

SUPPLEMENTARY MATERIAL

A differential requirement for ciliary transition zone proteins in human and mouse neural progenitor fate specification

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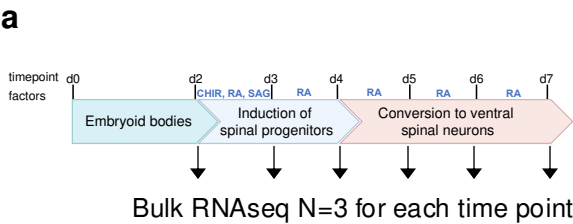
[#]These authors jointly supervised this work

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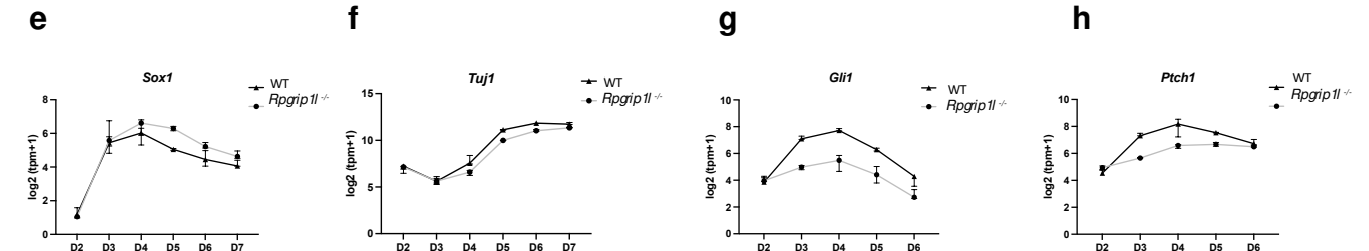
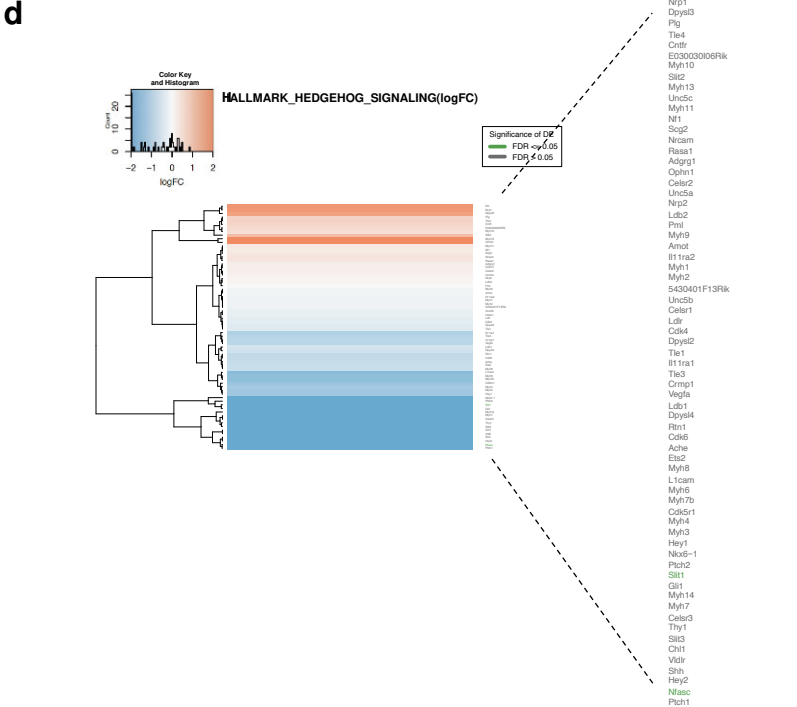
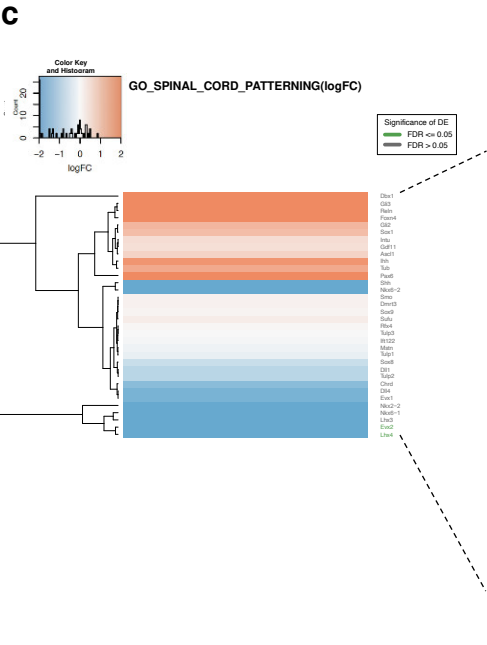
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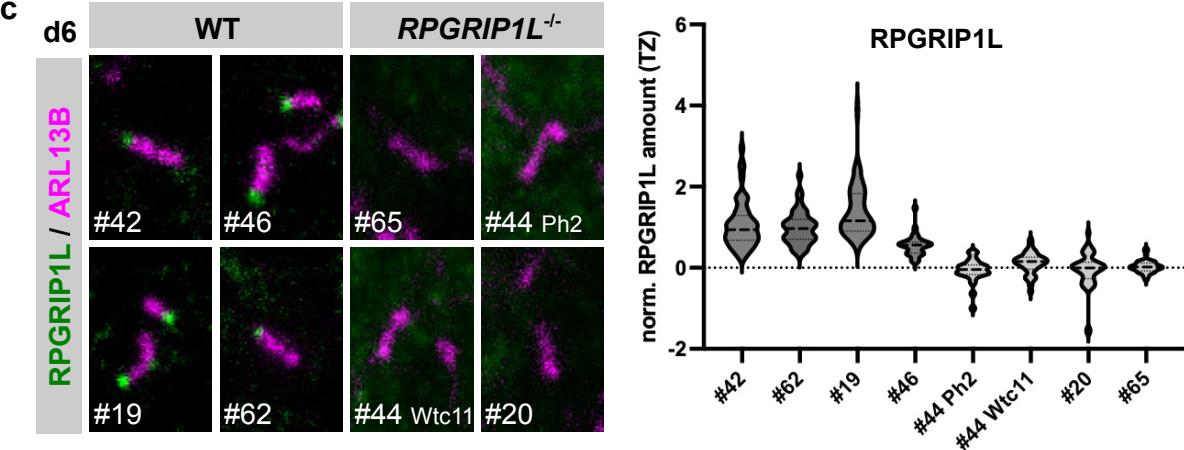
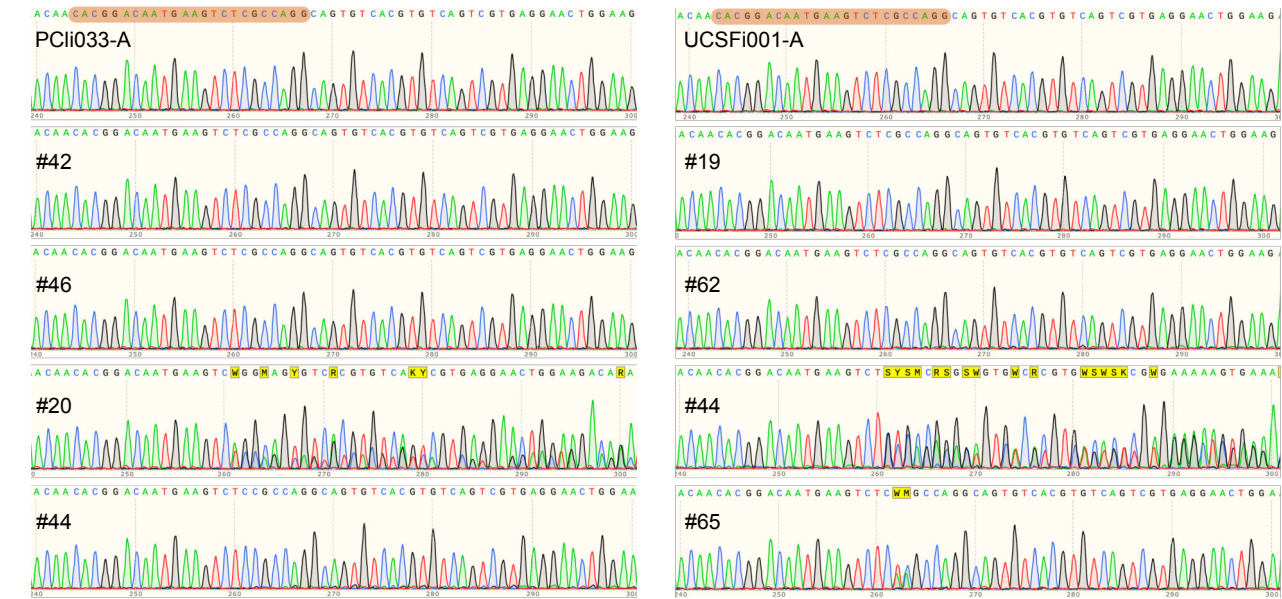
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ENSMUSG00000026468	Lhx4	LIM homeobox protein 4	-2.9380576	0.24563724	5.69E-33	6.37E-30
ENSMUSG00000031737	Irx5	Iroquois homeobox 5	2.50242649	0.22365503	4.63E-29	4.40E-26
ENSMUSG00000028736	Pax7	paired box 7	2.82332962	0.26018955	1.97E-27	1.70E-24
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ENSMUSG0000004231	Pax2	paired box 2	2.00227118	0.24969976	1.07E-15	3.23E-13
ENSMUSG00000035187	Nkx6-1	NK6 homeobox 1	-1.9061719	0.23892466	1.49E-15	4.35E-13
ENSMUSG00000026934	Lhx3	LIM homeobox protein 3	-1.9997871	0.26092015	1.80E-14	4.82E-12
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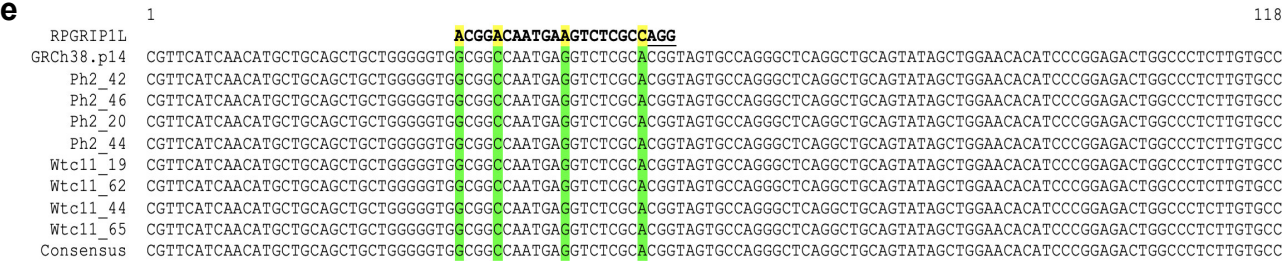
Supplementary Figure 1: Longitudinal transcriptomic analysis of WT and *Rpgrip1l*^{-/-} spinal organoids. **a** Diagram depicting the time points of spinal differentiation chosen for bulk RNAseq analysis. **b** Table listing selected top-differentially expressed genes between WT and *Rpgrip1l*^{-/-} day 4 spinal organoids obtained by DEseq2 analysis. **c, d** Ensemble of Gene Set Enrichment Analyses (EGSEA) performed on differentially expressed genes between WT and *Rpgrip1l*^{-/-} organoids on day 4. Heatmaps ranked 1st for c5 GO Gene Sets (c), and h Hallmark Signatures (d) are shown. **e-h** Temporal analysis of *Sox1* (NPs), *Tuj1* (early neurons), *Gli1* and *Patched1* (SHH targets) in the course of the differentiation of WT and *Rpgrip1l*^{-/-} organoids. Log2(tpm+1) data from bulk RNASeq analysis are displayed as mean ± SEM (N=3 independent experiments for each genotype).

