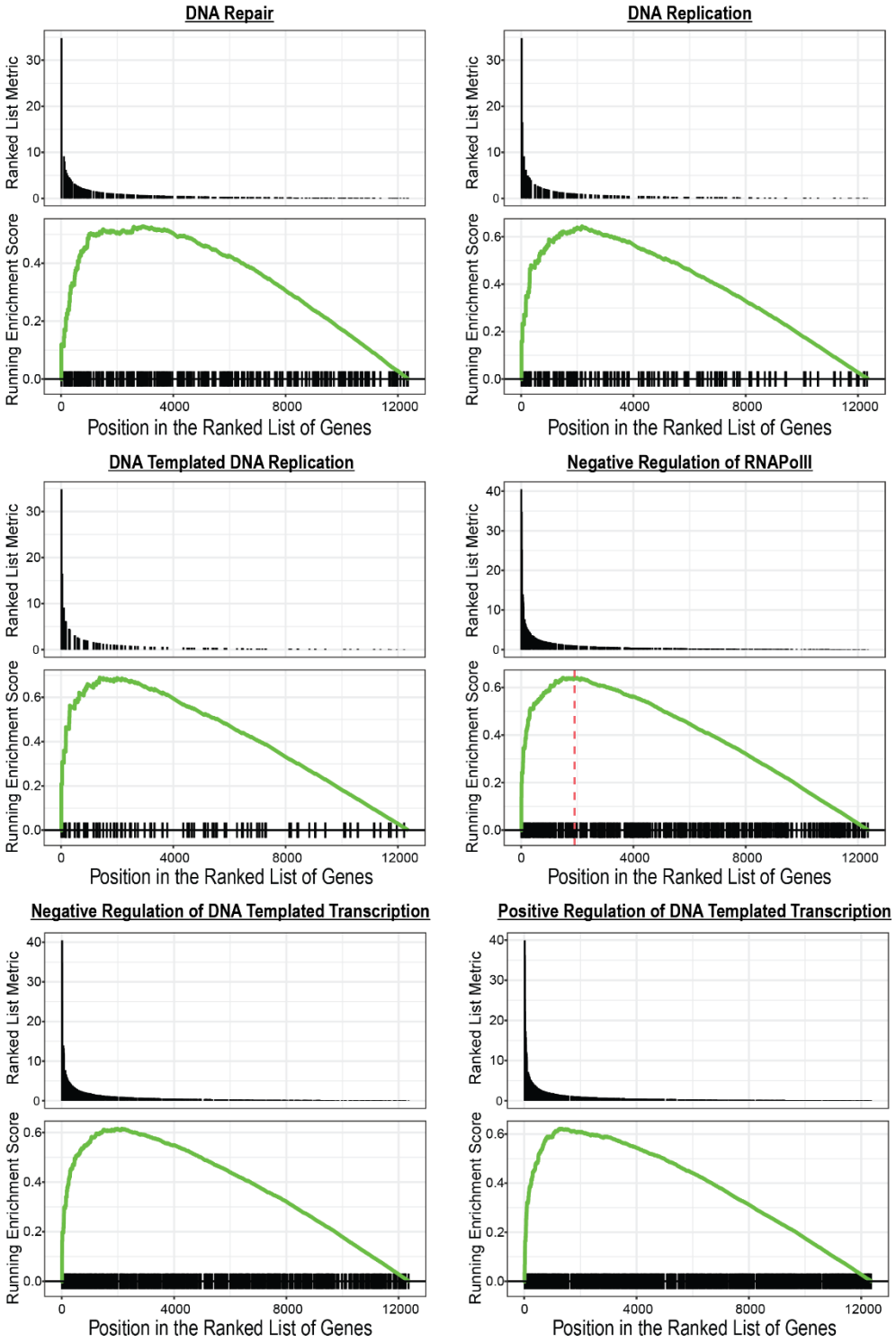


Supplementary Figure 10 – Supplemental data of transcriptomic analyses from control, CANVAS, and heterozygous isogenic correction patient iPSC-derived neurons.

