Supplemental Table S1: sg RNA sequences for targeting

| **Specific guide RNA** | **5' - 3'** |
|---|---|
| *VSX2*.sgRNA | GTCAAGGCGCGCTCAGATGC |
| *BRN3b*.sgRNA | AAGAGTCTTCTAAATGCCGG |
| *RCVRN*.sgRNA | AGGGAGGACAGCTGAACAGT |

Supplemental Table S2: Primers used for Gibson Assembly to make the HDR template

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| VSX2.5'HA | ATTGGGTACCGGGCCTCCTGTGAGAACAGTGTG | CCGCTTCCGTCGACCAAAGCCATGTCCTCCAGC |
| VSX2.3'HA | ATACGAAGTTATTAGGTGTAGGTCAAGGCGCGCTCA | CTCCACCGCGGTGGCGCCAGATTGGGTTGTTCAAGG |
| RCVRN.5'HA | CTATAGGGCGAATTGGGTACTGCCTTCCCCGCCAGGTC | GTCGACCTCGAGGGGGGGCCTGGCGTTCTTCATCTTTTCCTTCACTTTTTG |
| RCVRN.3'HA | ATACGAAGTTATTAGGTGTGAACACACATGCACACA | CTCCACCGCGGTGGCCAAAAGCTTATTCATCGGG |
| BRN3b.5'HA | GGCGAATTGGAGCTCCACCGCGGTGGCCGCCGAGGCTCTGGCAGC | ATACAGCACAGCATAGGTCCAGGGTTCTCCTCCACG |
| BRN3b.3'HA | CCACTAGTTCTAGAAATAGAAGACTCTTGGCCTCTCC | TTGATATCGAATTCCTGCAGCCCGGGGTGCATCGGTCATGCTTCC |

Supplemental Table S3: Primers used for CRISPR off-target screening

| Off-Target Screening VSX2-sgRNA | | | | |
|---|---|---|---|---|
| **Name** | **Gene** | **Sequence** | **PAM** | **Off-target Score*** |
| VSX2, Chr14 | | GTCAAGGCGCGCTCAGATGC | CGG | 100 |
| Chr19 non-gene sequence | | GTCAAGGCGTACTCAGATGC | GAG | 2.668116758 |
| Chr19 non-gene sequence | | GTGAAGAAGTGCTCAGATGC | CAG | 0.916843223 |
| Sytabulin, Chr8 | ENSG00000147642 | GTGAAGACACCCTCAGATGC | TGG | 0.349165048 |
| VEGF-A, Chr6 | ENSG00000112715 | GTCAAGGCGTGCTCCGATGG | GGG | 0.317986706 |
| KIF16B, Chr20 | ENSG00000089177 | GTCGAAGCGGGCTCCGATGC | AGG | 0.252128661 |
| STAT2, Chr12 | ENSG00000170581 | GTCAATGGGAGCTCTGATGC | AGG | 0.234357477 |

| Off-Target Screening BRN3b-sgRNA | | | | |
|---|---|---|---|---|
| **Name** | **Gene** | **Sequence** | **PAM** | **Off-target Score*** |
| BRN3b (POU4F2), Chr4 | ENSG00000151615 | AAGAGTCTTCTAAATGCCGG | CGG | 100 |
| RP11-1100L3.7, Chr12 | ENSG00000257663 | AGCAGTCTTCCAGATGCCGG | CAG | 0.371654759 |
| RP11-45M11.7, Ch6 | ENSG00000275846 | AAGCTCCTTCTAAATGCCAG | TAG | 0.351773802 |
| TC2N, Chr14 | ENSG00000165929 | TAAAGTCTTCTAAATGCCAA | TAG | 0.331943062 |
| FAM83F, Chr22 | ENSG00000133477 | AAGAGAATTGGAAATGCCGG | CAG | 0.302192873 |
| RP11-484K9.4, Chr3 | ENSG00000272844 | AAGACTCTTTGAAATGCCTG | CGG | 0.288247111 |
| RP11-321M21.1 , Chr18 | ENSG00000266774 | AATAGTCCTCCAAATGCTGG | CAG | 0.202171083 |

| Off-Target Screening RCVRN-sgRNA | | | | |
|---|---|---|---|---|
| **Name** | **Gene** | **Sequence** | **PAM** | **Off-target Score*** |
| Recoverin, Chr17 | ENSG00000109047 | AGGGAGGACAGCTGAACAGT | TGG | 100 |
| Chr4 non-gene sequence | | AGGGAGGCCAGCTGAAGAGT | GGG | 3.099576271 |
| Chr2 non-gene sequence | | GGAGAGGGCAGCTGAACAGT | TAG | 2.726928675 |
| Chr14 non-gene sequence | | AGAGAGATCAGCTGAACAGT | GGG | 1.740860136 |
| Chr17 non-gene sequence | | AGAAAGGACAGCTGAACTGT | AGG | 0.741790707 |
| Chr17 non-gene sequence | | AGTGAGGATAGCTGGACAGT | AGG | 0.541032634 |

* Off-target scores provided by Benchling.