Guide	Sequence + PAM	Crispr Score	Off Target
RPGRIP1L_exon3	ACGGACAATGAAGTCTCGCC AGG	92	0-0-0-8-38

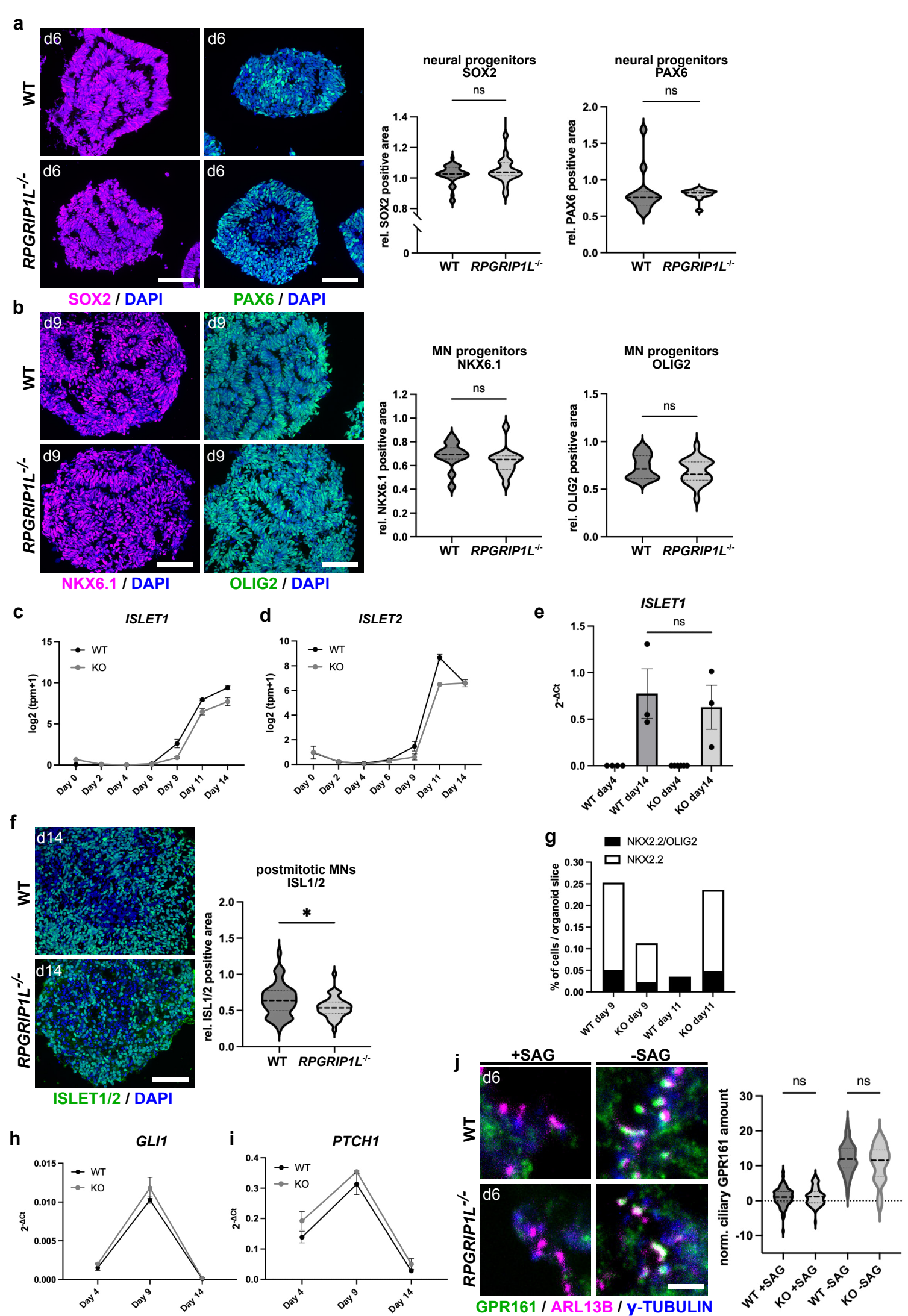
Cell line	Origin	# clone	Genotype	Method	Mutation
PCli033-A	PHENOCELL (PCI)	42	+/+	InDel	-
PCli033-A	PHENOCELL (PCI)	46	+/+	InDel	-
PCli033-A	PHENOCELL (PCI)	20	-/-	InDel	c.114_118del; p.Arg39Glyfs*8 c.116del; p.Arg39Profs*44
PCli033-A	PHENOCELL (PCI)	44	-/-	InDel	c.115dup; p.Arg39Profs*10 c.115dup; p.Arg39Profs*10
UCSFi001-A	Conklin Lab Gladstone/UCS	19	+/+	InDel	-
UCSFi001-A	Conklin Lab Gladstone/UCS	62	+/+	InDel	-
UCSFi001-A	Conklin Lab Gladstone/UCS	44	-/-	InDel	c.114_115insG; p.Arg39Alafs*10 c.116_117delinsT; p.Arg39Leufs*44
UCSFi001-A	Conklin Lab Gladstone/UCS	65	-/-	InDel	c.114_115dup; p.Arg39Leufs*45 c.114_115dup; p.Arg39Leufs*45



on/off target	CFD Score
ontarget_mm0_exon_RPGRIP1L_chr16_53730171_F	1.00
ontarget_mm0_exon_RPGRIP1L_chr16_53570171_R	1.00
mm4_exon_NPRL2/CYB561D2/XXcos-LUCA11.5_chr3_50385586_F	0.18
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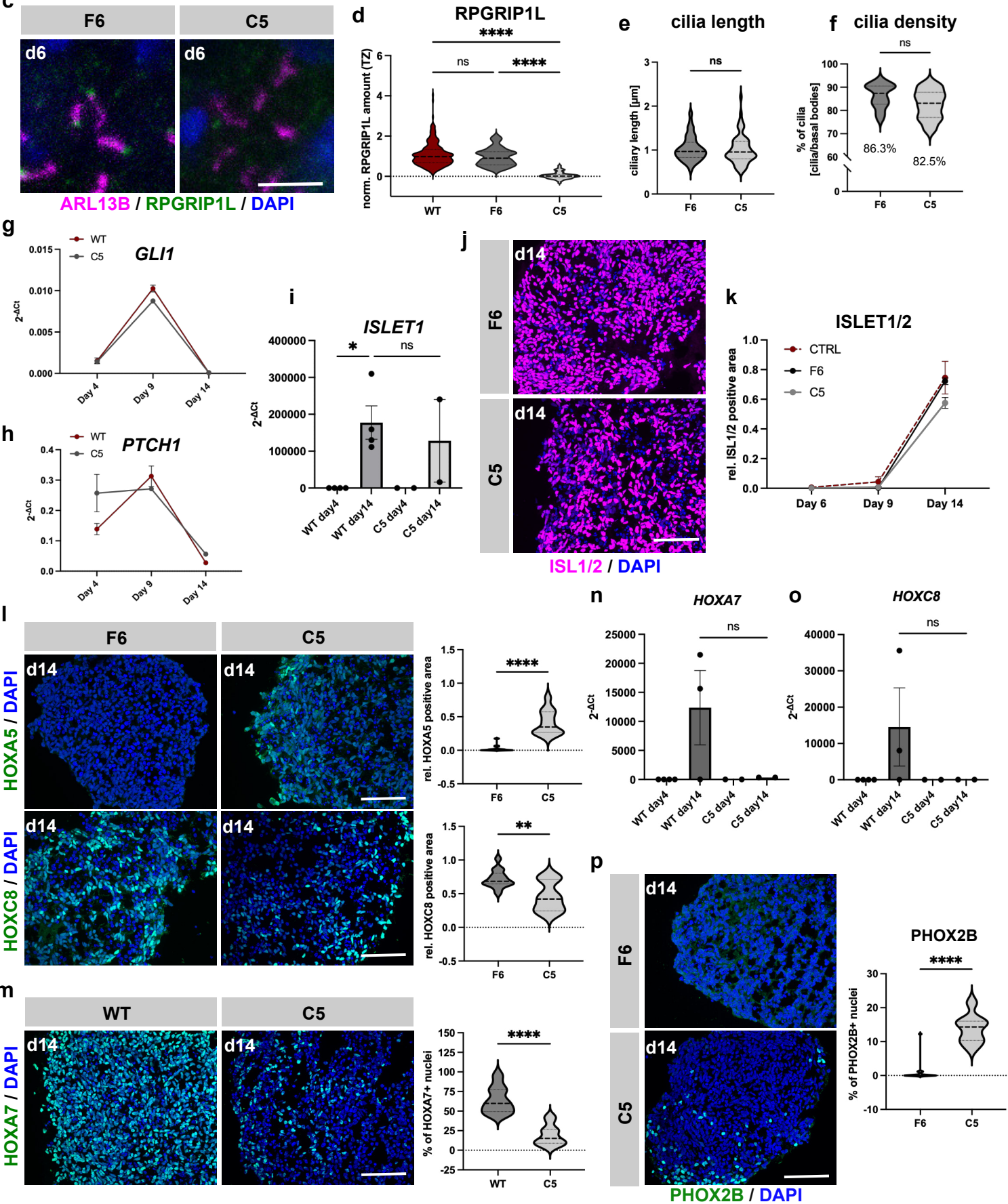
Supplementary Figure 2: Generation of RPGRIP1L-deficient hiPSC lines. **a** Sequence of the CRISPR-guide that was used for the generation of mutations in exon 3 of *RPGRIP1L*. The Crispr Score corresponds to the CFD specificity score based on the CFD off-target model. The Off Target field indicates the number of off-targets for each number of mismatches (8 off-targets with 3 mismatches and 38 off-targets with 4 mismatches). None of these mismatches are in the 12 bp adjacent to the PAM. **b** List of hiPSC clones that were used in this project. The original hiPSC line (PCli033-A or UCSFi001-A), clone number, genotype and specific mutation are indicated for each clone. Sequencing chromatograms are presented for each clone and original cell line. The CRISPR-guide sequence is indicated in orange. **c** Immunofluorescence of RPGRIP1L in WT and RPGRIP1L-deficient cilia in spinal organoids at day 6. Cilia are labeled by ARL13B and RPGRIP1L. Data are shown as median with quartiles. N=3 experimental replicates per clone. **d** Identified off-targets and corresponding CFD scores for the *RPGRIP1L_exon3* guide. **e** Off-target analyses via sequencing of the *NPRL2* locus. Sequence of the CRISPR-guide is indicated above. Bases highlighted in yellow and green indicate mismatches between the guide sequence and the off-target sequence. No mutation in *NPRL2* was detectable around the off-target site in WT and KO hiPSC clones.



Supplementary Figure 3: Phenotyping of WT and RPGRIP1L-deficient organoids. **a, b** IF analysis of day 6 neural progenitors and day 9 pMNs in WT and *RPGRIP1L*^{-/-} organoids. Quantifications are shown as median with quartiles. Statistics: unpaired t tests with Welch's correction. **c, d** Log2(tpm+1)-graphs show temporal expression of *ISLET1* and *ISLET2* in WT and *RPGRIP1L*^{-/-} organoids. Data are shown as mean ± SEM. **e** qPCR analysis of *ISLET1* in WT and *RPGRIP1L*^{-/-} organoids at days 4 and 14. Statistics: Kruskal-Wallis with Dunn's multiple comparison test. **f** IF analysis of MNs in WT and *RPGRIP1L*^{-/-} organoids at day 14. Quantifications are shown as median with quartiles. Statistics: unpaired t tests with Welch's correction (p=0.0125). **g** Quantification of the percentage of NKX2.2-positive and NKX2.2/OLIG2 double-positive cells per organoid section for WT and RPGRIP1L-deficient organoids at days 9 and 11. Statistics: Chi-square tests. **h, i** *GLII* and *PTCH1* qPCR analyses in WT and *RPGRIP1L*^{-/-} organoids. Data are shown as mean ± SEM. Statistics: Mann-Whitney tests at each time point. **j** IF analysis of ciliary GPR161 amount in WT and *RPGRIP1L*^{-/-} day 6 organoids treated either with SAG or DMSO. Quantifications are shown as median with quartiles. Statistics: Kruskal-Wallis with Dunn's multiple comparison test. **a-j** N: number of independent experiments, n: number of different WT or KO clones per experiment. For a, b, f, g and j, 2 WT and 2 KO clones from each iPSC line) (n=4 for each). For c and d, 2 WT and 1 KO clones from each line (n=4 WT and n=2 KO). For e, h, and i, 2 WT and 2 KO clones from each line (n=4 for each) at day 4; 2 WT and 2 KO PCli033-A clones and 1 WT and 1 KO UCSFi001-A clones (n=3 for each) at day 9 and day 14. N=3 for a, b, f; N=2 for g; N=1 for c, d, e, h, i, j. Scale bars: 100 μm in a, b, f; 2.5 μm in j.

