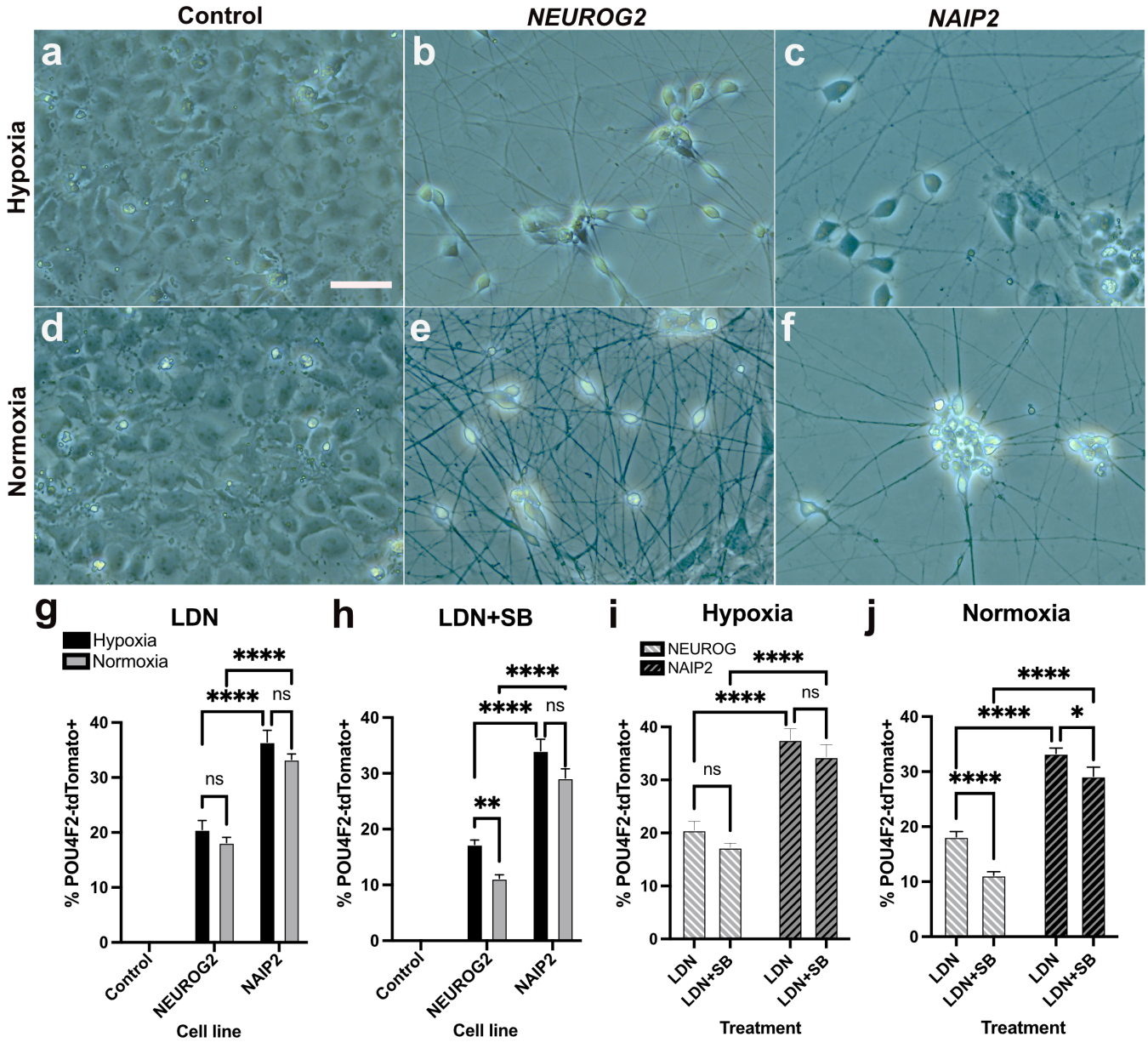
**Human Retinal Ganglion Cell Neurons Generated by Synchronous BMP inhibition and Transcription Factor Mediated Reprogramming.**

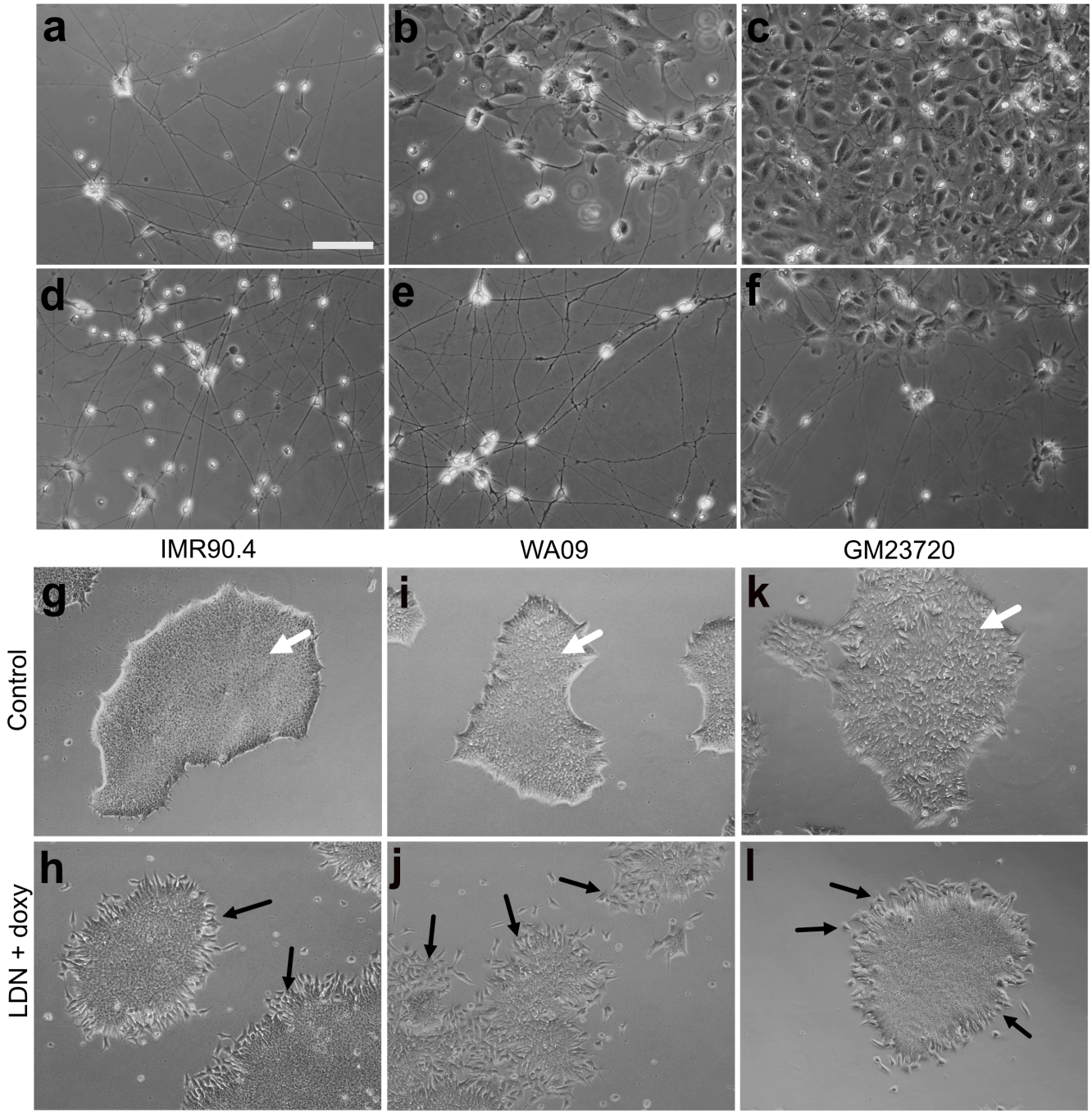
Devansh Agarwal[1,2‡], Nicholas Dash[2‡], Kevin W. Mazo[2], Manan Chopra[2], Maria P. Avila[2], Amit Patel[2], Ryan M. Wong[2], Cairang Jia[2], Hope Do[2], Jie Cheng[3], Colette Chiang[2], Shawna L. Jurlina[2], Mona Roshan[2], Michael W. Perry[4], Jong M. Rho[5], Risa Broyer[2], Cassidy D. Lee[2], Robert N. Weinreb[2], Cezar Gavrilovici[5], Nicholas W. Oesch[2,6], Derek S. Welsbie[2], Karl J. Wahlin[2*]