Supplemental Table S4: Primers flanking the sgRNA cut site used to generate PCR fragments for sequencing

| Set of primers used to screen for off-target cutting efficiency of **VSX2.sgRNA** (*) symbol at the end indicates that this primer is good to use as probe for sequencing | | | |
|---|---|---|---|
| Gene | Forward (5'-3') | Reverse (5'-3') | Size |
| *Sytabulin* | GCACCGCATGGCTTCTCACC (*) | GGCCCCATCAAAATAAAACCATC | 1.2kb |
| *VEGFA* | TGTGGCGGCCTCCCTTCATCTG (*) | CCCGCTCGCTCGCTCGCTCAC | 887bp |
| *Kinesis* | GCCTGGCACCCTTGACATT | AGCAGGCAGAGCATCCCATCC (*) | 913bp |
| *Stat2* | TTGAGGGGCTGGAGAAAGATAAGT (*) | TGGGGAGCAGAGACAAATAGAGAA | 906bp |
| Chr19 | CACTGCCCACTACCCACTACTAAG (*) | CGGGAGCAATATGGGAAATGGTC | 941bp |

| Set of primers used to screen for off-target of cutting efficiency **RCVRN.sgRNA** (*) symbol at the end indicates that this primer is good to use as probe for sequencing | | | |
|---|---|---|---|
| Gene | Forward (5'-3') | Reverse (5'-3') | Size |
| chr4 | TGTTCCCGGCCATTTGTA (*) | ATCTTGCCAGCATCCATTATCT | 844bp |
| chr2 | AAGCCCACTGGAAAGGTATGAACT (*) | AATGGGAAGGGGACTGAACAAA | 833bp |
| chr14 | AGTTTACGGGAGGGAGGTCAGC (*) | TGGCAGGGAGAAACAGTAGAA | 596bp |
| chr17 | GGGTGGCGGCAGCTTGATAAA (*) | CCCCGAGGATAGCACTGTTGG | 497bp |
| chr17 | GAGCCCCCGGAAGCACAAATACAG (*) | GGCAGGCGTCTCCGTTCTCACAC | 648bp |

| Set of primers used to screen for off-target cutting efficiency of **BRN3b.sgRNA** (*) symbol at the end indicates that this primer is good to use as probe for sequencing | | | |
|---|---|---|---|
| Gene | Forward (5'-3') | Reverse (5'-3') | Size |
| 1100L3.7 | CTTCCCGGCACCAAATCACTCTAC (*) | GCCCCTCCCCTGCTTATCTGG | 1.0kb |
| 45M11.7 | ACCCCTTTTATTCGTGCTCTATTG (*) | AGTCCCGCGTCCTGCTCTC | 1.0kb |
| FAM83F | TGGCCTTTTGCTTTTTCACACC | CACCCCCGGCGTCCTTTACCTG (*) | 854bp |
| RP11-484K9 | CCGTAGGGGGCGAGGAACC (*) | GTGAAGGCGGAAATACAAACAGTC | 691bp |
| RP11-321M21 | GGGGCAAGCTTCTCCACTATTATC | GTTCCATCCTGCGGCTCTTC (*) | 931bp |

Supplemental Table S5: Primers for RT-qPCR

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| Oct4 | TGTACTCCTCGGTCCCTTTC | TCCAGGTTTTCTTTCCCTAGC |
| NANOG | CAGTCTGGACACTGGCTGAA | CTCGCTGATTAGGCTCCAAC |
| PAX6 | CGGAGTGAATCAGCTCGGTG | CCGCTTATACTGGGCTATTTTGC |
| SIX3 | CCGGAAGAGTTGTCCATGTT | CGACTCGTGTTTGTTGATGG |
| LHX2 | CAAGATCTCGGACCGCTACT | CCGTGGTCAGCATCTTGTTA |
| VSX2 | TCATGGCGGAGTATGGGCT | TCCAGCGACTTTTTGTGCATC |
| BRN3b | CTCGCTCGAAGCCTACTTTG | GACGCGCACCACGTTTTTC |
| RCVRN | CCAGAGCATCTACGCCAAGTT | CCGTCGAGGTTGGAATCGAAG |
| MITF | GACATGCGCTGGAACAAGGGAACC | CCGGGGGACACTGAGGAAAGGAG |
| BEST-1 | AACTGAGCCTACCACACAACA | CGGATTCGACCTCCAAGCC |
| GAPDH | CAATGACCCCTTCATTGACC | GACAAGCTTCCCGTTCTCAG |