Guide	Sequence + PAM	Crispr Score	Off Target
RPGRIP1L_exon3	ACGGACAATGAAGTCTCGCC AGG	92	0-0-0-8-38
RPGRIP1L_exon27	TATGCTTAGTTAGACGTGAA AGG	96	0-0-1-5-61

Cell line	Origin	# clone	Genotype	Method	Mutation
PCli033-A	PHENOCELL (PCI)	F6	+/-	KO	+ / Δ ex3-ex27
PCli033-A	PHENOCELL (PCI)	C5	-/-	KO	Δ ex3-x27 / Δ ex3-ex27



Supplementary Figure 4: Phenotyping spinal organoids from full-deletion RPGRIP1L hiPSCs. a

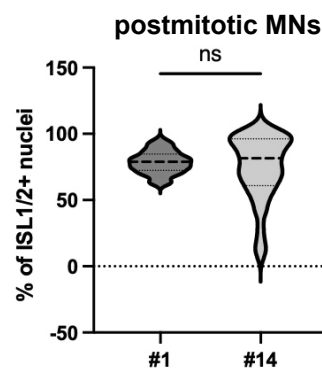
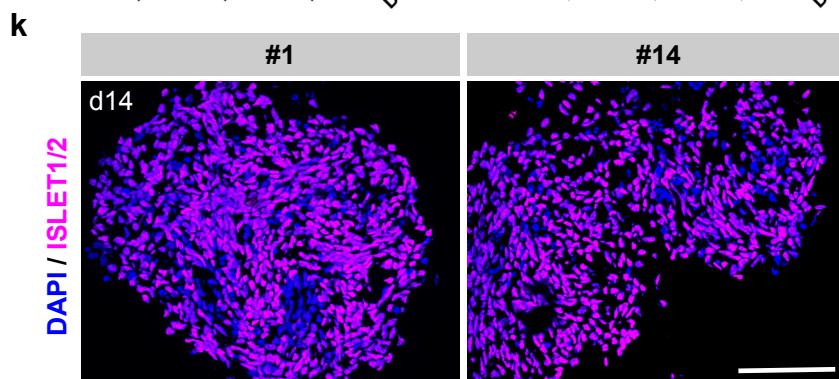
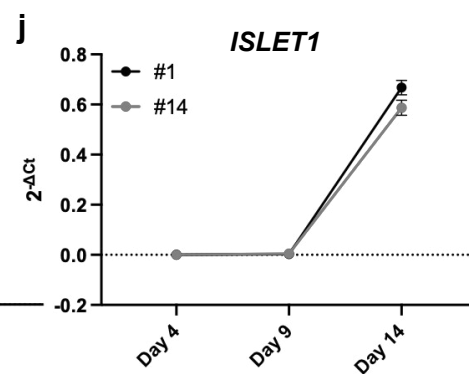
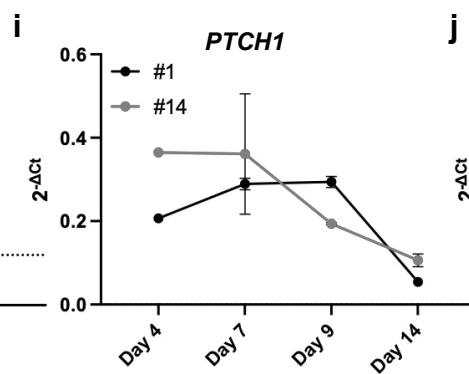
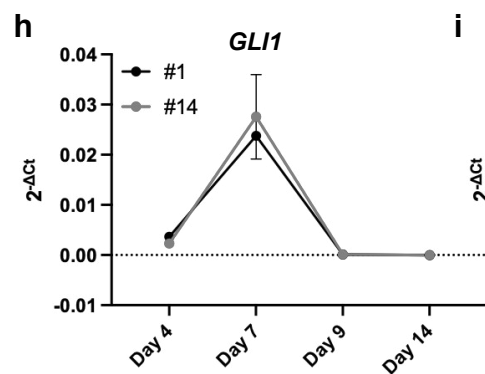
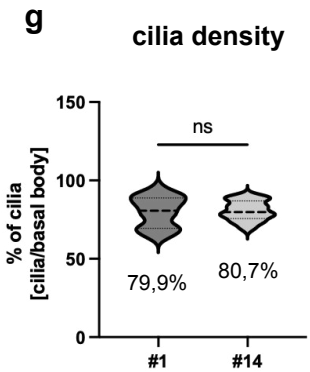
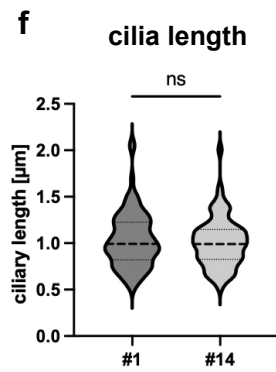
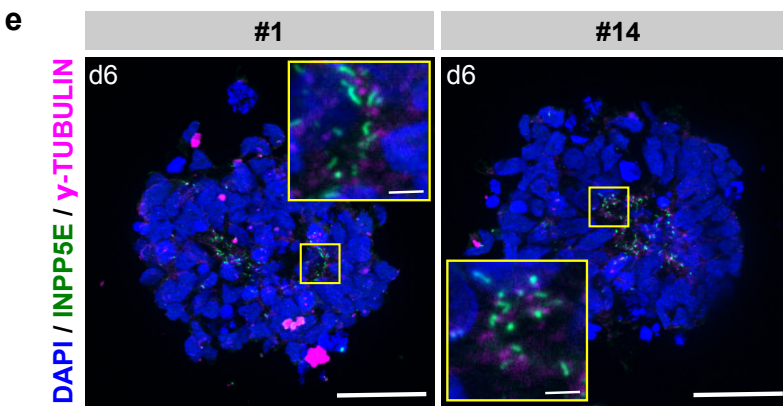
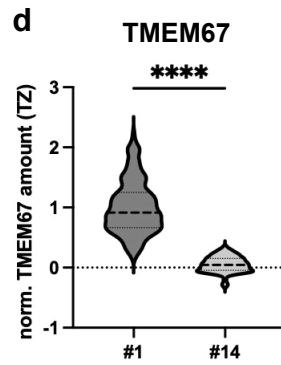
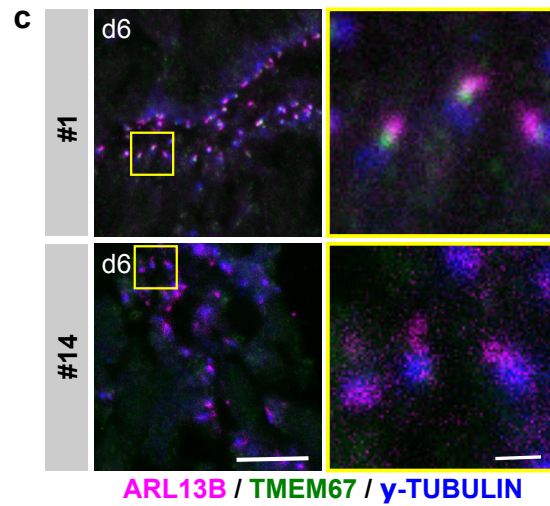
Sequences of the CRISPR-guides in RPGRIP1L exons 3 and 27. The CRISPR Scores are CFD specificity scores based on the CFD off-target model. Off-targets for each number of mismatches are indicated, one of which is in the 12 bp adjacent to the PAM. **b** The hiPSC line of origin, clone number, genotype and mutation are indicated for each clone. **c** Immunofluorescence for RPGRIP1L in *RPGRIP1L*^{+/+} and *RPGRIP1L*^{-/-} day 6 organoids. **d** Quantification of ciliary RPGRIP1L amounts in WT, *RPGRIP1L*^{+/+} and *RPGRIP1L*^{-/-} spinal organoids at day 6. WT is described in Supplementary Fig. 2. Data are shown as median with quartiles. Statistics: Kruskal-Wallis with Dunn's multiple comparison test ($p < 0.0001$). **e, f** Quantifications of ciliary length and density in *RPGRIP1L*^{+/+} and *RPGRIP1L*^{-/-} day 6 organoids. Data are shown as median with quartiles. Statistics: Mann-Whitney test. **g, h** *GLII* and *PTCH1* qPCR analyses in WT and *RPGRIP1L*^{-/-} organoids. Statistics: Mann-Whitney tests at each time point. **i** qPCR analysis of *ISLET1* in WT and *RPGRIP1L*^{-/-} organoids at day 4 and day 14. Statistics: Kruskal-Wallis and Dunn's multiple comparison test ($p=0.0268$). **j** Immunofluorescence analysis of MNs (ISLET1/2) in *RPGRIP1L*^{+/+} and *RPGRIP1L*^{-/-} organoids. **k** Quantifications of relative ISLET1/2 positive area in WT, *RPGRIP1L*^{+/+} and *RPGRIP1L*^{-/-} organoids. Data are shown as mean $\pm$ SEM. Statistics: Kruskal Wallis with Dunn's multiple comparison test. **l, m** Immunofluorescence analysis of HOXA5, HOXC8 and HOXA7 in WT (m), *RPGRIP1L*^{+/+} (l) and *RPGRIP1L*^{-/-} (l, m) day 14 organoids. Quantifications are shown as median with quartiles. Statistics: unpaired t tests with Welch's correction (l: ** $p=0.0089$; **** $p < 0.0001$) (m: $p < 0.0001$). **n, o** qPCR analyses of *HOXA7* and *HOXC8* in WT and *RPGRIP1L*^{-/-} organoids at days 4 and 14. Statistics: Kruskal-Wallis and Dunn's multiple comparison test. **p** Immunofluorescence analysis of PHOX2B in *RPGRIP1L*^{+/+} and *RPGRIP1L*^{-/-} day 14 organoids. Quantifications are shown as median with quartiles. Statistics: unpaired t tests with Welch's correction ($p < 0.0001$). **d-p** N: number of independent experiments; n: number of different clones analyzed per experiment. For d, k and m, 2 WT clones from each line ($n=4$). For WT, $N=4$ in d and $N=1$ in k and m. $N=3$ for F6 and C5. For g, h, i, n and o, 2 WT clones from each line at day 4 ($n=4$) and 2 PCli033-A and 1 UCSFi001-A WT clones ($n=3$) at day 9 and day 14. C5: $N=2$. In e, f, k, l and p, $N=3$ for F6 and C5. Scale bars: 2.5 μm in c and 250 μm in j, l, m, p.

a

Guide	Sequence + PAM	Crispr Score	Off Target
TMEM67_exon1	GGTTAGAGACCGGTTAACCC TGG	97	0-0-0-1-21
TMEM67_exon27	ATCTCGGCGCTCACTAGCAG TGG	94	0-0-0-0-29

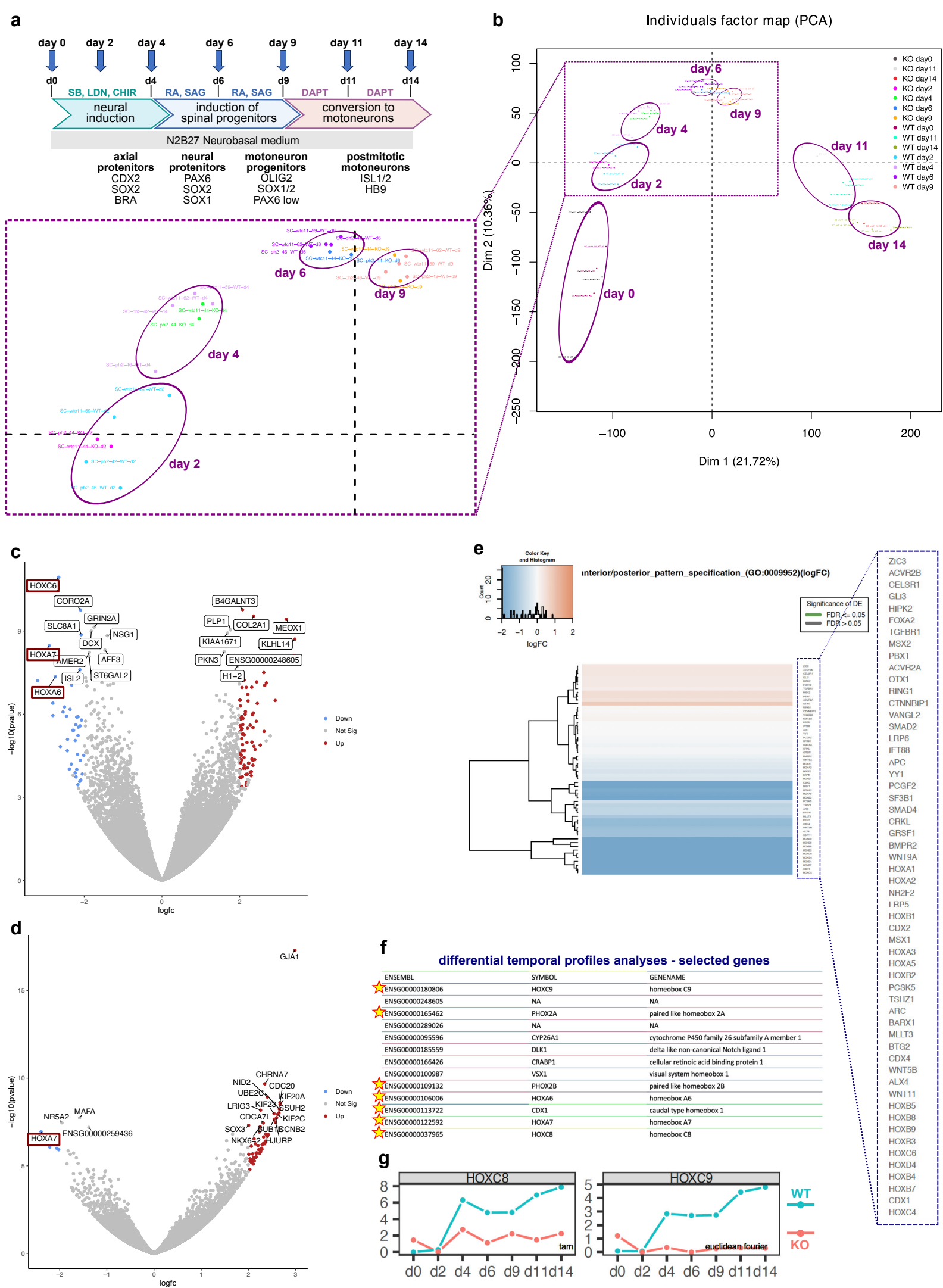
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Cell line	Origin	# clone	Genotype	Method	Mutation
PCli033-A	PHENOCELL (PCI)	#1	+/-	KO	+ / Δ ex1-ex27
PCli033-A	PHENOCELL (PCI)	#14	-/-	KO	Δ ex1-x27 / Δ ex1-ex27