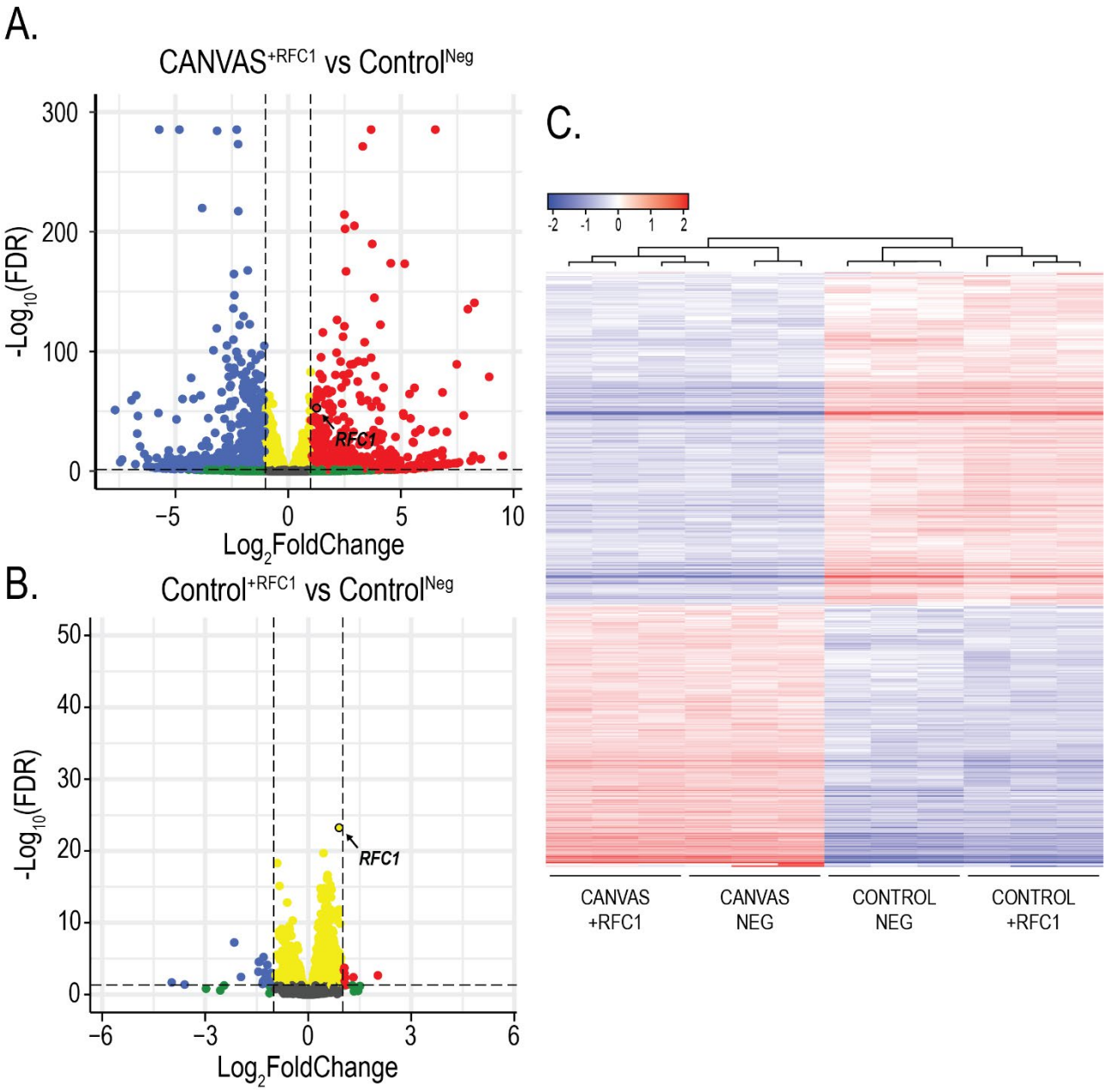
A) Principal component analyses of CANVAS (n=6) vs control (n=6) patient iPSC-derived neurons (left) with scree plot of explained variance with principal component axis of variance per sample shown for PC1 and PC2 (right). **B**) CANVAS (n=6), control (n=6), and Heterozygous Isogenic (n=2) patient iPSC-derived neurons (left) with principal component axis of variance per sample shown for PC1 and PC2 (right). **C**) Heatmap of normalized expression for the top 1000 genes differentially expressed in CANVAS, control, and CANVAS isogenic patient iPSC-derived neurons. **D**) Gene Ontology (GO) pathway analysis of the top up/downregulated Biological Process, Cellular Component, and Molecular Function for the genes that showed significant expression correction in heterozygous isogenic iPSC-derived neurons compared to CANVAS/control. **E**) Normalized read counts for *CAMK2B, GAP43, HOMER1, NEURL1, SYP, CHL1*, and *SHANK1* from control (n=6), CANVAS (n=6), and isogenic (n=2) iPSC-derived neurons.