***Supplementary Materials:***

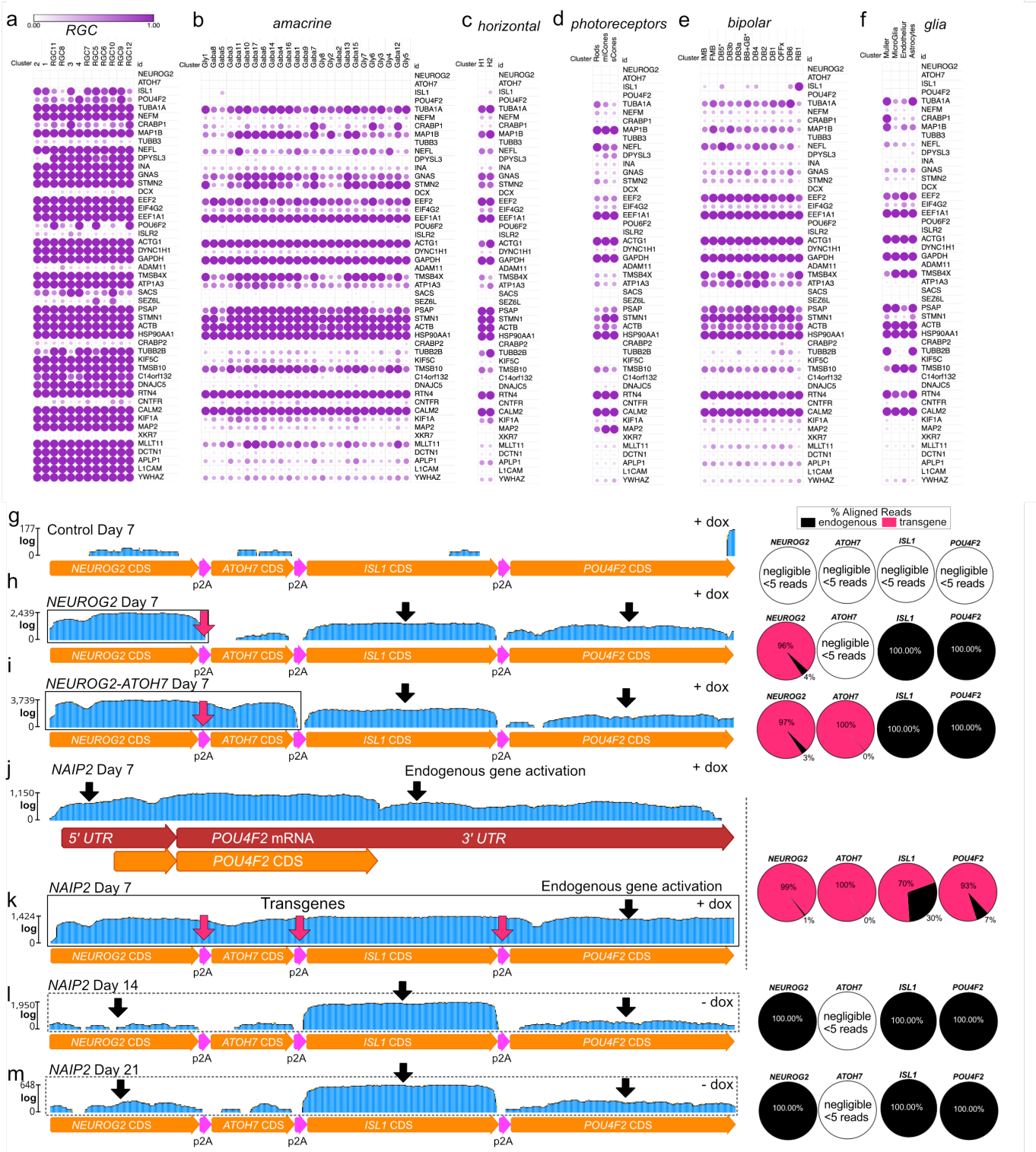



**Supplementary Figure 1.** ***The role of hypoxia and dual SMAD inhibition on RGC-iNs formation***. (**a**) Bright-field imaging of D6 normoxia (**a-c**) and hypoxia (**d-f**) conditioned control, NEUROG2, or NAIP2-nc (prior to colony selection) cells after treatment with LDN/dox. (**g**) Evaluation of POU4F2-tdTomato+ cells in control, NEUROG2, NAIP2-nc samples treated with (**g**) LDN or (**h**) LDN + SB431542 in hypoxia and normoxia. (**i**) Comparison between POU4F2-tdTomato+ cells after LDN or LDN193189 plus SB431542 under (**i**) hypoxia or (**j**) normoxia. Statistical significance was determined by ordinary two-way ANOVA with Tukey's multiple comparison and FDR <0.05. (n=3

biological replicates; P values: <0.05*, <0.01**, <0.0001***, <0.00001****; error bars: SEM). Scale=100μm. NAIP2-nc indicates non-clonally selected NAIP2 PSCs.

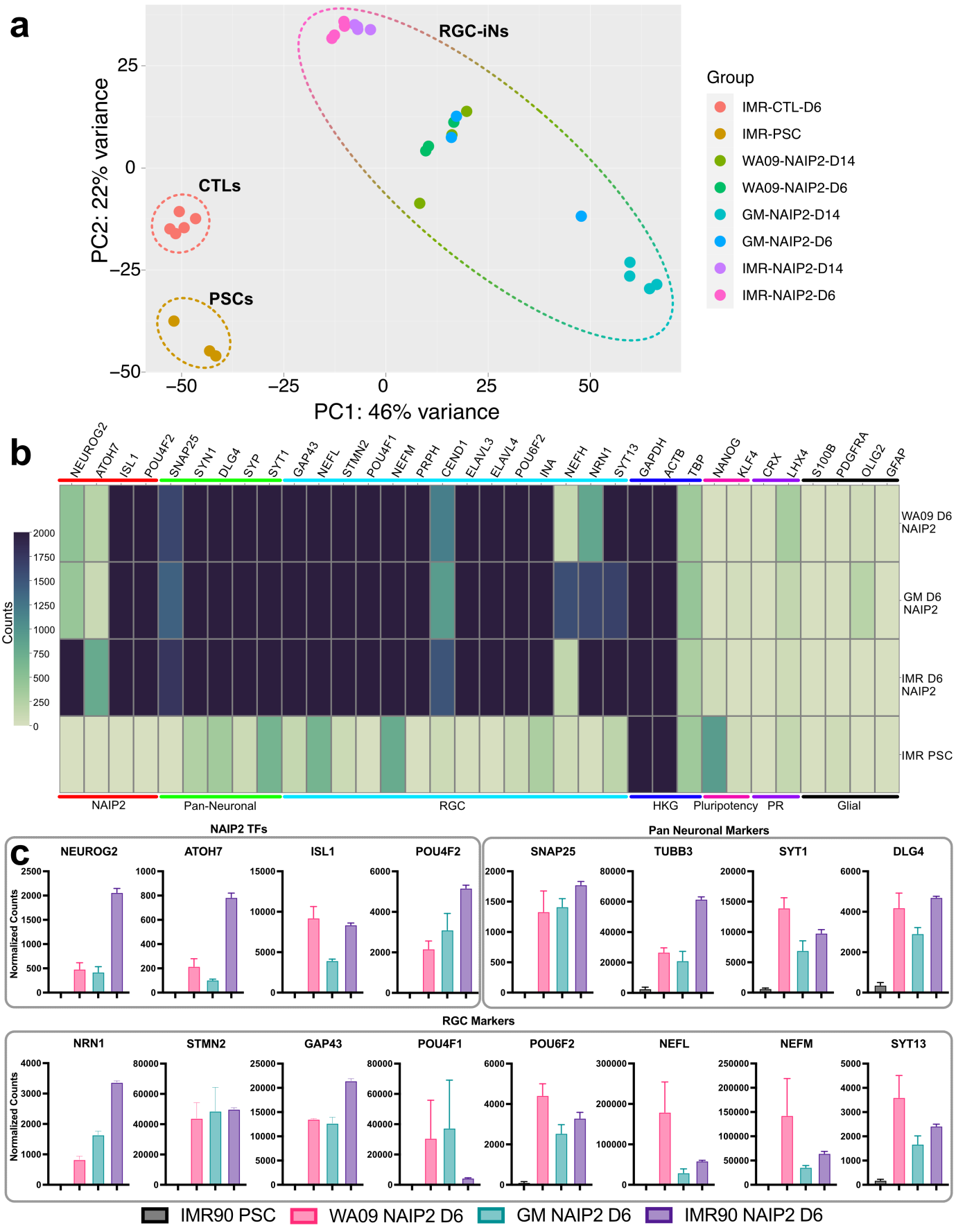



**Supplementary Figure 2**. ***Clonal enrichment of neuronal producing clones following zeocin selection and manual isolation and confirmation of neuronal induction by LDN/dox.*** (**a-f**) Individual NAIP2 clones in differentiated (NAIP2c1-6) PSCs following zeocin selection and manual clonal selection. Scale=200µm. Morphology of NAIP2 cells in three genetic backgrounds (IMR90.4, WA09, GM23720) in untreated controls (**g,i,k**) and after 24 hours of LDN/dox treatment (**h,j,l**).