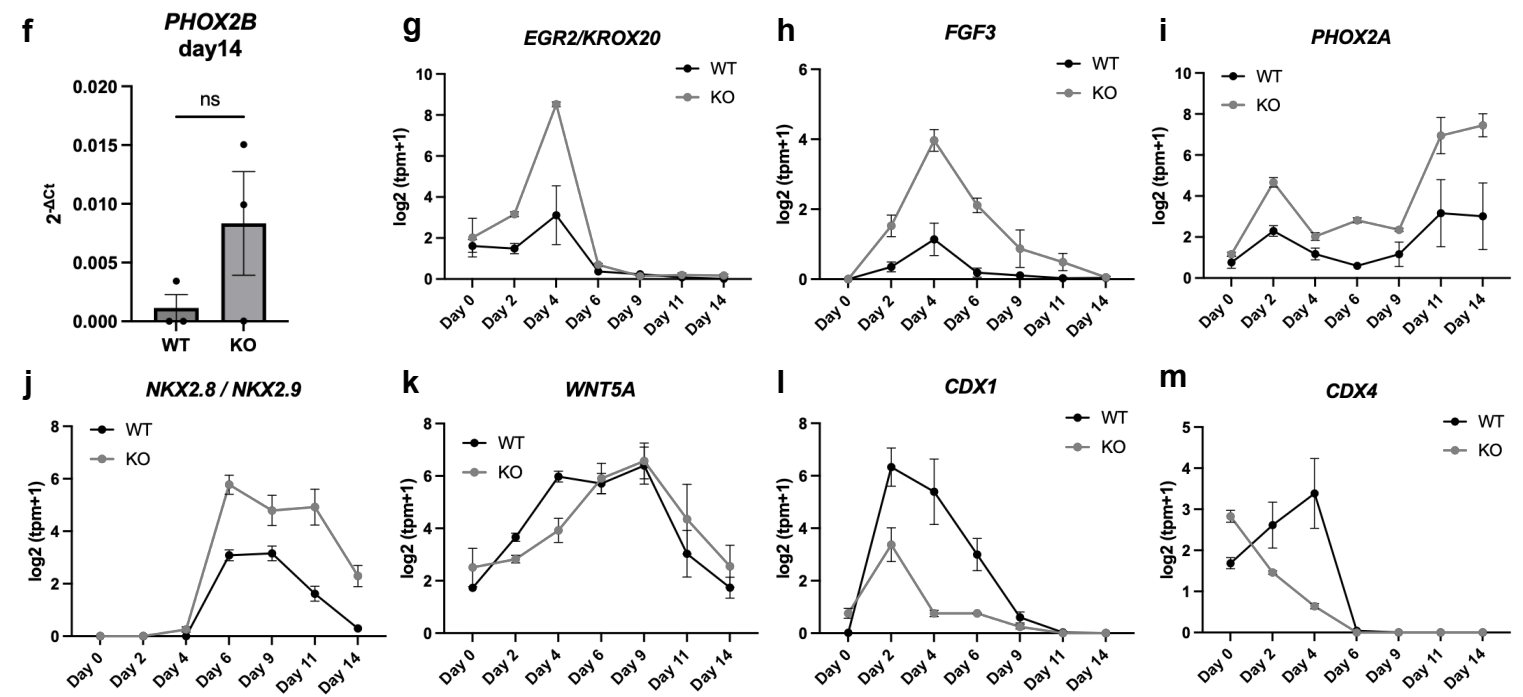
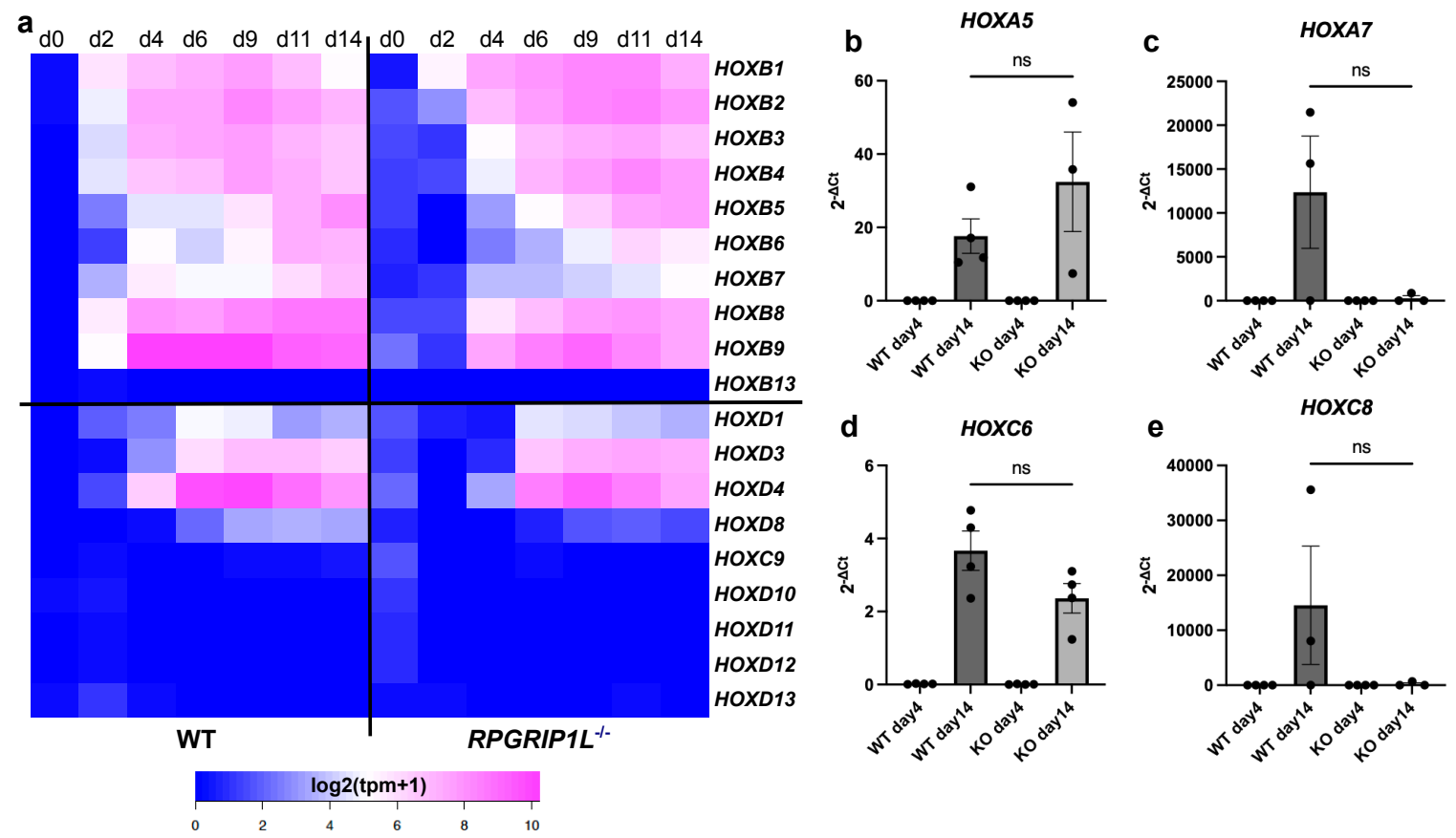


Supplementary Figure 5: Spinal MN differentiation of TMEM67-deficient hiPSC lines. a

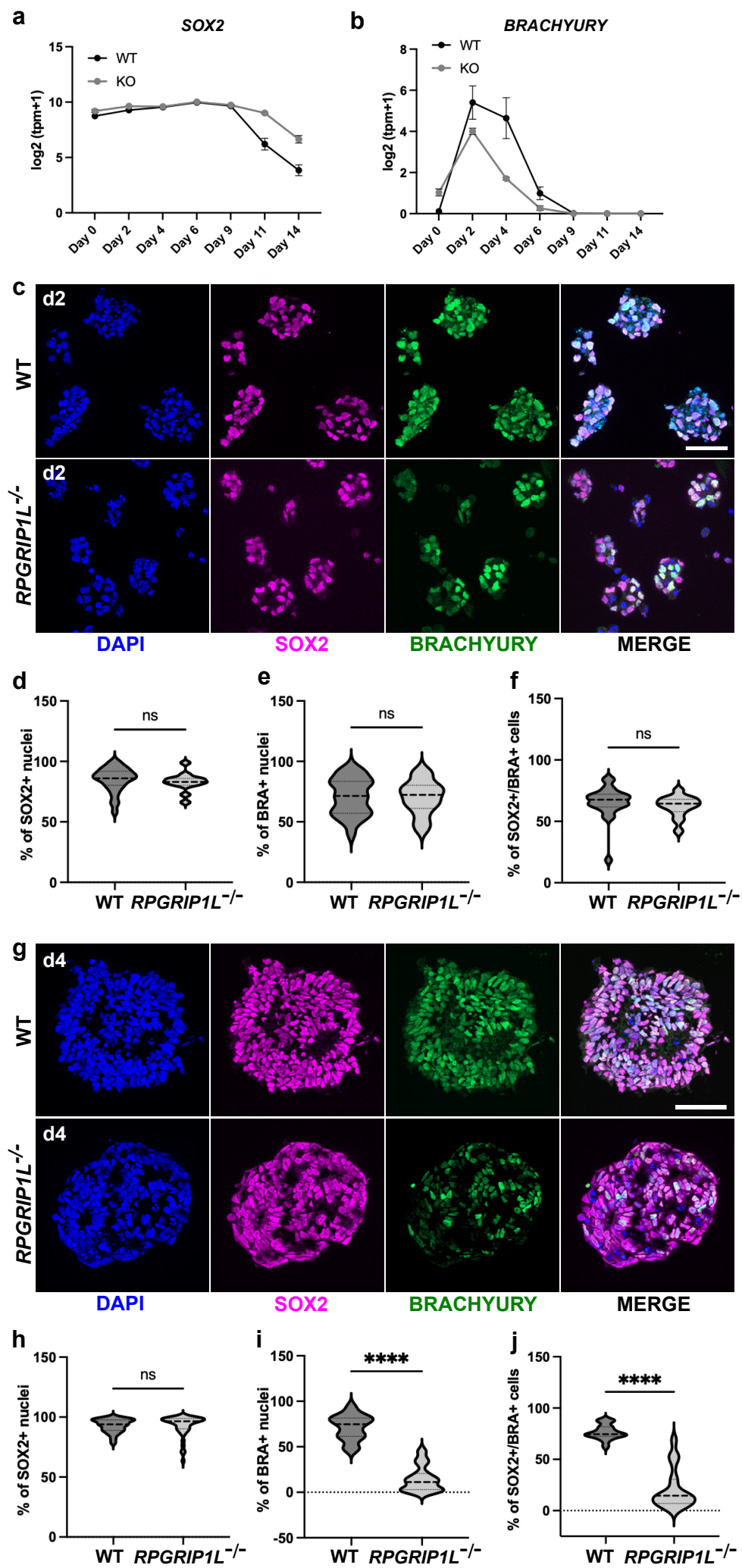
Sequences of the CRISPR-guides in exons 1 and 27 used for the generation of a full-deletion hiPSC line. The CRISPR Score is the CFD specificity score based on the CFD off-target model. The Off Target field indicates the number of off-targets for each number of mismatches, none of which are in the 12 bp adjacent to the PAM. **b** List of hiPSC lines used. The iPSC line of origin, clone number, genotype and specific mutation are indicated for each clone. **c** Immunofluorescence for TMEM67 in *TMEM67^{+/-}* and *TMEM67^{-/-}* day 6 organoids. Cilia are labeled by ARL13B and basal bodies by γ -TUBULIN. TMEM67 is labeled in green. **d** Quantification of the ciliary TMEM67 amount in *TMEM67^{+/-}* and *TMEM67^{-/-}* spinal organoids at day 6. Data are shown as median with quartiles. Asterisks denote statistical significance according to unpaired t tests with Welch's correction ($P < 0.0001$). **e** Immunofluorescence staining of primary cilia in *TMEM67^{+/-}* and *TMEM67^{-/-}* spinal organoids. Cilia are labeled by INPP5E and basal bodies by γ -TUBULIN. **f, g** Quantification of cilia length and density in *TMEM67^{+/-}* and *TMEM67^{-/-}* spinal organoids at day 6. Statistics: unpaired t tests with Welch's correction. **h, i, j** qPCR analyses of *GLII*, *PTCH1* and *ISLET1* gene expression profiles in *TMEM67^{+/-}* and *TMEM67^{-/-}* spinal organoids over time. Data are presented as mean $\pm$ SEM. Statistics: Mann-Whitney tests. **k** Immunofluorescence analysis of MNs in *TMEM67^{+/-}* and *TMEM67^{-/-}* day 14 organoids. Quantifications are shown as median with quartiles. Statistics: unpaired t test with Welch's correction. **c-k** N: number of independent experimental replicates. N=3 for #1 and #14 in c, f, g, h-k. Scale bars: 10 μ m in c, 50 μ m in e and 150 μ m in k. For magnified images (yellow squares): 1 μ m in c, 2 μ m in e.