Supplementary Figure 11 – Supplemental data to RFC1 knockdown RNASeq analyses.
A) Principal component analyses of CANVAS (n=6), control (n=3), control *shControl* (n=3), and control *shRFC1* (n=3) patient iPSC-derived neurons. **B**) Scree plots of explained variance with principal component axis of variance per sample shown for PC1 and PC2. **C**) Normalized read counts for CAMK2B, GAP43, HOMER1, NEURL1, SYP, CHL1, and SHANK1 from CANVAS (n=6), *shControl* (n=3), and *shRFC1* (n=3) iPSC-derived neurons.



Supplementary Figure 12 – Gene set enrichment analysis (GSEA) of RFC1-associated functions in RFC1 knockdown control iPSC-derived neurons. Gene set enrichment analysis (GSEA) of the RFC1-associated functions DNA repair, DNA replication, DNA templated DNA replication, negative regulation of RNA Pol II, and positive and negative regulation of DNA templated transcription in RFC1 knockdown control iPSC-derived neurons.



Supplementary Figure 13 –RFC1 reprovision RNASeq analyses.
A) Volcano plot of -Log_{10}FDR vs Log_2(Fold Change) for CANVAS patient-derived neurons transduced with either full-length RFC1 CDS lentivirus or control lentivirus (n=3/group), RFC1 labelled. **B**) Volcano plot of -Log_{10}FDR vs Log_2(Fold Change) for control-derived neurons transduced with either full-length RFC1 CDS lentivirus or control lentivirus (n=3/group), RFC1 labelled. **C**) Heatmap of normalized expression for the top 1000 genes differentially expressed in CANVAS, control, and CANVAS isogenic patient iPSC-derived neurons.