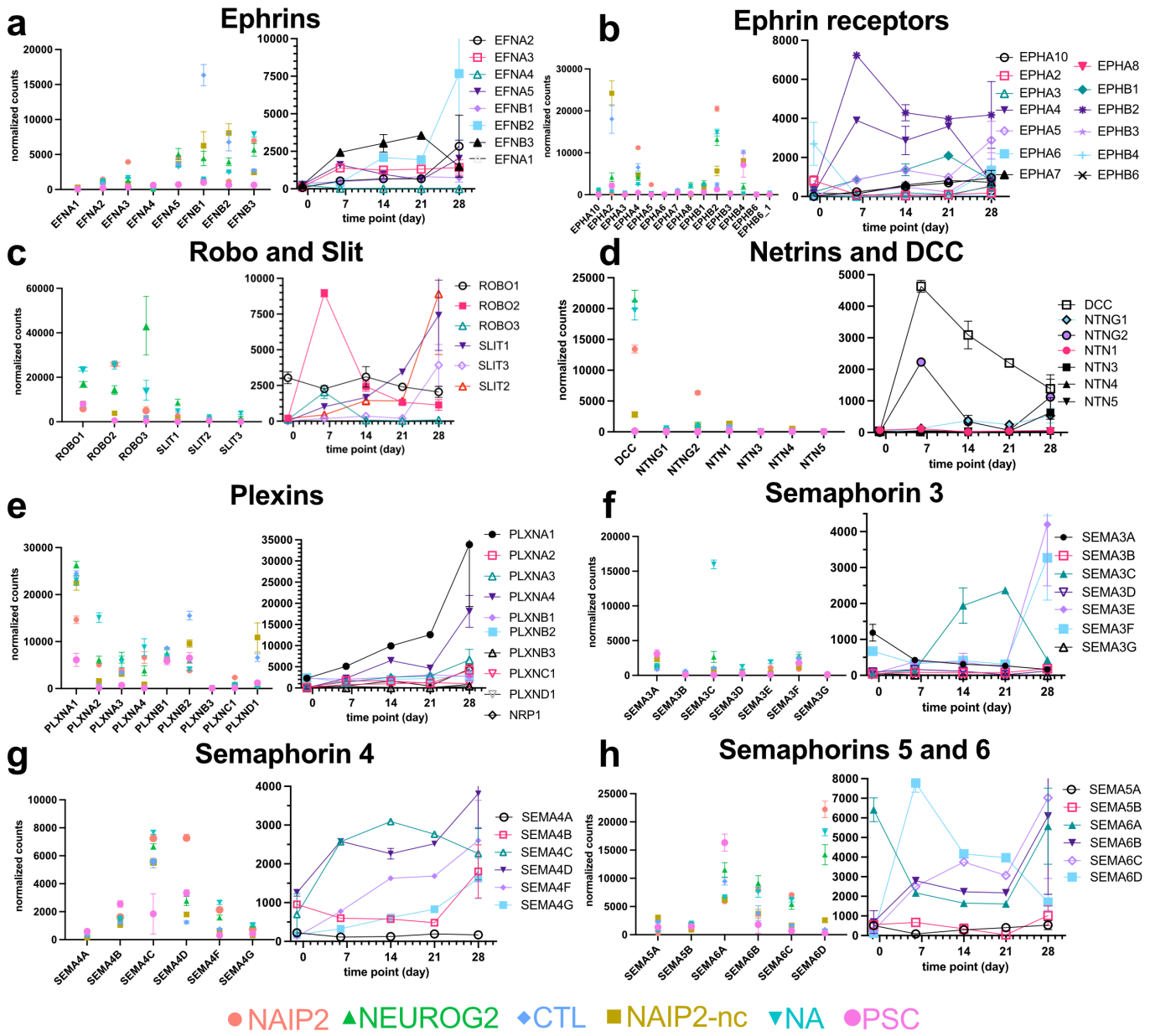



**Supplementary Figure 3.** ***Endogenous and transgene expression and correlation of RGC-iN bulk RNA-seq to human foveal and peripheral retina RGC cell clusters.*** Correlation of the top 50 RGC-iN expressed genes from bulk RNA-seq on day 7 relative to RGC cell clusters (**a**) identified by scRNA-seq of the human fovea and peripheral retina (https://singlecell.broadinstitute.org/; Yan et al., 2020). Amacrine (**b**), horizontal (**c**), photoreceptor (**d**), bipolar (**e**) and glial (**f**) cell clusters were also presented to demonstrate contrast to RGCs. RNA-seq coverage maps (left) and pie charts (right) illustrating % endogenous versus transgene aligned reads identified using the Geneious RNA-seq aligner with one-week old (**g**) control, (**h**) NEUROG2, (**i**) NEUROG2-ATOH7 cells aligned to the *NAIP2* gene

cassette or (**j**) NAIP2 expressed transcripts aligned to the endogenous *POU4F2* gene. (**k-m**) NAIP2 transcripts from RGC-iNs after 1, -2 and -3 weeks. Black arrows (**j**) indicate endogenous gene expression mapped to the 5 prime or 3 prime untranslated regions, while pink arrows (**h,i,k**) indicate p2A sequences that map to the NAIP2 transgene cassette.

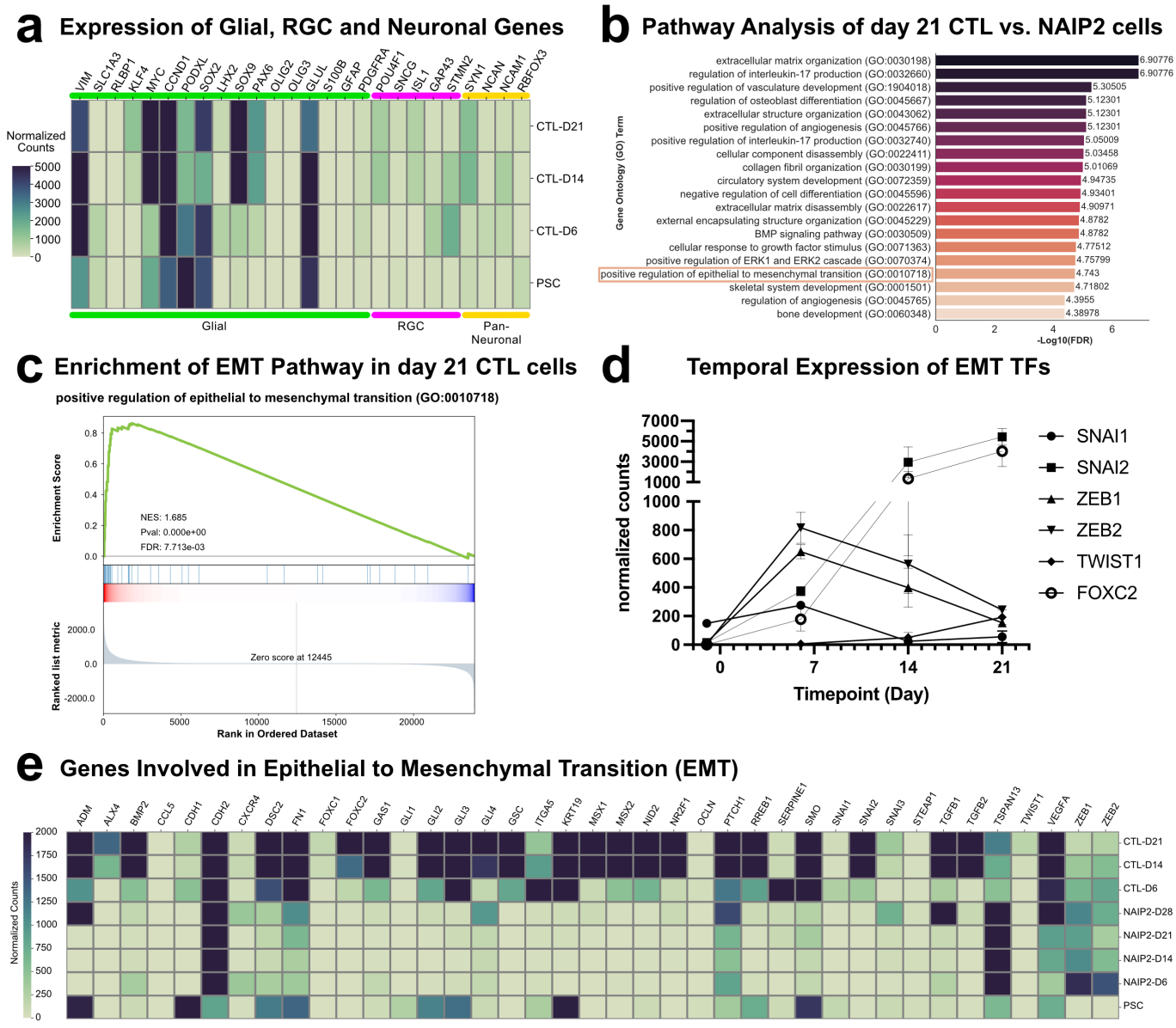

a
RGC-iNs
CTLs
PSCs
PC2: 22% variance
PC1: 46% variance
Group
IMR-CTL-D6
IMR-PSC
WA09-NAIP2-D14
WA09-NAIP2-D6
GM-NAIP2-D14
GM-NAIP2-D6
IMR-NAIP2-D14
IMR-NAIP2-D6
b
NEUROG2 ATOH7 ISL1 POU4F2 SNAP25 SYN1 DLG4 SYP SYT1 GAP43 NEFL STMN2 POU4F1 NEFM PRPH CEND1 ELAVL3 ELAVL4 POU6F2 INA NEFH NRN1 SYT13 GAPDH ACTB TBP NANOG KLF4 CRX LHX4 S100B PDGFRA OLIG2 GFAP
Counts
WA09 D6 NAIP2
GM D6 NAIP2
IMR D6 NAIP2
IMR PSC
NAIP2
Pan-Neuronal
RGC
HKG
Pluripotency
PR
Glial
c
NAIP2 TFs
Pan Neuronal Markers
RGC Markers
Normalized Counts
NEUROG2
ATOH7
ISL1
POU4F2
SNAP25
TUBB3
SYT1
DLG4
NRN1
STMN2
GAP43
POU4F1
POU6F2
NEFL
NEFM
SYT13
IMR90 PSC
WA09 NAIP2 D6
GM NAIP2 D6
IMR90 NAIP2 D6

**Supplementary Figure 4.** ***Comparison of gene expression profiles among RGC-iNs derived from PSC lines across various genetic backgrounds.*** (**a**) Principal Component Analysis (PCA) plot of PSCs, day 6/14 IMR90-CTL, IMR90-NAIP2, GM-NAIP2, WA09-NAIP2 RGC-iNs. (**b**) Heatmap indicating the expression of selected markers for housekeeping (HKGs), pluripotency, NAIP2 cassette, photoreceptor (PRs), glial, RGC, and pan-neuronal genes. (**c**) Bar graphs showcasing the expression of NAIP2 transcription factors (TFs), pan-neuronal marker and RGC marker genes in IMR90-PSCs, WA09-NAIP2-D6, GM-NAIP2-D6 and IMR90-NAIP2-D6 RGC-iNs (n=3 for IMR90-PSCs/WA09-NAIP2-D6/WA09-NAIP2-D14/GM-NAIP2-D6, n=4 for GM-NAIP2-D14/IMR90-NAIP2-D6/IMR90-NAIP2-D14, n=5 for CTL, error bars=SEM).