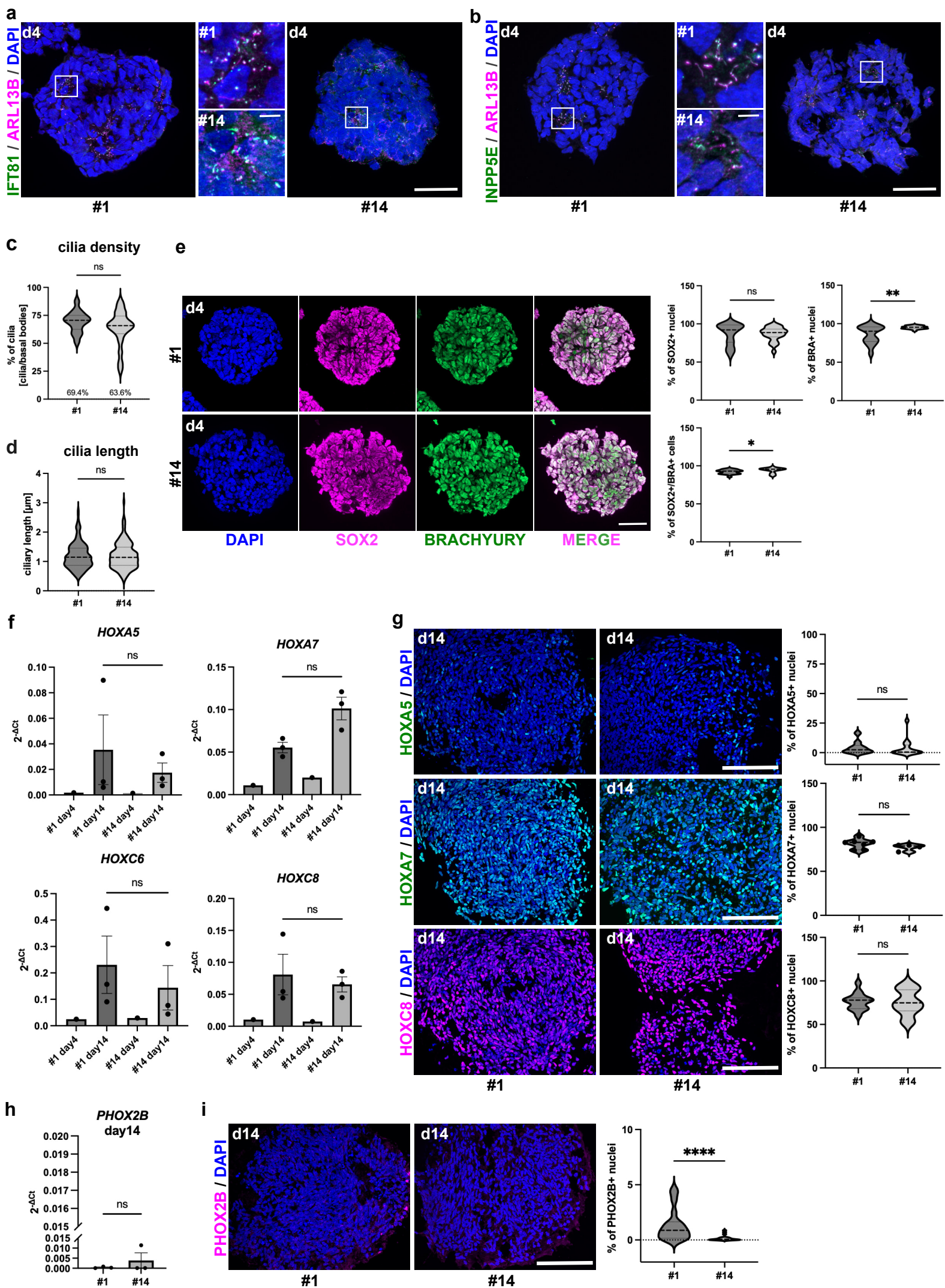
Supplementary Figure 6: Bulk RNAseq analysis indicates altered antero-posterior patterning in RPGRIP1L-deficient spinal organoids. **a** Schematic summary of the human spinal 3D differentiation approach. Samples for bulk RNAseq analyses were collected at indicated time points. The clones were generated from two independent iPCS lines, PCIi033-1 and UCSFi001-A. 2 WT clones and 1 KO clone from each line were analyzed (WT: n=4, KO: n=2). **b** PCA analyses performed on WT and KO samples show the variation of data over time. The zoom-in on the left shows that WT and KO samples cluster closely together at single time points. **c, d** Volcano plots of RNAseq data on day 11 (c) and day 14 (d). The threshold for significance was set to 0.01 and the LogFC threshold to 2. **e** EGSEA at day 2 of spinal differentiation shows downregulated *anterior/posterior_pattern_specification* (GO:0009952) in RPGRIP1L-deficient organoids. Genes that correspond to this GO term are depicted on the right. **f** List of genes with different temporal expression profiles based on euclidean distance measurements between WT and *RPGRIP1L* KO organoids over the entire time course of differentiation. Asterisks indicate genes that are related to antero-posterior patterning. **g** Examples of genes with different temporal expression profiles. *HOXC8* was captured via time alignment measurement (tam) and *HOXC9* via euclidean measurement.



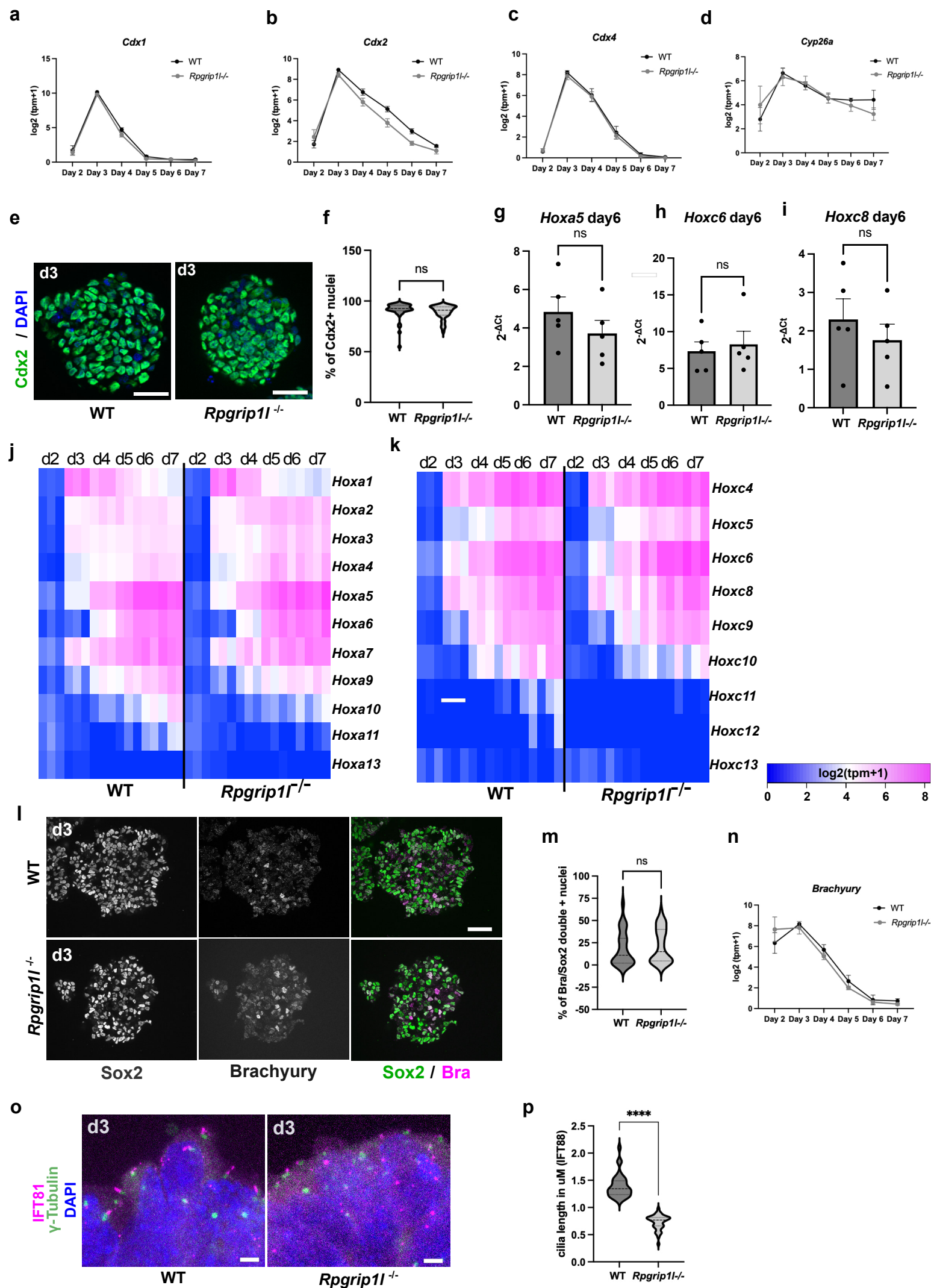
Supplementary Figure 7: Antero-posterior patterning defects in RPGRIP1L-deficient spinal organoids. **a** Heatmap showing *HOXB* and *HOXD* gene expressions in WT and RPGRIP1L-deficient spinal organoids over time. The graph was generated from $\log_2(\text{tpm}+1)$ files of bulk RNASeq analysis. **b-e** qPCR analyses of *HOXA5*, *HOXA7*, *HOXC6* and *HOXC8* in WT and RPGRIP1L-deficient organoids at day 4 and day 14. Kruskal-Wallis and Dunn's multiple comparison tests were performed for statistical analyses. **f** qPCR analysis of *PHOX2B* in WT and RPGRIP1L-deficient organoids at day 14. Unpaired t test with Welch's correction was performed for statistical analyses. **g-m** $\log_2(\text{tpm}+1)$ graphs show expression profiles of *EGR2/KROX20* (g) *FGF3* (h), *PHOX2A* (i), *NKX2.8/NKX2.9* (j), *WNT5A* (k), *CDX1* (l) and *CDX4* (m) over time. Data are shown as mean $\pm$ SEM. **a-m** N=1 number of experimental replicates per clone. n: number of different WT or KO clones analyzed per experiment. For a and g-m, 2 WT clones and 1 KO clone from each line (n=4 for WT and n=2 for KO). For b-e, 2 WT clones and 2 KO clones of each iPSC line at day 4 (n=4 for each genotype), and 2 WT and 2 KO clones in the PCLi033-A background and 2 WT and 1 KO clone in the UCSFi001-A background (n=4 for WT and n=3 for KO) at day 14. For f, 2 WT and 2 KO clones in the PCLi033-A background and 1 WT and 1 KO clone in the UCSFi001-A background (n=3 for each genotype).