Purpose	Primers	Reagents	Conditions
NHEJ CRISPR	Fwd gRNA: GAGAATAGCAACGGTGTAGCTGG Rev gRNA: TCATTTTCTGAAATACGGACAGG	20uM HiFi Cas9 (IDT) tracR-ATTO550 (IDT) 1:1 gRNA(total) 120ul R-Buffer	1450V, 10ms, 3-pulses
Non-Edited Screening Repeat-Flanking PCR	Fwd: CTGAAGTGATTGGCCTGTCTCCC Rev: CACTGGATCAAGGACAGAGTCA	2X Faststart Master Mix (Roche) Primers 0.5 M gDNA 50 ng	95°C 4 min [95°C 30s, 60°C 30s, 72°C 60s] x35 72 C 5 min
Edited Screening Repeat-Spanning PCR	Fwd: GGGTGGTGGCTGTCTCATC Rev: CACTGGATCAAGGACAGAGTCA		
Repeat-Primed PCR	Fwd: FAM-TCAAGTGATACTCCAGCTACACCGT Anchor: CAGGAAACAGCTATGACC **(AAAAG)11 Allele** Rev1: CAGGAAACAGCTATGACCAACAGAGCAAGACTCTGTTTCAAAAAAGAAAAGAAAAGAAAAGAAAA Rev2: CAGGAAACAGCTATGACCAACAGAGCAAGACTCTGTTTCAAAAAGAAAAGAAAAGAAAAGAAAA Rev3: CAGGAAACAGCTATGACCAACAGAGCAAGACTCTGTTTCAAAAGAAAAGAAAAGAAAAGAAAA **(AAGGG)exp Allele** Rev1: CAGGAAACAGCTATGACCAACAGAGCAAGACTCTGTTTCAAAAAAGGGAAGGGAAGGGAAGGGAA Rev2: CAGGAAACAGCTATGACCAACAGAGCAAGACTCTGTTTCAAAAAGGGAAGGGAAGGGAAGGGAA Rev3: CAGGAAACAGCTATGACCAACAGAGCAAGACTCTGTTTCAAAAGGGAAGGGAAGGGAAGGGAA	2X Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher) Fw Primer 0.5 M Anchor 0.5 M Rv primers (Rv1:Rv2:Rv3=1:1:1) 0.05 M DMSO 3% gDNA 50 ng	98°C 3 min [98°C 10s, 65°C 15s, 72°C 60s] x35 72°C 5 min
Intron 2 Retention Screening	Exon 2 Fwd: CATTCGGAAATTCTTTGGAGTA Exon 3 Rev: ATCCTCTTTACGGGAGCTATTTAC Intron 2 Rev: TCAATGCAAAATTATACCCAGA	2X Faststart Master Mix (Roche) Primers 0.5 M gDNA 50 ng	95°C 4 min [95°C 30s, 59°C 30s, 72°C 60s] x35 72 C 5 min
qRT-PCR	RFC1: FAM-MGB Taqman Probes (Thermo Hs01099126_m1, Exon14-15, Amplicon Length 70 bp) Actin: VIC-MGB-PL Taqman Probes (Thermo Hs99999903_m1, Exon 1, Amplicon Length 171 bp)	TaqMan™ 2X Universal PCR Master Mix (ThermoFisher) 1X RFC1 FAM Taqman Probes 1X Actin VIC Taqman Probes 2 µl cDNA	95°C 20s [95°C 5s, 60°C 20s] x40

Supplementary Table 1 – Table of PCR primer sequences, reagents, and thermocycling conditions used.

Anti-Nanoluc-3F	
Nluc-3xF-DNAp50-B1DI-P1	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTTTGTAGCCGGCTGTCTGTCGCCAGTCCCCAACGAAATCTT CGAGTGTGAAATATAGCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
Nluc-3xF-DNAp50-B1DI-P2	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTTTGGATCGGAGTTACGGACACCCCGAGATTCTGAAACAAA CTGGACACACATATAGCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
Nluc-3xF-DNAp50-B1DI-P3	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTAAATTTTTTCGATCTGGCCCATTTGGTCGCCGCTCAGACC TTCATACGGGATATAGCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
Nluc-3xF-DNAp50-B1DI-P4	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTCGTAACCCCGTCGATTACCAGTGTGCCATAGTGCAGGATC ACCTTAAAGTATATAGCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
Anti-CCCTT 5' Anchor	
CCCTT-P1	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTAAGGGAAGGGAAGGGAAGGGAAGGGAAGGGAAGGGAAG GGAGCATGTTCTAAAGAGAATATA GCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
Anti-CTTTT 5' Anchor	
CTTTT-P1	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTAAAAGAAAAGAAAAGAAAAGAAAAGAAAAGAAAAGAAAAG AGCATGTTCTAAAGAGAATATAGCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
Anti-AAGGG 5' Anchor	
AAGGG-P1	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTT TTTGAAACAGAGTCATATAGCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
AAGGG-P2	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTT TTGAAACAGAGTC ATATA GCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
AAGGG-P3	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTCCCTTT TGAAACAGAGTC ATATA GCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
Anti-AAAAG 5' Anchor	
AAAAG-P1	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTTTTT GAAACAGAGTCATATAGCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
AAAAG-P2	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTTTTG AAACAGAGTCATATAGCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG
AAAAG-P3	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGATATTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTTTGA AACAGAGTCATATA GCATTCTTTCTTGAGGAGGGCAGCAAACGGGAAGAG

Supplementary Table 2 – Table HCR probe sequences used.