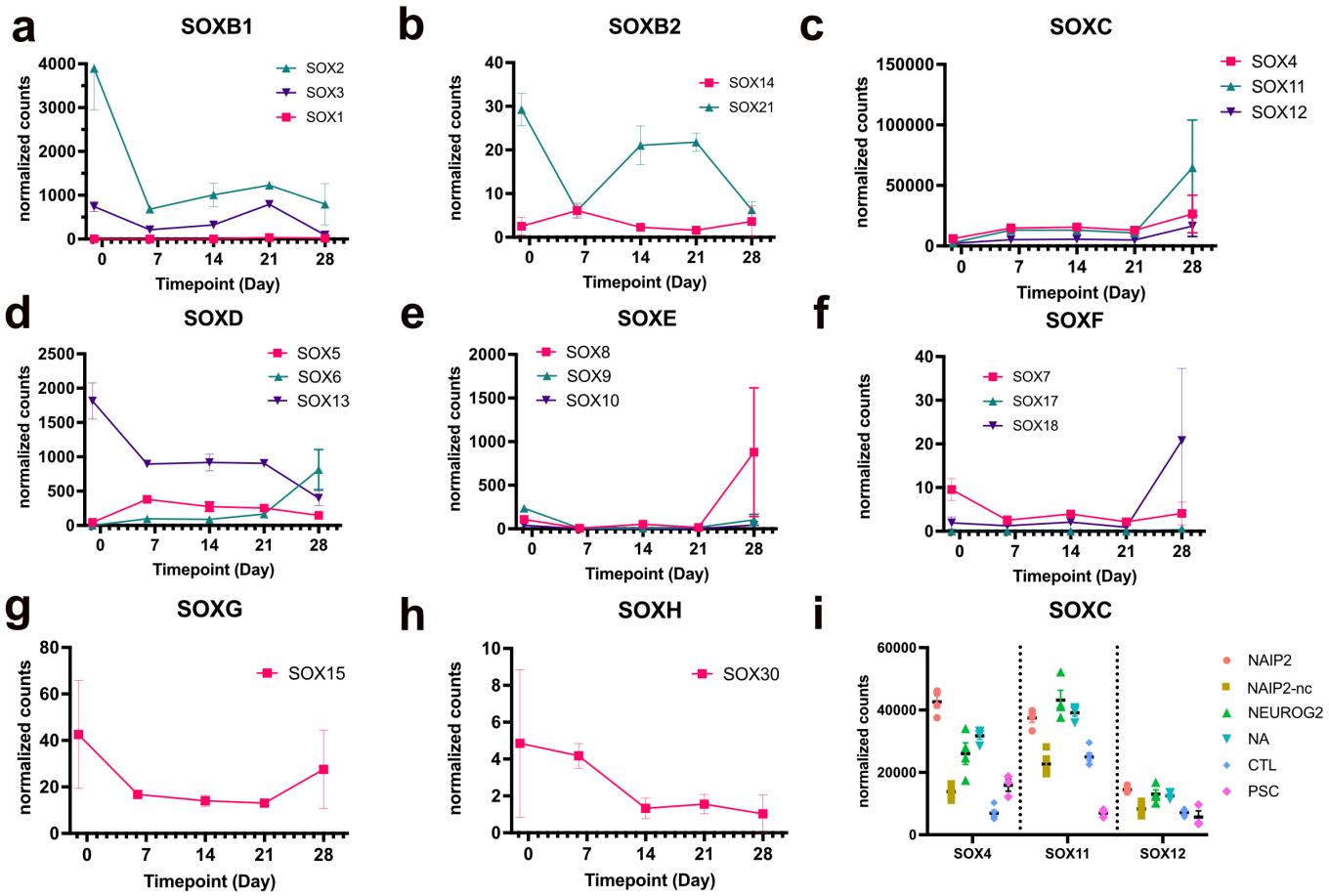



**Supplementary Figure 5.** ***Axon guidance cue expression in RGC-iNs over time.*** The first plot in each panel (**a-h**) represents bulk RNA-seq normalized counts compared between IMR90.4 NAIP2, NAIP2-nc, NEUROG2, NA, CTL samples differentiated for one week or undifferentiated PSCs. NAIP2-nc indicates non-clonally selected NAIP2 PSCs (n=3 for PSCs, n=4 for NAIP2/NEUROG2/NA, n=5 for NAIP2-nc /CTL, error bars=SEM). The second plot in each

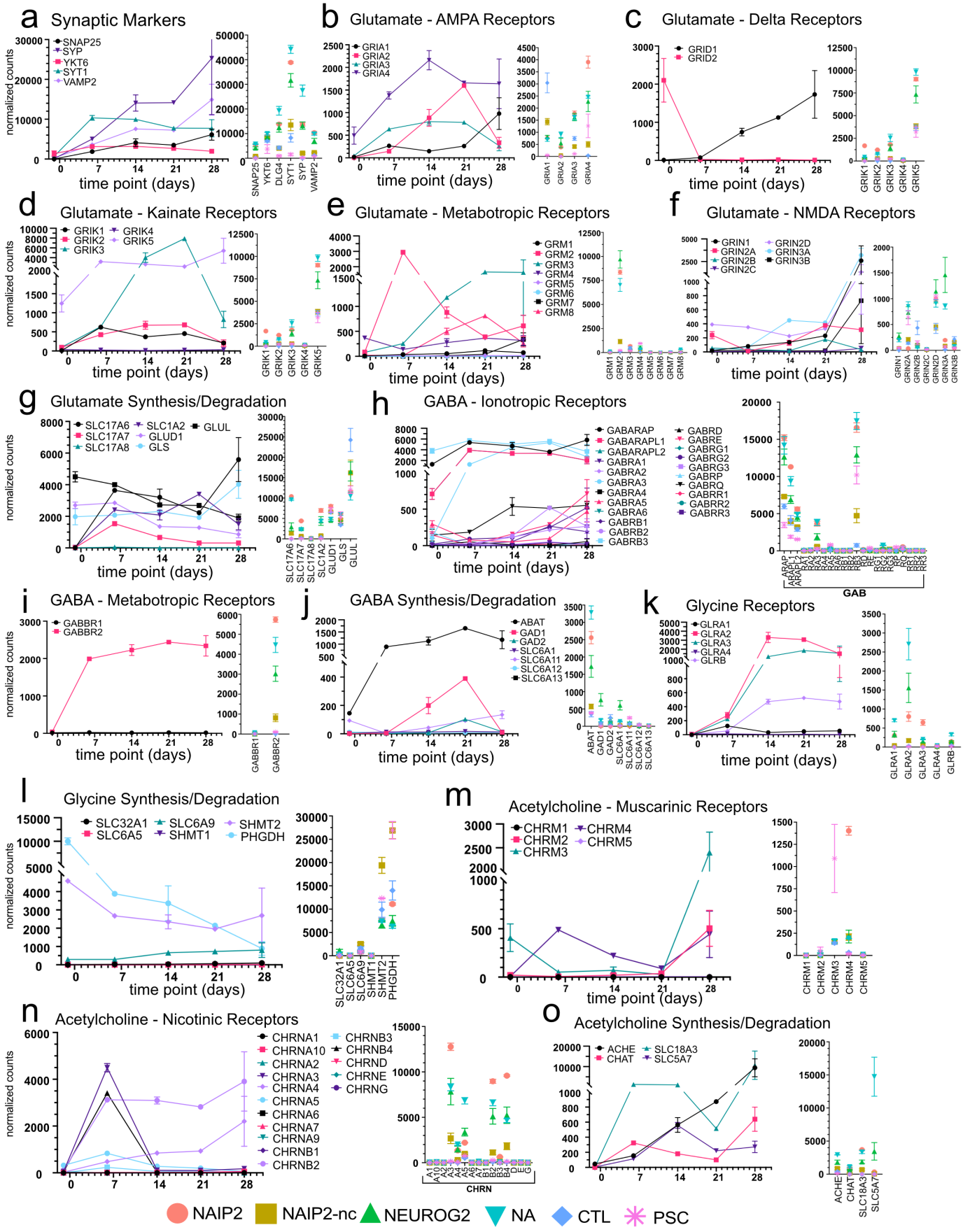
panel (**a-h**) represents bulk RNA-seq normalized counts compared between IMR90.4 NAIP2 iPSCs at 0, 1, 2, 3 and 4 weeks (n=3 for PSCs, n=4 for all other treatment groups, error bars=SEM).