Supplementary Figure 8: hiPSCs adopt an NMP-like axial fate during early stages of ventral spinal differentiation. **a, b** Log2(tpm+1) graphs generated from bulk RNASeq analyses show the expression profiles of *SOX2* and *BRACHYURY* over time. Data are shown as mean $\pm$ SEM. **c, g** Immunofluorescence of SOX2 and BRACHYURY in WT and RPGRIP1L-deficient spinal organoids at day 2 and day 4. Scale bars: 150 μ m. **d-f, h-j** Quantifications show the percentage of SOX2, BRACHYURY and SOX2/BRACHYURY positive nuclei per organoid at day 2 and day 4. Data are shown as median with quartiles. Statistics: unpaired t tests with Welch's correction ($p < 0.0001$). **a-j** n: number of different WT or KO clones analyzed per experiment. For a and b, 2 WT clones and 1 KO clone from each line (n=4 for WT and n=3 for KO). For d-f and h-j, 2 WT clones and 2 KO clones from each iPSC line (n=4 for each genotype). For d-f, number of independent experimental replicates N=1 and for h-i, number of independent experimental replicates N=3.

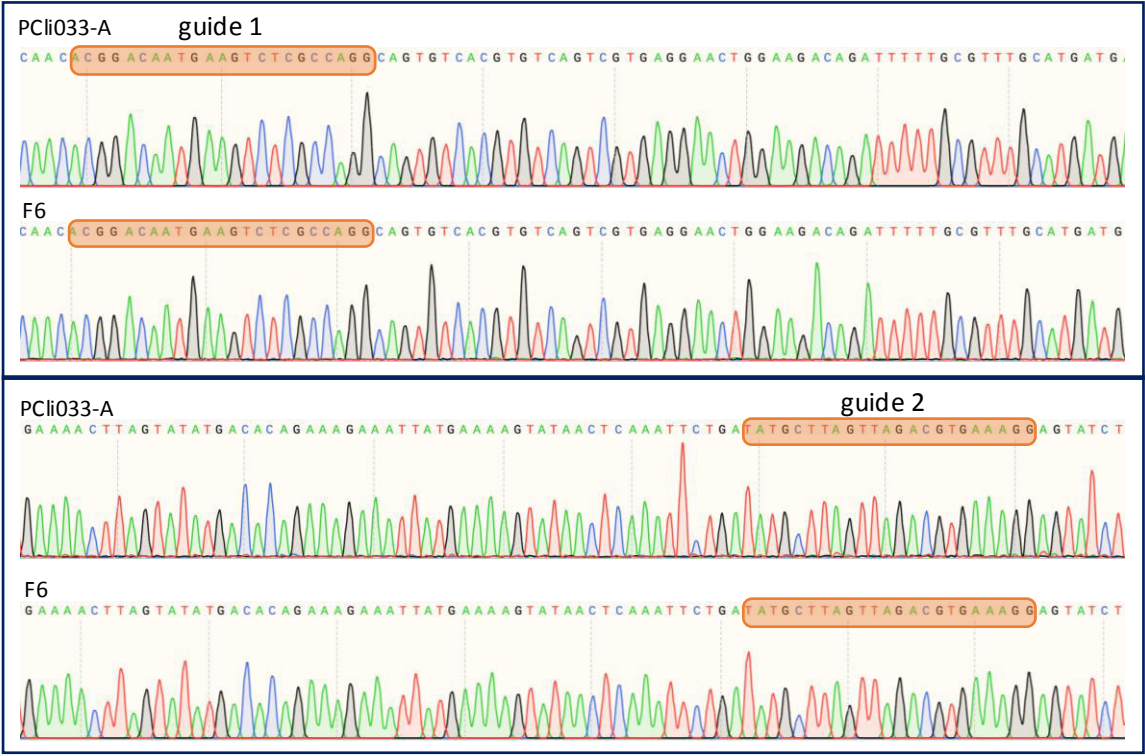


Supplementary Figure 9: TMEM67-deficient spinal organoids display unaltered axial progenitor specification and correct antero-posterior patterning. **a, b** Immunofluorescence of cilia in *TMEM67*^{+/-} and *TMEM67*^{-/-} day 4 organoids. Cilia are labeled by IFT81 and ARL13B (a) or by INPP5E and ARL13B (b). **c, d** Quantification of cilia density and ciliary length in *TMEM67*^{+/-} and *TMEM67*^{-/-} day 4 organoids. Data are shown as median with quartiles. Statistics: unpaired t test with Welch's correction (c) and Mann-Whitney test (d). **e** Immunofluorescence of SOX2 and BRACHYURY in *TMEM67*^{+/-} and *TMEM67*^{-/-} spinal organoids at day 4. Data are shown as median with quartiles. Statistics: unpaired t tests with Welch's correction (*p=0.0424, **p=0.0057). **f** qPCR analyses of *HOXA5*, *HOXA7*, *HOXC6* and *HOXC8* in *TMEM67*^{+/-} and *TMEM67*^{-/-} organoids at day 4 and day 14. Statistics: Mann-Whitney tests. **g** Immunofluorescence of *HOXA5*, *HOXA7* and *HOXC8* in *TMEM67*^{+/-} and *TMEM67*^{-/-} spinal organoids at day 14. Quantifications show the percentages of *HOXA5*, *HOXA7* and *HOXC8* positive nuclei per organoid. Data are shown as median with quartiles. Statistics: unpaired t test with Welch's correction (*HOXC8*) and Mann-Whitney tests (*HOXA5*, *HOXA7*). **h** qPCR analysis of *PHOX2B* in *TMEM67*^{+/-} and *TMEM67*^{-/-} spinal organoids at day 14. Statistics: Mann-Whitney test. **i** Immunofluorescence of *PHOX2B* in *TMEM67*^{+/-} and *TMEM67*^{-/-} spinal organoids at day 14. Quantifications show the percentage of *PHOX2B* positive nuclei per organoid. Data are shown as median with quartiles. Statistics: unpaired t tests with Welch's correction (p < 0.0001). **a-i** N: number of independent experimental replicates. N=3 for both clones in c, d, e, g, h and i; N=3 for both clones in f at day 14 and N=1 for both clones in f at day 4. Scale bars: 50 μm in a, b, e; 150 μm in g, i. For magnified areas (white squares): Scale bars: 5 μm in a, b.

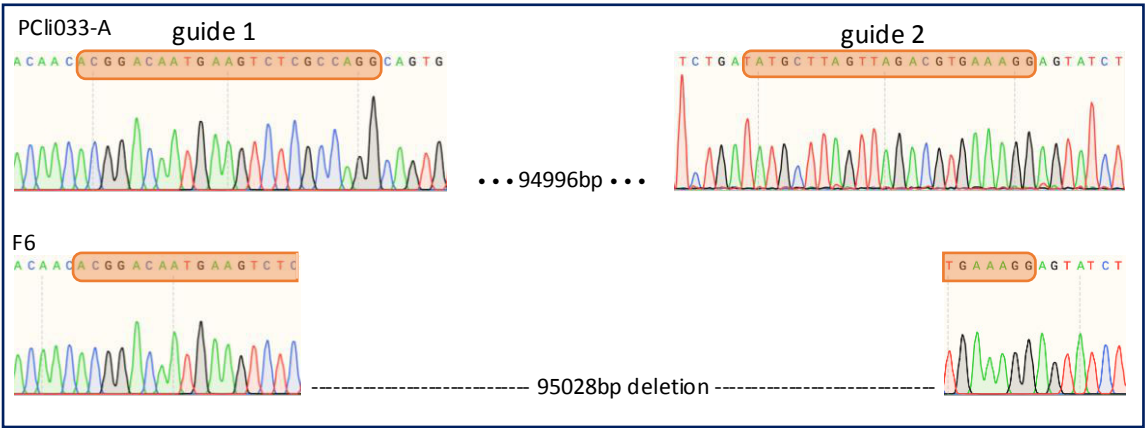


Supplementary Figure 10: Mouse *Rpgrip11*-deficient spinal organoids do not display spinal to cranial specification defects. a-d, n Temporal analysis of *Cdx1*, *Cdx2*, *Cdx4* (spinal identity), *Cyp26a1* (cranial identity) and *Brachyury* (NMPs) expression in the course of the differentiation of WT and *Rpgrip11*^{-/-} organoids. Log₂(tpm+1) data from bulk RNASeq analysis are displayed as mean ± SEM (N=3 for each genotype). **e** Immunofluorescence for Cdx2 on sections from WT and *Rpgrip11*^{-/-} organoids at day 3. **f** Percentage of nuclei positive for Cdx2 in WT and *Rpgrip11*^{-/-} day 3 organoids. Data are shown as violin plots, median quartiles are indicated as dotted lines. Statistics: two-sided unpaired t test with Welch's correction. N=3 independent experiments, n=1 clone per genotype. **g-i** qPCR analyses of indicated *Hox* genes from WT and *Rpgrip11* KO organoids at day 6. Data are displayed as mean ± SEM. Statistics: two-sided Mann-Whitney test. N=5 independent experiments; n=1 clone per genotype. **j, k** Heatmap depicting the expression log₂(tpm+1) of selected *Hox* genes in WT and *Rpgrip11* KO spinal organoids over time. **l** Immunofluorescence of Sox2 and Brachyury in WT and *Rpgrip11*^{-/-} organoids at day 3. **m** Percentage of nuclei double positive for Sox2 and Brachyury in WT and *Rpgrip11*^{-/-} day 3 organoids. Data are shown as violin plots, median quartiles are indicated as dotted lines. Statistics: two-sided unpaired t test with Welch's correction. N=2 independent experiments; n=1 clone per genotype. **o** Immunofluorescence for the indicated ciliary markers on sections of day 3 WT and *Rpgrip11*^{-/-} organoids. **p** Quantifications of ciliary staining. Data shown are mean ± SEM. Statistics: unpaired t tests with Welch's correction (P < 0.0001). N=2 independent experiments. Scale bars, 10 μm in e and 1.2 μm in o.

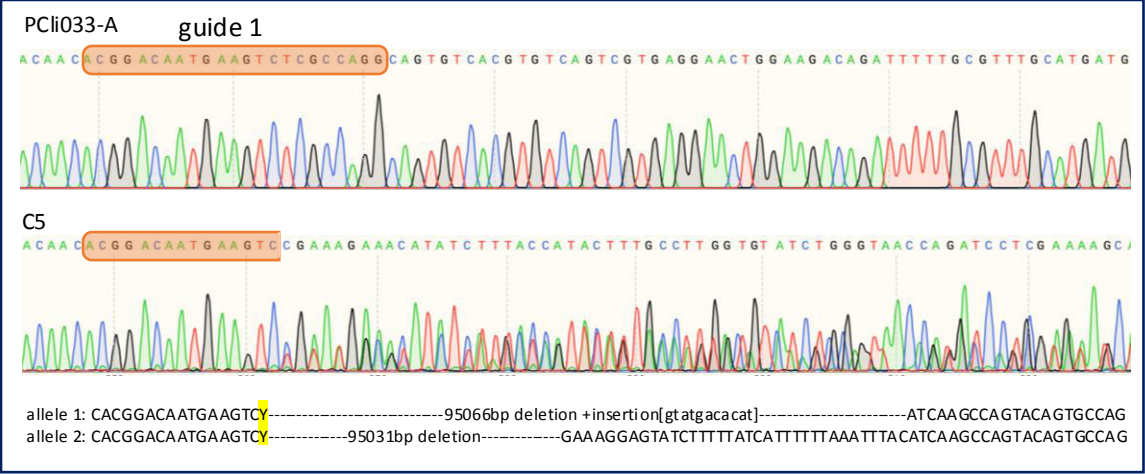
a RPGRIP1L_clone F6_allele1_no modification



b RPGRIP1L_clone F6_allele2_deletion

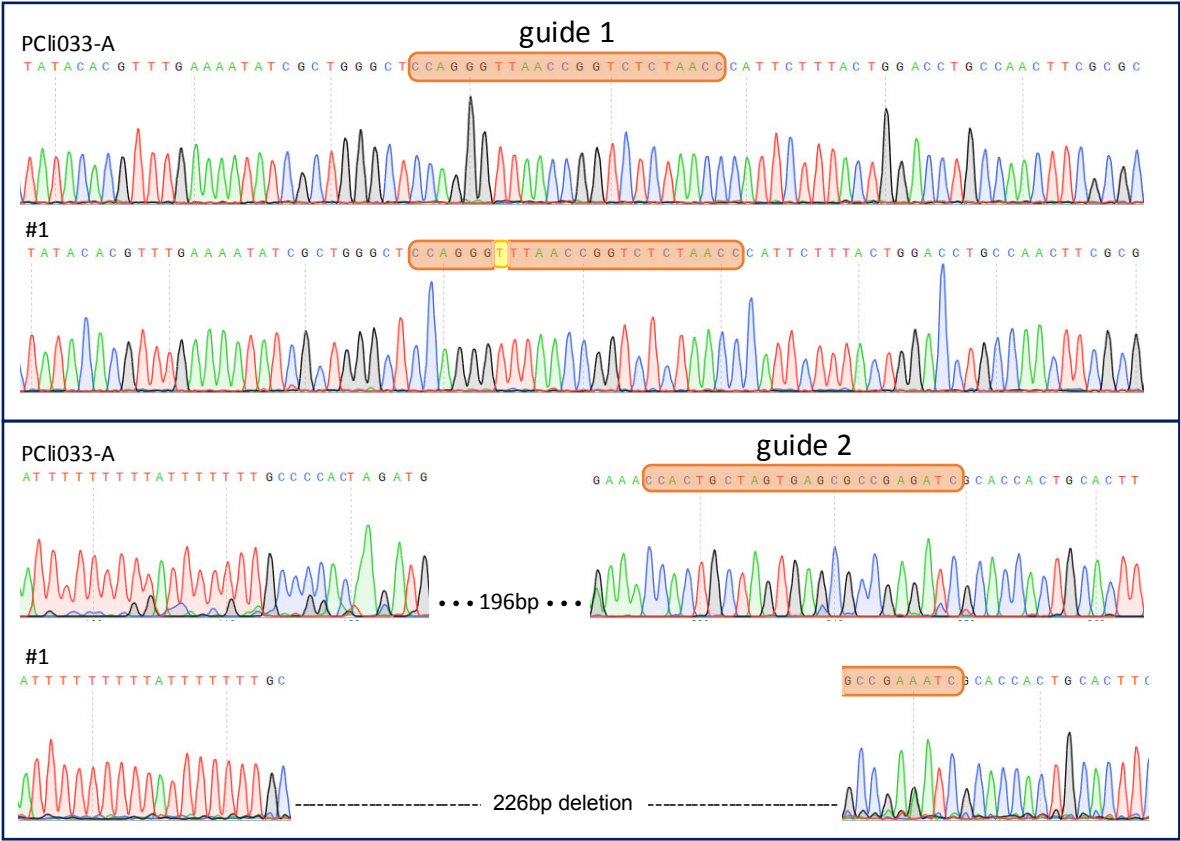


c RPGRIP1L_clone C5_allele1+2_deletion

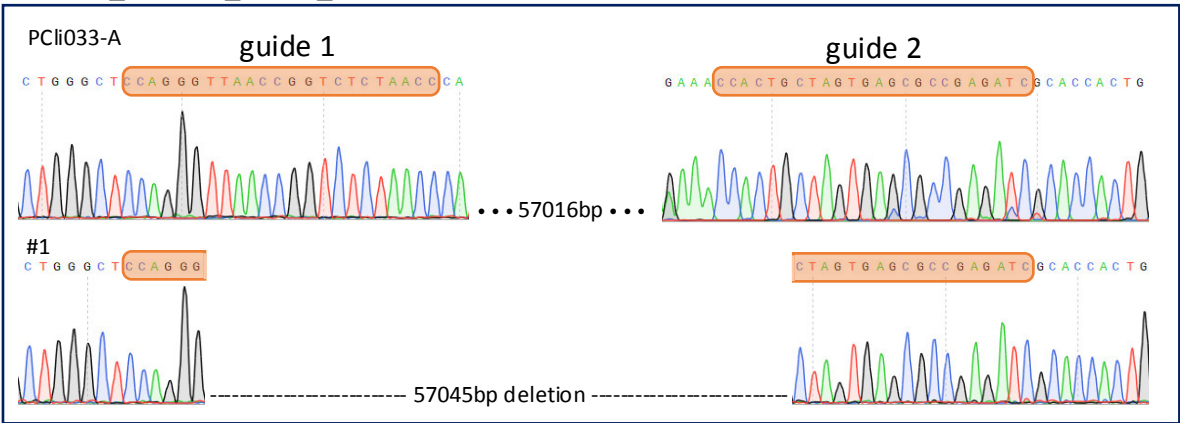


Supplementary Figure S11: Sequencing chromatograms for the full-deletion RPGRIP1L hiPSC clones. a-c Sequencing chromatograms for clone F6 (HET) and C5 (KO) compared to the PCLi033-A control sequence. CRISPR-guides targeting exon 3 (guide 1) and exon 27 (guide 2) are highlighted in orange. **a** The WT allele of clone F6 shows no modification. **b** The mutant allele of clone F6 shows a full deletion of 95028 bps. **c** The KO clone C5 harbors big deletions on both alleles. On one allele 95031 bps are deleted, on the other allele 95066 bps were deleted with a random insertion of 11 bps.

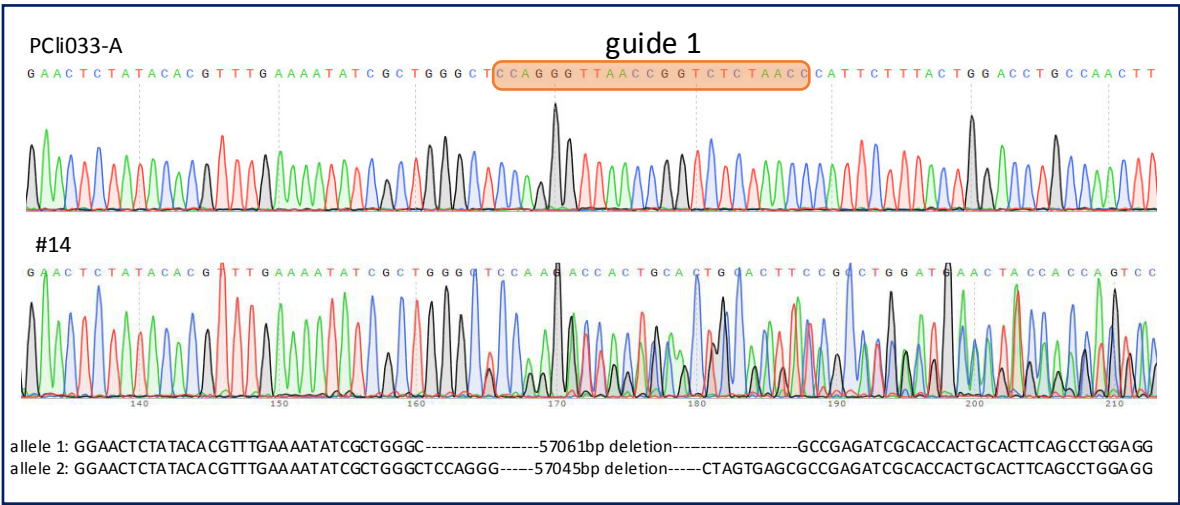
a TMEM67_clone #1_allele1_InDels in non-coding regions



b TMEM67_clone #1_allele2_deletion



c TMEM67_clone #14_allele1+2_deletions



Supplementary Figure S12: Sequencing chromatograms for the full-deletion TMEM67 hiPSC clones. a-c Sequencing chromatograms for clone #1 (HET) and #14 (KO) compared to the PCli033-A control sequence. CRISPR-guides targeting the 5'UTR (guide 1) and intron 27 (guide 2) are highlighted in orange. **a** The WT allele of clone #1 shows an 1bp insertion (highlighted in yellow) in a non-coding region of the 5'UTR and a deletion of 226 bp in a non-coding region in intron 27. **b** The mutant allele of clone #1 shows a deletion of 57045 bps. **c** The KO clone #14 harbors big deletions on both alleles. On one allele 75045 bps are deleted, on the other allele 75061 bps.

qPCR			
Gene	species	Forward	Reverse
<i>GLI1</i>	human	CCCAGTACATGCTGGTGGTT	GCTTTACTGCAGCCCTCGT
<i>PTCH1</i>	human	GATGGGGCCATCTCCACATT	CGCCGCAAAGAAGTACCTTACA
<i>ISLET1</i>	human	TGTTTGAAATGTGCGGAGTG	GCATTTGATCCCGTACAACC
<i>HOXA5</i>	human	GGAGATCATAGTTCCGTGAGC	GCTGAGATCCATGCATTGT
<i>HOXA7</i>	human	CCCTGGATGCGGTCTTCA	CCTTCGTCCTTATGCTCTTTCT
<i>HOXC6</i>	human	ACAGACCTCAATCGCTCAGG	GTACCGCGAGTAGATCTGGC
<i>HOXC8</i>	human	CCTCCGCCAACACTAACAGT	CAAGGTCTGATACCGGCTGT
<i>PHOX2B</i>	human	AACCCGATAAGGACCACTTTTG	AGAGTTTGTAAGGAAGTGC
<i>GAPDH</i>	human	CAACGGATTTGGTCGTATTGG	GCAACAATATCCACTTTACCAGAGTTA
<i>Gli1</i>	mouse	TTATGGAGCAGCCAGAGAGA	GAGCCCGCTTCTTTGTTAAT
<i>Ptch1</i>	mouse	TGACAAAGCCGACTACATGC	AGCGTACTCGATGGGCTCT
<i>Hoxa5</i>	mouse	CAGATCTACCCCTGGATGCG	GTCTGGTAGCGAGTGTAGGC
<i>Hoxc6</i>	mouse	ACACAGACCTCAATCGCTCAG	CGAGTTAGGTAGCGGTTGAAG
<i>Hoxc8</i>	mouse	CGTCTCCCAGCCTCATGTTT	CGGCGCTTTCTGGTCAAATA
<i>Olig2</i>	mouse	TCCCCAGAACCCGATGATCTT	CGTGGACGAGGACACAGTC
<i>Dbx1</i>	mouse	CAACAGACCCACCAC	AGGAGCTGGCACTCT
<i>Tbp</i>	mouse	AGAACAATCCAGACTAGCAGC	GGGAACCTCACATCACAGCTC
Sequencing			
Gene_region	on/off-target	Forward	Reverse
RPGRIP1L exon3	on-target	TTTTATCCTCCTGCTTGCGG	AAGAACGGCACTTGAATACATTT
RPGRIP1L exon27	on-target	TGTAATGGTGACCCAGGGATGT	AGAAGGAACATAGCCTGTTTGC
TMEM67 5'UTR	on-target	TGGGTTTTAAGTCAGTGGGGT	GTTGTTGTCGCACTTCTCCG
TMEM67 intron27	on-target	TGAAGTGGGACACAGTGGAAG	ATCCATGTTTATTGGCAGTGG
NPRL2 exon8	off-target	CAAGTCCCTGCAAGAGGCAT	CTCTGCTCTTCCCAGATCAC

Supplementary Table 1: List of qPCR and sequencing primers.