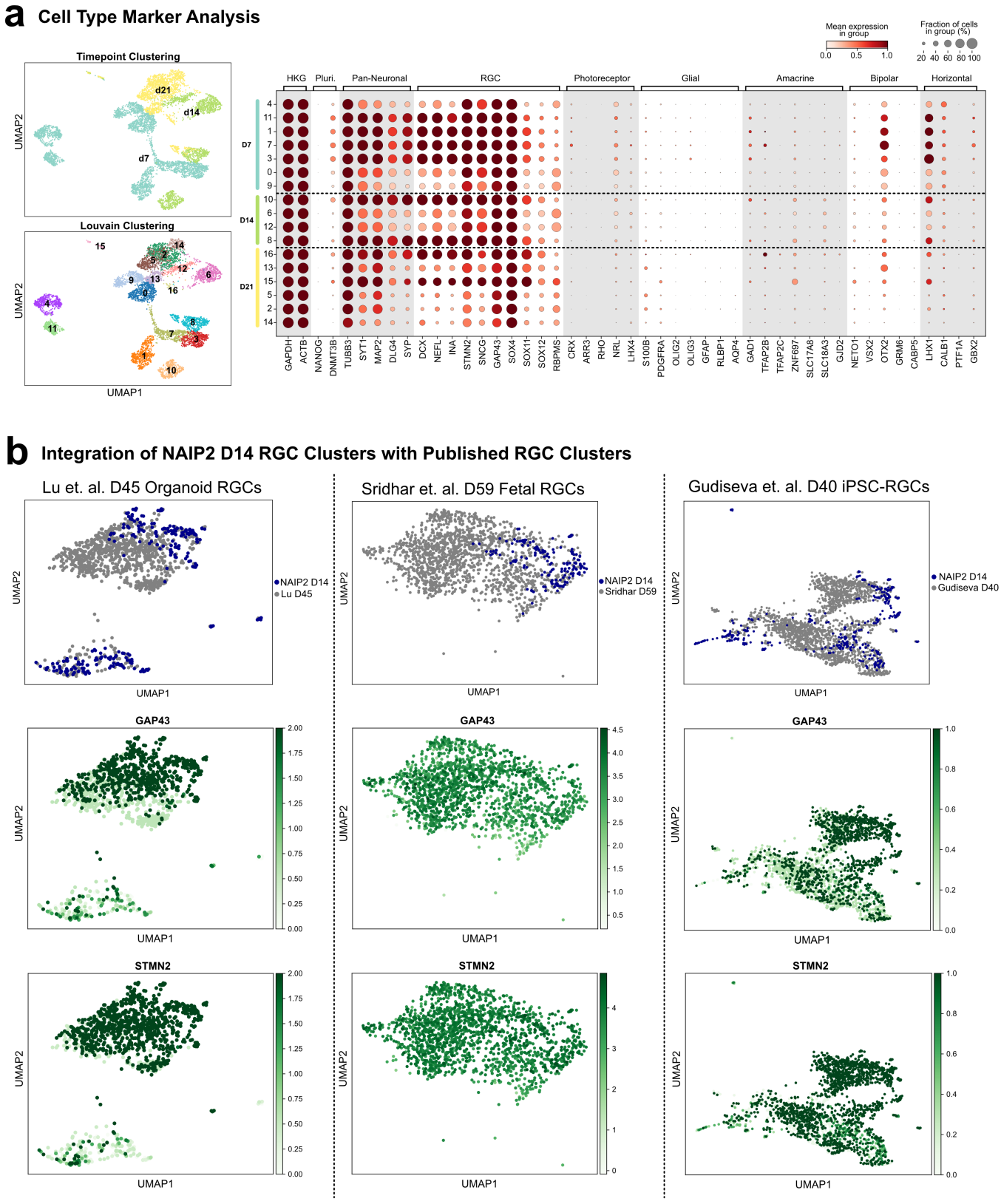

**Supplementary Figure 6.** ***Gene expression in CTL cells over time.*** (**a**) Heatmap showing expression of glial, RGC and pan-neuronal genes in PSCs, CTL -D6, -D14 and -D21 samples. Gene set enrichment analysis of day 21 CTL versus NAIP2 cells indicating (**b**) bar plot of the top 20 upregulated pathways ranked by –log10(FDR) and (**c**) rank/enrichment of the epithelial to mesenchymal transition (EMT) pathway. (**d**) Temporal expression of EMT transcription factors in PSCs, CTL-D6, -D14 and -D21 samples (n=3 for PSCs, n=4 for CTL-D14/-D21, n=5 for CTL-D6, error bars=SEM). (**e**) Heatmap showing expression of genes involved in EMT in PSCs, NAIP2-D6, -D14, -D21, -D28, and CTL-D6, -D14 and -D21 samples.




**Supplementary Figure 7.** ***Comparison of SOX family gene expression in RGC-iNs.*** (**a-h**) Bulk RNA-seq normalized counts for IMR90.4 NAIP2 iPSCs at 0, 1, 2, 3 and 4 weeks (n=3 for PSCs, n=4 for all other samples, error bars=SEM). (**i**) Bulk RNA-seq normalized counts compared between IMR90.4 NAIP2, NAIP2-nc, NEUROG2, NA, CTL samples differentiated for one week or undifferentiated PSCs. NAIP2-nc indicates non-clonally selected NAIP2 PSCs.

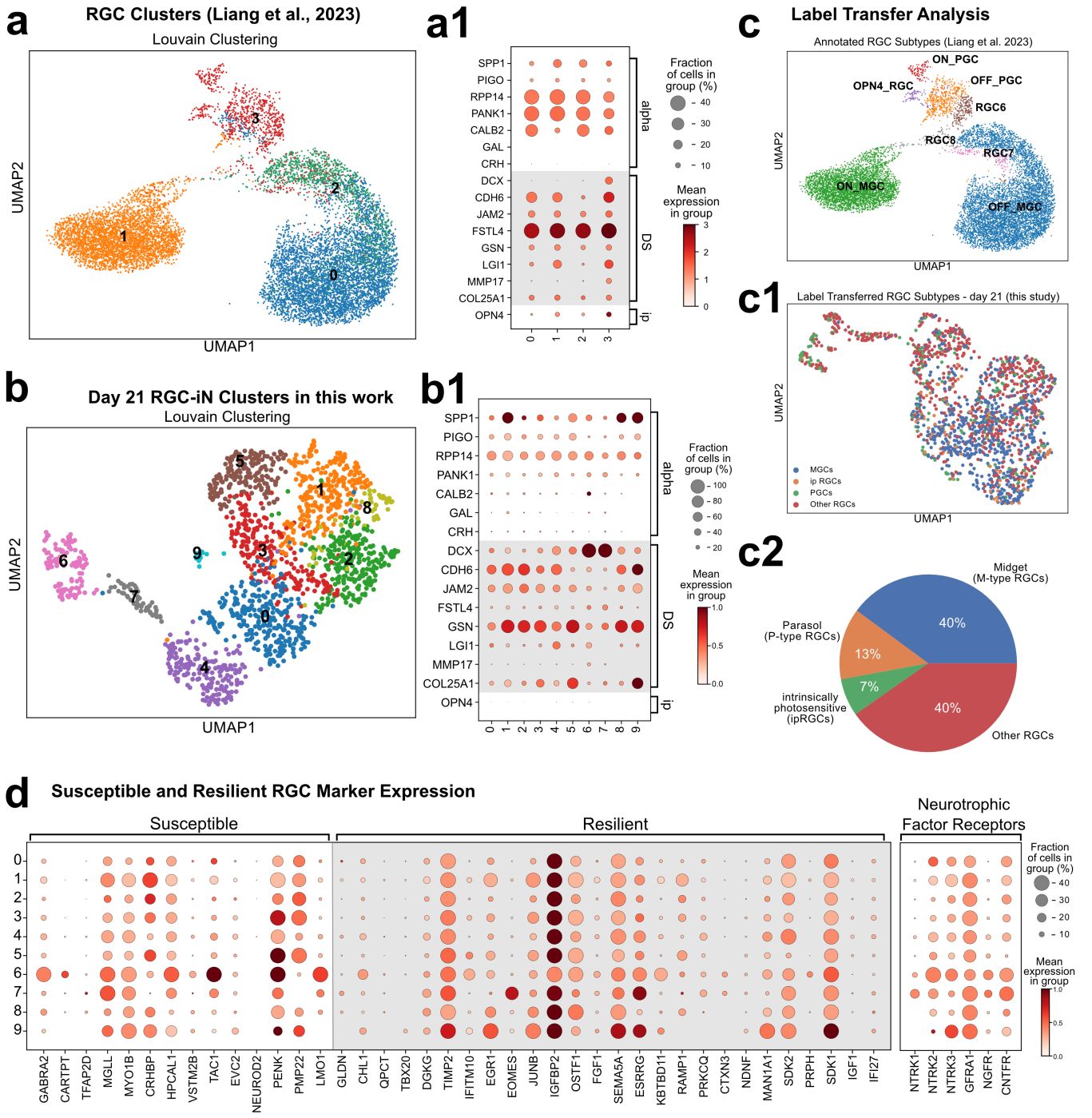

a
Synaptic Markers
SNAP25
SYP
YKT6
SYT1
VAMP2
normalized counts
time point (days)
b
Glutamate - AMPA Receptors
GRIA1
GRIA2
GRIA3
GRIA4
c
Glutamate - Delta Receptors
GRID1
GRID2
GRIK1
GRIK2
GRIK3
GRIK4
GRIK5
d
Glutamate - Kainate Receptors
e
Glutamate - Metabotropic Receptors
GRM1
GRM2
GRM3
GRM4
GRM5
GRM6
GRM7
GRM8
f
Glutamate - NMDA Receptors
GRIN1
GRIN2A
GRIN2B
GRIN2C
GRIN2D
GRIN3A
GRIN3B
g
Glutamate Synthesis/Degradation
SLC17A6
SLC17A7
SLC17A8
SLC1A2
GLUD1
GLS
GLUL
h
GABA - Ionotropic Receptors
GABARAP
GABARAPL1
GABARAPL2
GABRA1
GABRA2
GABRA3
GABRA4
GABRA5
GABRA6
GABRB1
GABRB2
GABRB3
GABRD
GABRE
GABRG1
GABRG2
GABRG3
GABRP
GABRQ
GABRR1
GABRR2
GABRR3
GAB
i
GABA - Metabotropic Receptors
GABBR1
GABBR2
j
GABA Synthesis/Degradation
ABAT
GAD1
GAD2
SLC6A1
SLC6A11
SLC6A12
SLC6A13
k
Glycine Receptors
GLRA1
GLRA2
GLRA3
GLRA4
GLRB
l
Glycine Synthesis/Degradation
SLC32A1
SLC6A5
SLC6A9
SHMT1
SHMT2
PHGDH
m
Acetylcholine - Muscarinic Receptors
CHRM1
CHRM2
CHRM3
CHRM4
CHRM5
n
Acetylcholine - Nicotinic Receptors
CHRNA1
CHRNA10
CHRNA2
CHRNA3
CHRNA4
CHRNA5
CHRNA6
CHRNA7
CHRNA9
CHRNB1
CHRNB2
CHRNB3
CHRNB4
CHRND
CHRNE
CHRNG
CHRN
o
Acetylcholine Synthesis/Degradation
ACHE
CHAT
SLC18A3
SLC5A7
NAIP2
NAIP2-nc
NEUROG2
NA
CTL
PSC

**Supplementary Figure 8.** ***Comparison of neurotransmitter receptor and synthesis/degradation machinery in RGC-iNs over time.*** The first plot in each panel (**a-o; left**) represents bulk RNA-seq normalized counts compared between IMR90.4 NAIP2 iPSCs at 0, 1, 2, 3 and 4 weeks (n=3 for PSCs, n=4 for all other samples, error bars=SEM). The second plot in each panel (**a-o; right**) represents bulk RNA-seq normalized counts compared between IMR90.4 NAIP2, NAIP2-nc, NEUROG2, NA and CTL samples differentiated for one week or undifferentiated PSCs. NAIP2-nc indicates non-clonally selected NAIP2 PSCs. (n=3 for PSCs, n=4 for NAIP2/NEUROG2/NA, n=5 for NAIP2-nc /CTL, error bars=SEM).



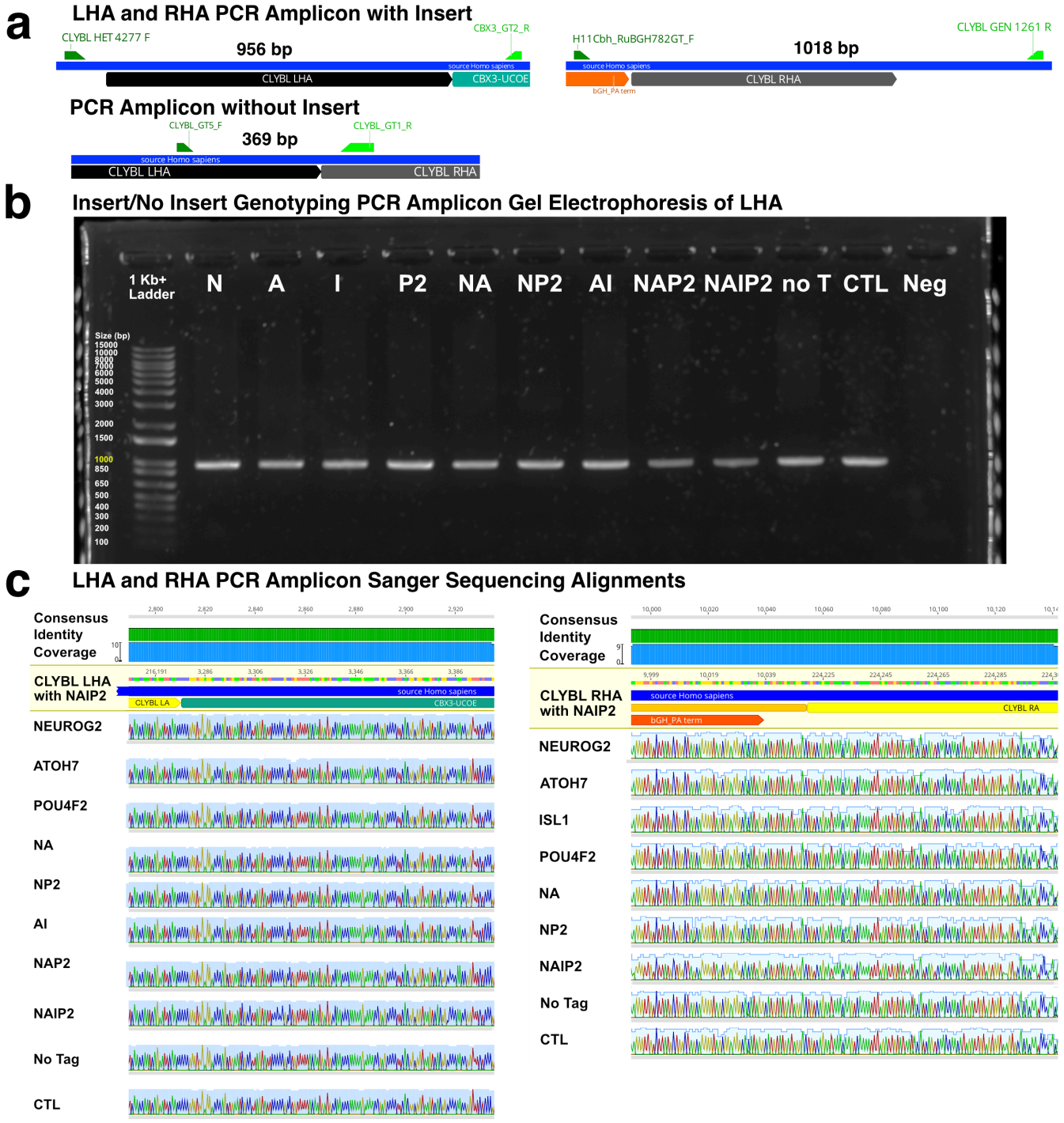

**Supplementary Figure 9.** ***Cell type marker and integrative data scRNA-seq analysis.*** (**a,** left) UMAPs of RGC-iNs colored by timepoint (top) and Louvain clusters (bottom). (**a,** right) Dotplot showing expression of various cell type marker genes (housekeeping [HKG], pluripotency [pluri.], pan-neuronal, RGC, photoreceptor, glial, amacrine, bipolar and horizontal cells) in each cluster of RGC-iNs across day 7, 14 and 21 timepoints. (**b**) UMAPs highlighting

the integration of NAIP2 day 14 RGC-iN clusters with published RGC clusters from day 45 retinal organoid RGCs (left), day 59 fetal RGCs (center) and day 40 iPSC-RGCs (right).




**Supplementary Figure 10.** ***RGC-subtypes in published scRNA-seq datasets compared with this work.*** UMAP with Louvain clustering of human RGCs from Liang et al., 2023 (**a**, left) and dotplot (**a1**, right) showing expression of various RGC-subtype marker genes in each cluster. UMAP with Louvain clustering of day 21 RGC-iNs from this work (**b**, left) and dotplot (**b1**, right) showing expression of various RGC-subtype marker genes in each cluster. (**c**) Label transfer analysis with annotated UMAP of RGC subtypes from Liang et al., 2023 (**c**, top), UMAP of label transferred RGC subtypes in day 21 RGC-iNs (**c1**, middle) and RGC-iNs subtype proportions (**c2**, bottom) in this

study. Dotplot highlighting expression of (**d**) susceptible/resilient RGC markers and neurotrophic factor receptors in day 21 RGC-iNs.




**Supplementary Figure 11.** ***Validation of transgene cassette insertions.*** (**a**) Diagrammatic representation of the left (LHA) and right (RHA) homology arm PCR amplicons with the transgene insert and the PCR amplicon without the insert. (**b**) Gel electrophoresis images of PCR genotyping LHA insert amplicons (956 bp) showing the presence of the transgene cassette, (**c**) Sanger sequencing verification of inserts with sequences that flank the inserts on the left and right homology arm of the CLYBL safe harbor site.

**Supplementary Table 1.** ***Sample characteristics and conditions.*** Table comparing the differentiation conditions of the various samples including transgene cassette, small molecule treatment, dox treatment, clonal selection and number or replicates.

| Condition | CTL (control) | NEUROG2 | NA | NAIP2-nc | NAIP2 | PSC |
|---|---|---|---|---|---|---|
| **Transgene Cassette** | Empty | NEUROG2 | NEUROG2-p2A-ATOH7 | NEUROG2-p2A-ATOH7-p2A-ISL1-p2A-POU4F2 | NEUROG2-p2A-ATOH7-p2A-ISL1-p2A-POU4F2 | NEUROG2-p2A-ATOH7-p2A-ISL1-p2A-POU4F2 |
| **Reporter** | *SIX6*-p2A-h2b-eGFP/*POU4F2*-p2A-tdTomato | *SIX6*-p2A-h2b-eGFP/*POU4F2*-p2A-tdTomato | *SIX6*-p2A-h2b-eGFP/*POU4F2*-p2A-tdTomato | *SIX6*-p2A-h2b-eGFP/*POU4F2*-p2A-tdTomato | *SIX6*-p2A-h2b-eGFP/*POU4F2*-p2A-tdTomato | *SIX6*-p2A-h2b-eGFP/*POU4F2*-p2A-tdTomato |
| **Small Molecule Treatment Duration** | LDN (D-1 to D0) | LDN (D-1 to D0) | LDN (D-1 to D0) | LDN (D-1 to D0) | LDN (D-1 to D0) | None |
| **Dox Treatment Duration** | D-1 to D6 | D-1 to D6 | D-1 to D6 | D-1 to D6 | D-1 to D6 | None |
| **Clonal Selection Performed** | Yes | Yes | Yes | No | Yes | not applicable |
| **Number of Replicates** | 5 | 4 | 4 | 4 | 4 | 3 |
| **Timepoints Sequenced (Bulk RNAseq)** | D6, D14, D21 | D6 | D6 | D6 | D6, D14, D21, D28 | D-1 |
| **Timepoints Sequenced (scRNAseq** | None | None | None | None | D6, D14, D21 | None |