primary Antibody	host	dilution IF	dilution WB	company	reference
Ac3	rabbit	1:500	/	Abcam	ab125093
Arl13b	mouse IgG2a	1:400	/	Antibody Incorporated	75-287
Brachyury	rabbit	1:500	/	Cell Signaling/Ozyme	81694
Brachyury	goat	1:500	/	R&D systems	AF2085
Cdx2	rabbit	1:1000	/	Abcam	ab76541
Gpr161	rabbit	1:200	/	Proteintech	13398-1-AP
Hoxa5	rabbit	1:300	/	Sigma	HPA029319
Hoxa7-CoraLite®	mouse IgG1	1:250	/	Proteintech	CL488-67112
Hoxc8	rabbit	1:300	/	Sigma	HPA028911
Ift81	rabbit	1:200	/	Proteintech	11744-1-AP
Ift88	rabbit	1:300	/	Proteintech	13967-1-AP
Inpp5e	rabbit	1:300	/	Proteintech	17797-1-AP
Islet1/2	mouse IgG2b	1:50	/	DSHB	39.4D5
Nkx2.2	mouse IgG2b	1:50	/	DSHB	74.5A5
Nkx6.1	mouse IgG1	1:50	/	DSHB	F55A12
Olig2	rabbit	1:200	/	Millipore	AB9610
Pax6	rabbit	1:200	/	BioLegend	PRB-278P
Pax6	mouse IgG1	1:50	/	DSHB	PAX6
FoxA2	mouse IgG1	1:50	/	DSHB	4C7
Phox2b	rabbit	1:500	/	kindly provided by JF Brunet	/
RPGRIP1L	rabbit	1:300	/	homemade anti-human	against human RID-domain (Uniprot:A0A087WX34)
Rpgrip11	rabbit	1:300	1:1000	homemade anti-mouse	against mouse RID-domain (PMID:26150391)
Sox2	rabbit	1:200	/	Millipore	AB5603
Sox2	mouse	1:200	/	Abcam	ab79351
Tmem67	rabbit	1:200	/	Proteintech	13975-1-AP
Tuj1	mouse IgG2a	1:300	/	BioLegend	MMS-435P
γ -Tubulin	mouse IgG1	1:200	/	Sigma	T6557
Actin	mouse IgG2a	/	1:2000	Sigma	A4700
secondary Antibody	host	dilution IF	dilution WB	conjugate	company
Mouse IgG1	goat	1:400	/	Alexa488	Molecular Probes
Mouse IgG1	goat	1:400	/	Alexa633	Molecular Probes
Mouse IgG2a	goat	1:400	/	Alexa488	Molecular Probes
Mouse IgG2a	goat	1:400	/	Alexa594	Molecular Probes
Mouse IgG2a	goat	1:400	/	Alexa633	Molecular Probes
Mouse IgG2b	goat	1:400	/	Alexa488	Molecular Probes
Mouse IgG2b	goat	1:400	/	Alexa594	Molecular Probes
Mouse IgG2b	goat	1:400	/	Alexa633	Molecular Probes
Rabbit	goat	1:400	/	Alexa488	Molecular Probes
Rabbit	goat	1:400	/	Alexa594	Molecular Probes
Rabbit	goat	1:400	/	Alexa633	Molecular Probes
HRP anti mouse	goat	/	1:5000	Jackson Immuno	115-035-003
HRP anti rabbit	goat	/	1:5000	Jackson Immuno	111-035-003

Table S2: List of primary and secondary antibodies.

DESEQ DATA	day 0		day 2		day 4		day 6		day 9		day 11		day 14	
Gene	FoldCh ange	adj. p	FoldCh ange	adj. p	FoldCh ange	adj. p	FoldCh ange	adj. p	FoldCh ange	adj. p	FoldCh ange	adj. p	FoldCh ange	adj. p
DBX2	- 0.18694 1274465 912	0.99999 275578 2949	- 0.03711 7431320 5926	NA	1.41884 1998405 11	0.999843 5049474 57	0.09250 6575149 0877	0.52047 2144065 023	0.00798 0855643 73704	NA	0.11079 0002483 988	0.98435 638032 2919	- 0.07514 5212275 4046	NA
OLIG2	- 0.31580 6948843 472	0.99999 275578 2949	0.03539 9612488 1901	NA	0.68151 4889354 778	0.999843 5049474 57	0.71078 9498123 939	0.06426 1810082 3034	0.40666 8408138 64	0.90942 565282 5697	0.31602 3578776 84	0.98435 638032 2919	0.64332 3797608 997	0.671402 6303798
NKX6.1	0.92682 2337069 594	0.99999 275578 2949	0.00734 2893645 58429	0.99992 8945743 403	0.70524 5466655 836	0.425437 0586971 56	0.59606 0127469 382	0.00878 6766298 31874	0.17543 4500469 697	0.93482 339580 8711	0.22205 2033077 543	0.98435 638032 2919	1.10416 6607081 51	0.064984 5505305 754
NKX2.2	0.09486 3909983 811	0.99999 275578 2949	0.05028 4433923 495	NA	2.41443 3317137 91	0.347702 1344074 33	0.03221 4806757 3956	0.01020 3922241 3872	0.99319 146825 0953	0.38419 6617234 371	0.98435 638032 2919	0.98435 638032 2919	0.63506 7717328 612	0.709330 1397292 74
TUJ1 TUBB3	0.13741 9404338 622	0.99999 275578 2949	0.24148 3869308 178	0.99992 8945743 403	0.03176 8350272 2379	0.999843 5049474 57	0.52563 1334403 643	0.25749 6529147 253	0.27369 4825533 952	0.90942 565282 5697	0.09733 8082115 3323	0.98435 638032 2919	0.06776 2369833 1345	0.957692 9385902 44
MAP2	- 0.20577 5069959 076	0.99999 275578 2949	0.10777 1137786 243	0.99992 8945743 403	1.34152 2439698 27	0.888767 6441911 76	0.19880 2426775 524	0.68529 3011358 499	0.32225 3307368 812	0.87644 195964 6297	0.43552 0269274 038	0.98435 638032 2919	0.35161 3473045 83	0.636772 7476425 11
GLI1	0.10734 8391158 355	0.99999 275578 2949	0.06570 6174128 6489	0.99992 8945743 403	0.08844 5754450 264	0.999843 5049474 57	0.30702 2477791 36	0.24018 4014366 194	0.07003 8796270 4957	0.96414 451877 9646	0.75650 9009289 376	0.98435 638032 2919	1.25234 8031070 15	0.170444 8623686 04
PTCH1	0.45158 4059635 72	0.99999 275578 2949	0.31613 6534051 952	0.99992 8945743 403	0.61093 9002622 102	0.526213 1886297 42	0.13994 9286836 129	0.75572 7717404 031	0.22359 1429465 98	0.90942 565282 5697	0.55581 2858617 782	0.98435 638032 2919	0.69715 2660458 381	0.208639 1937522 2
HOXA5	0.12563 1104181 62	NA	0.50323 9015795 25	0.99992 8945743 403	2.46522 1108747 07	0.051631 0734416 811	1.22052 5745387 96	5.34932 9138672 71e-05	0.65919 2644229 813	0.51243 154792 4915	0.55639 8599108 139	0.98435 638032 2919	1.14892 5431497 02	0.220367 1137368 23
HOXA7	0.11572 7942300 252	NA	0.10143 8888914 404	0.58960 3710550 248	5.41237 1207152 77	0.000289 6397824 85042	1.10057 4523686 97	0.00513 9334729 16255	0.33518 8007070 526	0.90942 565282 5697	0.75791 6994373 097	0.98435 638032 2919	2.39220 2259970 12	0.000113 9018007 44846
HOXC4	0.30985 0083082 869	NA	0.78116 6324093 198	0.28923 9960890 192	2.84180 8726882 52	0.018029 6345543 299	0.39257 7233337 781	0.39206 7033039 252	0.22940 8453740 531	0.90942 565282 5697	0.30279 9164750 074	0.98435 638032 2919	0.41544 0681607 327	0.496103 8358627 32
HOXC8	0.13425 1027070 905	NA	0.16194 1762212 616	0.25701 1344792 264	3.42079 4670120 08	0.045813 9095374 785	0.59225 2430538 549	0.13501 1324244 442	0.21490 2101465 428	0.90942 565282 5697	0.41477 4824152 828	NA	0.91796 6761409 756	0.438594 6620681 28
PHOX2B	0.03373 3340295 3211	0.99999 275578 2949	0.03487 8524297 4024	NA	2.85559 0416550 1	0.846306 4561798 9	0.04615 4160768 9378	NA	0.01725 4293991 7648	NA	0.09459 3019958 8227	0.98435 638032 2919	0.11642 4616601 268	NA
TBX20	0.12466 9956005 961	0.99999 275578 2949	0.01871 9786221 9268	0.78799 6701551 123	0.42073 2376159 818	0.781136 9367186 22	0.03421 2272794 0005	0.62013 9992890 314	0.04526 6876524 2352	NA	0.11061 2880906 62	NA	0.18341 5733869 837	NA
CDX2	0.24860 7089675 479	NA	1.29943 4265251 91	0.00107 5791285 65606	2.24652 4280060 32	0.083222 5387857 653	0.35572 6588106 836	0.54157 0770017 432	0.34525 3143672 659	0.90942 565282 5697	0.59664 2769504 026	0.98435 638032 2919	0.61463 0377234 082	0.708967 9919308 06
ISLET1	0.94884 1702088 542	0.99999 275578 2949	0.00547 6490562 72241	0.96121 3551665 762	0.03440 6701036 8778	0.993616 7932417 23	0.05468 5921817 5328	0.72095 7207140 202	0.68148 2383121 564	0.36912 714792 612	0.29873 0236430 02	0.98435 638032 2919	1.33284 0267076 56	0.027631 2324956 031
ISLET2	- 0.20701 5658263 226	0.99999 275578 2949	- 0.00185 0980432 27171	0.98974 8530403 228	1.44770 0663338 46	0.703986 6492101 52	0.06154 6918569 8836	0.76630 0687081 257	0.33073 3479275 233	0.90942 565282 5697	0.48811 3893192 837	0.98435 638032 2919	0.12422 8083384 201	0.946707 9819959 83
EGR2 KROX20	0.29647 7052907 205	0.99999 275578 2949	1.06272 4802479 36	0.04866 5900156 5123	2.91915 5416861 5	NA	0.24564 3938619 576	0.29367 7834028 938	0.10455 0590356 454	NA	0.10889 2152049 011	0.98435 638032 2919	0.39081 3013872 423	NA
FGF3	NA	NA	0.55642 3648752 666	0.73570 2481996 449	3.09182 4701617 46	0.717950 8045515 41	0.80612 2850752 378	0.01296 6617955 9297	0.27362 5730917 9	NA	0.58554 7517413 279	0.98435 638032 2919	0.04283 7323715 8474	NA
PHOX2A	- 0.03807 6996560 9032	0.99999 275578 2949	1.50603 0307200 89	9.46439 3932042 05e-05	1.10018 2940109 39	0.973364 0918162 74	1.62209 6511516 49	3.16195 9134622 27e-06	0.06612 6860601 9388	0.95408 728830 1341	0.10096 3222325 493	0.98435 638032 2919	0.20727 7613695 511	NA
NKX2.9 NKX2.8	NA	NA	NA	NA	4.03729 5113001 33	NA	0.11809 0880194 09	NA	0.14014 7176942 807	0.93556 287278 4137	0.30652 9304682 024	0.98435 638032 2919	0.25407 5335345 996	0.906275 9676638 14
WNT5A	0.78711 3881184 649	NA	- 0.68425	0.41630 0398219 02	- 1.98916	0.001813 9443686	0.31478 8358362 476	0.60681 3448913 638	0.07881 1557587 6228	0.96400 422826 1934	0.37760 8669084 226	0.98435 638032 2919	1.24760 8379611 97	0.165005 1812220 7

			6853388 062		8226581 27										
CDX1	0.45940 4032345 429	0.99999 275578 2949	- 0.29104 2959350 702	0.99992 8945743 403	- 7.30679 3380275 81	0.001813 9443686 9744	- 0.91161 9632746 876	0.02231 0174598 7616	- 0.15895 6667613 704	NA	- 0.02718 9003471 6007	0.98435 638032 2919	NA	NA	
CDX4	0.91966 2900406 647	0.99999 275578 2949	- 0.55126 4535430 092	0.99992 8945743 403	- 4.93232 0037771	0.031231 2419994 349	- 0.02128 5734227 0105	NA	NA	NA	NA	NA	NA	NA	
SOX2	0.26602 2598665 522	0.99999 275578 2949	1.00974 3961220 85	0.07188 1371451 3949	0.00686 4941096 60109	0.999843 5049474 57	0.02911 7164874 9504	0.95256 2519878 802	0.06792 7804310 804	0.96234 670386 8404	0.94514 7320877 804	0.98435 638032 2919	1.81470 6217781 32	0.006212 7654211 9315	
BRA/TBXT	0.86616 0290851 194	0.99999 275578 2949	- 0.30583 3451368 79	0.99992 8945743 403	- 4.23651 6046024 46	0.059753 6628456 613	0.29577 3791704 23	0.48884 9938833 062	- 0.00726 0121803 5299	NA	NA	NA	NA	NA	

Table S3: Selected DESeq analyses of bulk RNAseq data. FoldChanges and adjusted p-values for gene expressions presented as log(tpm+1) plots in the manuscript are shown for each analysis time point. DESeq2 implements a two-sided Wald test of the Log2FC values. Adjustment of p-values for multiple testing (p-adj) was performed using the Benjamini and Hochberg procedure.