**Supplementary Table 2.** ***Key Resources Table.*** Table describing the various reagents and resources used in this work with their purpose in parentheses.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| ***Antibodies and Imaging (for immunostaining and verification of specific protein expression)*** | | |
| Calcein-AM (cell permeable dye for assessing cell viability) | Thermo Fisher Scientific | Cat# C3100MP |
| Chicken polyclonal anti-TAU | PhosphoSolutions | Cat# 1998-TAU, RRID: AB_2492256 |
| Chicken polyclonal anti-MAP2 | PhosphoSolutions | Cat# 1100-MAP2, RRID: AB_2492141 |
| Click-It EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 dye (for assessing cell proliferation) | Thermo Fisher Scientific | Cat# C10340 |
| Donkey anti-Mouse IgG (H+L) Alexa Fluor™ 488 | Thermo Fisher Scientific | Cat# A-21202, RRID: AB_141607 |
| Donkey anti-Goat IgG (H+L) Alexa Fluor™ 488 | Thermo Fisher Scientific | Cat# A-11055, RRID: AB_2534102 |
| Goat polyclonal anti-pan BRN3 | Santa Cruz Biotechnology | Cat# sc-6026, RRID: AB_673441 |
| Goat anti-Rabbit IgG (H+L) Alexa Fluor™ 488 | Thermo Fisher Scientific | Cat# A-11034, RRID: AB_2576217 |
| Goat anti-Chicken IgY (H+L) Alexa Fluor™ Plus 647 | Thermo Fisher Scientific | Cat# A32933, RRID: AB_2762845 |
| Hoechst 33342 (nuclear stain) | Thermo Fisher Scientific | Cat# H1399 |
| Mouse monoclonal anti-ISL1 | DSHB | Cat# 39.4D5, RRID: AB_2314683 |
| Mouse monoclonal anti-PAX6 | DSHB | Cat# pax6, RRID: AB_528427 |
| Mouse monoclonal anti-MAP2 | BioLegend | Cat# 801807, RRID: AB_2721423 |
| Mouse monoclonal anti-TUJ1 | Covance | Cat# MMS-435P, RRID: AB_2313773 |
| Mouse monoclonal anti-BRN3A | Santa Cruz Biotechnology | Cat# sc-8429, RRID: AB_626765 |

| | | |
|---|---|---|
| Rabbit polyclonal anti-GLAST (SLC1A3) | Novus | Cat# NB100-1869, RRID: AB_2190597 |
| Rabbit monoclonal anti-VIM | Abcam | Cat# ab92547, RRID: AB_10562134 |
| Rabbit monoclonal anti-pCREB | Cell Signaling Technology | Cat# 9198, RRID: AB_2561044 |
| ***Bacterial and virus strains*** | | |
| NEB® Stable Competent E. coli (bacteria for plasmid growth) | New England BioLabs | Cat# C3040I |
| ***Chemicals, peptides, and recombinant proteins*** | | |
| All-trans retinoic acid (ATRA) (for enhancing cell survival) | Sigma-Aldrich | Cat# R2625 |
| Accutase (single cell passaging of hPSCs) | Sigma-Aldrich | Cat# A6964 |
| B27 vitamin A (-) (neural supplement) | Thermo Fisher Scientific | Cat# 12587010 |
| B27 vitamin (neural supplement) | Thermo Fisher Scientific | Cat# 17504044 |
| BDNF (growth factor for RGC growth and survival) | Qkine | Cat# Qk050 |
| Blebbistatin (ROCK inhibitor for improving cell survival) | Sigma-Aldrich | Cat# B0560 |
| BrainPhys Neuronal Medium (basal media for supporting long-term growth of neurons) | StemCell Technologies | Cat# 05790 |
| CultureOne supplement (for enhancing neural conversion) | Thermo Fisher Scientific | Cat# A3320201 |
| DMEM (basal media) | Thermo Fisher Scientific | Cat# 11965 |
| DMEM/F12 50:50 (basal media) | Thermo Fisher Scientific | Cat# 11330 |
| Doxycycline hyclate (antibiotic for transgene induction) | Sigma-Aldrich | Cat# D5207 |
| DpnI enzyme (methylation sensitive enzyme for removing parental plasmid DNA) | New England BioLabs | Cat# R0176L |
| F12 (basal media) | Thermo Fisher Scientific | Cat# 11765 |
| Fetal Bovine Serum, qualified, heat inactivated (serum for maintenance of cultured cells) | Thermo Fisher Scientific | Cat# 16140071 |
| GDNF (growth factor for enhancing RGC growth and neuronal survival) | Qkine | Cat# Qk051 |
| GlutaMAX (stable form of glutamine as energy source for dividing cells) | Thermo Fisher Scientific | Cat# 35050061 |
| GNE-3511 (neuroprotective DLK/LZK inhibitor) | Sigma-Aldrich | Cat# 5.33168 |
| Insulin-Human Recombinant (N2 supplement component) | Roche | Cat# 11376497001 |
| IWR-1-endo (WNT pathway inhibitor to promote an anterior neural fate) | EMD Millipore | Cat# 681669 |
| L-ascorbic acid (N2 supplement component) | Sigma-Aldrich | Cat# A8960 |
| LDN-193189 (pre-patterning BMP pathway inhibitor) | Sigma-Aldrich | Cat# SML0559 |
| Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (cell attachment/differentiation of hPSCs) | Corning | Cat# 354230 |
| mTeSR1 (maintenance and propagation of hPSCs) | Stem Cell Technologies | Cat# 85850 |
| N-2 Supplement (neural supplement) | Thermo Fisher Scientific | Cat# 17502048 |
| NEBuilder® HiFi DNA Assembly Mastermix (assembly of DNA fragments) | New England BioLabs | Cat# E2621S |
| NEAA (non-essential amino acids for supporting neuronal growth) | Thermo Fisher Scientific | Cat# 11140 |
| Nicotinamide (NIC) (vitamin B3 supplement to enhance differentiation) | Sigma-Aldrich | Cat# 72340 |
| Poly-L-ornithine (PLO) hydrobromide (for neural attachment) | Sigma Aldrich | Cat# P3655 |
| Phusion Flash Polymerase (DNA amplification) | Thermo Fisher Scientific | Cat# F548L |
| PF-06260933 dihydrochloride (neuroprotective inhibitor of MAP4K4) | Sigma-Aldrich | Cat# PZ0272 |
| Smoothened Agonist (SAG) (Sonic hedgehog agonist for retinal organoid differentiation) | EMD Millipore | Cat# 566660 |
| Sodium pyruvate (carbon source supporting cellular metabolism) | Thermo Fisher Scientific | Cat# 11360 |
| Sodium selenite (N2 supplement component) | Sigma-Aldrich | Cat# S5261 |
| SuperScript IV reverse transcriptase (for converting mRNA into cDNA) | Thermo Fisher Scientific | Cat# 18090050 |

| | | |
|---|---|---|
| Taurine (amino acid for enhancing cell survival) | Sigma-Aldrich | Cat# T8691 |
| Thiazovivin (ROCK inhibitor for cell survival) | LC Labs | Cat# T-9753 |
| Transferrin - Human holo (N2 supplement component) | Sigma-Aldrich | Cat# T0665 |
| Zeocin (antibiotic for selection of hPSCs with stably integrated gene cassette) | InvivoGen | Cat# ant-zn-1p |
| ***Critical commercial assays*** | | |
| CellTiter-Glo (ATP based cell viability assay) | Promega | Cat# G7570 |
| DNA Clean & Concentrator-5 (for PCR DNA purification) | Zymo Research | Cat# #D4013 |
| PureLink HiPure Plasmid Midiprep Kit (for transfection grade plasmid DNA preparation) | Thermo Fisher Scientific | Cat# K210004 |
| PureLink RNA Mini Kit (for RNA extraction) | Thermo Fisher Scientific | Cat# 12183020 |
| Quick-RNA Miniprep Kit (for RNA extraction) | Zymo Research | Cat# R1054 |
| ZymoPURE II Plasmid Midiprep Kit (for transfection grade plasmid DNA preparation) | Zymo Research | Cat# D4200 |
| ZymoPURE Plasmid Miniprep Kit (for plasmid DNA preparation) | Zymo Research | Cat# D4210 |
| ***Deposited data*** | | |
| Raw bulk RNA-seq datasets | Sequence Read Archive | PRJNA885885 |
| Raw single cell RNA-seq datasets | Sequence Read Archive | PRJNA973095 |
| ***Experimental models: Cell lines*** | | |
| Human: Passage 54 IMR90-4 iPSCs | WiCell | Cat# ips-imr90-4, RRID: CVCL_C437 |
| Human: Passage 35 WA09 (H9) ESCs (NIH # NIHhESC-10-0062) | WiCell | Cat# WA09, RRID: CVCL_9773 |
| Human: Passage 48 GM23720 iPSCs | Coriell | Cat# GM23720, RRID: CVCL_T818 |
| ***Oligonucleotides*** | | |
| H11Cbh_RuBGH782GT_F: GCATCGCATTGTCTGAGTAGGTGTCATTCTATTC | This paper | N/A |
| CLYBL_GEN_1261_R: TTACGGCTCTGTTGGAGAGTCCAGTATTGAATTAG | This paper | N/A |
| CLYBL_HET_4277_F: CATTCAGACAAGTCAGTAGGGCCATCTTAGATCATCCAGCCCTA | This paper | N/A |
| CBX3_GT2_R: ATTTGACTAGAAGTTGATTCGGGTGTTTCCGGAA | This paper | N/A |
| CLYBL_HET_2300F: CTTCTTTGTTCTTCCCCAAGTCTTTCTTTCTAGACGAAACTACTT | This paper | N/A |
| CLYBL_HET_2300 R: GAGAGCTCAATCCTCATCTTACATAGAAGGAAGTCAGTAGATAAT | This paper | N/A |
| ***Recombinant DNA*** | | |
| pY026 | Zetsche et al.[1] | RRID: Addgene_84741 |
| AAVS1-Neo-M2rtTA | DeKelver et al.[2] | RRID: Addgene_60843 |
| P789_pY026_EnAsCpf1_CLYBL_T1_catRNA | This paper | RRID: Addgene_202760 |
| P931_CLYBL-CBX3-Cbh-Zeo-TetO-NAIP2 | This paper | RRID: Addgene_202761 |
| P932_CLYBL-CBX3-Cbh-Zeo-TetO-NEUROG2 | This paper | RRID: Addgene_202762 |
| ***Software and algorithms*** | | |
| Galaxy Bioinformatics Platform | Afgan et al.[3] | https://galaxyproject.org/, RRID: SCR_006281 |
| Python version 3.8.9 | Python Software Foundation | https://www.python.org/, RRID:SCR_008394 |
| R version 4.1.2 | The R Foundation | https://www.r-project.org/, RRID:SCR_001905 |
| ImageJ (Fiji) version 2.9.0 | NIH | https://imagej.net/software/fiji/, RRID:SCR_002285 |
| Affinity Designer version 2.1.0 | Serif Ltd. | https://affinity.serif.com/, RRID:SCR_016952 |
| GraphPad Prism 9 version 9.1.1 | GraphPad Software | https://www.graphpad.com/scientific-software/prism/, RRID:SCR_002798 |
| Geneious Prime 2022 | Biomatters | https://www.geneious.com/prime/, RRID:SCR_010519 |

| *Other* | | |
|---|---|---|
| Leica DMi1 Inverted Microscope | Leica Microsystems | https://www.leica-microsystems.com/ |
| ImageXpress Micro Confocal High-Content Imaging System | Molecular Devices | https://www.moleculardevices.com/ |

References for Supplementary Table 2:

1. Zetsche, B. *et al.* Multiplex gene editing by CRISPR–Cpf1 using a single crRNA array. *Nat Biotechnol* **35**, 31–34 (2017).
2. DeKelver, R. C. *et al.* Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Res.* **20**, 1133–1142 (2010).
3. Afgan, E. *et al.* The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Research* **46**, W537–W544 (2018).

**Supplementary Table 3**. ***SRA Accession Numbers***. Table of RNA-seq sample SRA accession numbers, names, age, cell line, replicate details and cell types.

| SRA Accession | Sample Name | Age | Cell Line | Replicate | Cell Type |
|---|---|---|---|---|---|
| SAMN31111428 | PSC_1 | 0 days | IMR-90.4 | PSC biological replicate 1 | Pluripotent stem cells |
| SAMN31111429 | PSC_2 | 0 days | IMR-90.4 | PSC biological replicate 2 | Pluripotent stem cells |
| SAMN31111430 | PSC_3 | 0 days | IMR-90.4 | PSC biological replicate 3 | Pluripotent stem cells |
| SAMN31111431 | CTL_1 | 7 days | IMR-90.4 | Control biological replicate 1 | Control neural cells |
| SAMN31111432 | CTL_2 | 7 days | IMR-90.4 | Control biological replicate 2 | Control neural cells |
| SAMN31111433 | CTL_3 | 7 days | IMR-90.4 | Control biological replicate 3 | Control neural cells |
| SAMN31111434 | CTL_4 | 7 days | IMR-90.4 | Control biological replicate 4 | Control neural cells |
| SAMN31111435 | CTL_5 | 7 days | IMR-90.4 | Control biological replicate 5 | Control neural cells |
| SAMN31111436 | NAIB_1 | 7 days | IMR-90.4 | NAIB D6 biological replicate 1 | Unpurified D6 retinal ganglion cells |
| SAMN31111437 | NAIB_2 | 7 days | IMR-90.4 | NAIB D6 biological replicate 2 | Unpurified D6 retinal ganglion cells |
| SAMN31111438 | NAIB_3 | 7 days | IMR-90.4 | NAIB D6 biological replicate 3 | Unpurified D6 retinal ganglion cells |
| SAMN31111439 | NAIB_4 | 7 days | IMR-90.4 | NAIB D6 biological replicate 4 | Unpurified D6 retinal ganglion cells |
| SAMN31111440 | NAIB_5 | 7 days | IMR-90.4 | NAIB D6 biological replicate 5 | Unpurified D6 retinal ganglion cells |
| SAMN31111441 | NEUROG2_1 | 7 days | IMR-90.4 | NEUROG2 D6 biological replicate 1 | D6 Neural cells |
| SAMN31111442 | NEUROG2_2 | 7 days | IMR-90.4 | NEUROG2 D6 biological replicate 2 | D6 Neural cells |
| SAMN31111443 | NEUROG2_3 | 7 days | IMR-90.4 | NEUROG2 D6 biological replicate 3 | D6 Neural cells |
| SAMN31111444 | NEUROG2_4 | 7 days | IMR-90.4 | NEUROG2 D6 biological replicate 4 | D6 Neural cells |
| SAMN31111445 | NEUROG2_5 | 7 days | IMR-90.4 | NEUROG2 D6 biological replicate 5 | D6 Neural cells |
| SAMN31111446 | NA_1 | 7 days | IMR-90.4 | NA D6 biological replicate 1 | D6 Neural cells |
| SAMN31111447 | NA_2 | 7 days | IMR-90.4 | NA D6 biological replicate 2 | D6 Neural cells |
| SAMN31111448 | NA_3 | 7 days | IMR-90.4 | NA D6 biological replicate 3 | D6 Neural cells |
| SAMN31111449 | NA_4 | 7 days | IMR-90.4 | NA D6 biological replicate 4 | D6 Neural cells |

| | | | | | |
|---|---|---|---|---|---|
| SAMN31111450 | NAIB_C1_D6_1 | 7 days | IMR-90.4 | NAIB C1 D6 biological replicate 1 | Purified D6 retinal ganglion cells |
| SAMN31111451 | NAIB_C1_D6_2 | 7 days | IMR-90.4 | NAIB C1 D6 biological replicate 2 | Purified D6 retinal ganglion cells |
| SAMN31111452 | NAIB_C1_D6_3 | 7 days | IMR-90.4 | NAIB C1 D6 biological replicate 3 | Purified D6 retinal ganglion cells |
| SAMN31111453 | NAIB_C1_D6_4 | 7 days | IMR-90.4 | NAIB C1 D6 biological replicate 4 | Purified D6 retinal ganglion cells |
| SAMN31111454 | NAIB_C1_D14_1 | 14 days | IMR-90.4 | NAIB C1 D14 biological replicate 1 | Purified D14 retinal ganglion cells |
| SAMN31111455 | NAIB_C1_D14_2 | 14 days | IMR-90.4 | NAIB C1 D14 biological replicate 2 | Purified D14 retinal ganglion cells |
| SAMN31111456 | NAIB_C1_D14_3 | 14 days | IMR-90.4 | NAIB C1 D14 biological replicate 3 | Purified D14 retinal ganglion cells |
| SAMN31111457 | NAIB_C1_D14_4 | 14 days | IMR-90.4 | NAIB C1 D14 biological replicate 4 | Purified D14 retinal ganglion cells |
| SAMN31111458 | NAIB_C1_D21_1 | 21 days | IMR-90.4 | NAIB C1 D21 biological replicate 1 | Purified D21 retinal ganglion cells |
| SAMN31111459 | NAIB_C1_D21_2 | 21 days | IMR-90.4 | NAIB C1 D21 biological replicate 2 | Purified D21 retinal ganglion cells |
| SAMN31111460 | NAIB_C1_D21_3 | 21 days | IMR-90.4 | NAIB C1 D21 biological replicate 3 | Purified D21 retinal ganglion cells |
| SAMN31111461 | NAIB_C1_D21_4 | 21 days | IMR-90.4 | NAIB C1 D21 biological replicate 4 | Purified D21 retinal ganglion cells |
| SAMN35158738 | NAIP2-D28-1 | 28 days | IMR-90.4 | NAIP2 D28 biological replicate 1 | Purified D28 retinal ganglion cells |
| SAMN35158739 | NAIP2-D28-2 | 28 days | IMR-90.4 | NAIP2 D28 biological replicate 2 | Purified D28 retinal ganglion cells |
| SAMN35158740 | NAIP2-D28-3 | 28 days | IMR-90.4 | NAIP2 D28 biological replicate 3 | Purified D28 retinal ganglion cells |
| SAMN35158741 | NAIP2-D28-4 | 28 days | IMR-90.4 | NAIP2 D28 biological replicate 4 | Purified D28 retinal ganglion cells |
| SAMN35119890 | NAIP2-D7-1 | 7 days | GM23720 | NAIP2 D7 biological replicate 1 | Purified D7 retinal ganglion cells |
| SAMN35119891 | NAIP2-D7-2 | 7 days | GM23720 | NAIP2 D7 biological replicate 2 | Purified D7 retinal ganglion cells |
| SAMN35119892 | NAIP2-D7-3 | 7 days | GM23720 | NAIP2 D7 biological replicate 3 | Purified D7 retinal ganglion cells |
| SAMN35119893 | NAIP2-D14-1 | 14 days | GM23720 | NAIP2 D14 biological replicate 1 | Purified D14 retinal ganglion cells |
| SAMN35119894 | NAIP2-D14-2 | 14 days | GM23720 | NAIP2 D14 biological replicate 2 | Purified D14 retinal ganglion cells |
| SAMN35119895 | NAIP2-D21-1 | 21 days | GM23720 | NAIP2 D21 biological replicate 1 | Purified D21 retinal ganglion cells |
| SAMN35119896 | NAIP2-D21-2 | 21 days | GM23720 | NAIP2 D21 biological replicate 2 | Purified D21 retinal ganglion cells |
| SAMN35119897 | NAIP2-D21-3 | 21 days | GM23720 | NAIP2 D21 biological replicate 3 | Purified D21 retinal ganglion cells |