Supplemental Table S6: Antibodies used for immunohistochemistry

| Antibodies | Supplier | Species | Type | Dilution | Reference |
|---|---|---|---|---|---|
| VSX2 | Millipore | Sheep | Polyclonal | 1:500 | ab9016 |
| BRN3 (labeled BRN3a, BRN3b, and BRN3c) | Santa Cruz | Goat | Polyclonal | 1:1000 | sc-6026X |
| GFP (also labeled Cerulean) | Abcam | Rabbit | Polyclonal | 1:4000 | ab6556 |
| MCM2 | Abcam | Rabbit | Polyclonal | 1:1000 | ab4461 |
| PROX-1 | Millipore | Rabbit | Polyclonal | 1:2000 | ab5475 |
| CRALBP | Abcam | Mouse | Monoclonal | 1:500 | ab15051 |
| AP2-α | DSHB | Mouse | Monoclonal | 1:35 | 3B5a |
| RCVRN | Millipore | Rabbit | Polyclonal | 1:500 | ab5585 |
| mCherry | Millipore | Chicken | Polyclonal | 1:500 | ab356481 |
| PAX6 | Santa Cruz | Mouse | Polyclonal | 1:100 | Sc-32766 |
| SIX3 | Santa Cruz | Mouse | Polyclonal | 1:100 | Sc-365519 |
| RX | Santa Cruz | Mouse | Polyclonal | 1:150 | Sc-271889 |
| Alexa Fluor 546 Donkey anti-Goat IgG (H+L) | Thermofisher | | | 1:500 | A11056 |
| Alexa Fluor 546 Donkey anti-Sheep IgG (H+L) | Thermofisher | | | 1:500 | A20198 |
| Alexa Fluor 546 Donkey anti-Rabbit IgG (H+L) | Thermofisher | | | 1:500 | A10040 |
| Alexa Fluor 546 Donkey anti-Mouse IgG (H+L) | Thermofisher | | | 1:500 | A10036 |
| Alexa Fluor 488 Donkey anti-Sheep IgG (H+L) | Thermofisher | | | 1:500 | A11015 |
| Alexa Fluor 488 Donkey anti-Rabbit IgG (H+L) | Thermofisher | | | 1:500 | A32790 |
| Alexa Fluor 546 Goat anti-Chicken IgG (H+L) | Thermofisher | | | 1:500 | A11040 |
| Alexa Fluor 488 Donkey anti-Rabbit IgG (H+L) | Thermofisher | | | 1:500 | A32790 |
| Alexa Fluor 488 Goat anti-Rabbit IgG (H+L) | Thermofisher | | | 1:500 | A32731 |

Supplemental Table S7: RT-qPCR primers for FACS sorted cells

| **Gene** | **Forward (5'- 3')** | **Reverse (5' - 3')** |
|---|---|---|
| Cerulean | AAGCTGACCCTGAAGTTCATCTGC | CTTGTAGTTGCCGTCGTCCTTGAA |
| VSX2 | TCATGGCGGAGTATGGGCT | TCCAGCGACTTTTTGTGCATC |
| mCherry | GATAACATGGCCATCATCAAGGA | CGTGGCCGTTCACGGAG |
| RCVRN | CCAGAGCATCTACGCCAAGTT | CCGTCGAGGTTGGAATCGAAG |
| eGFP | GACCAAAAGATCATGGTGAGC | GAACTTCAGGGTCAGCTTGC |
| BRN3b | CTCGCTCGAAGCCTACTTTG | GACGCGCACCACGTTTTTC |
| GAPDH | CAATGACCCCTTCATTGACC | GACAAGCTTCCCGTTCTCAG |

Supplemental Table S8: Primers used for genotyping in figures 1 and 2

| Location | Forward (5'-3') | Reverse (5'-3') | Size |
|---|---|---|---|
| Outside *VSX2* 5'HA to Cerulean | CCAAGTGGAGGAAGCGGGAGAAGT (FW1) | CGGCGGCGGTCACGAAC (RV1) | 2053bp |
| Puro to outside *VSX2* 3'HA | GCGTTGGCTACCCGTGAT (FW2) | GCCCCAGCTCCTTATTCC (RV2) | 1870bp |
| Outside *BRN3b* 5'HA to eGFP | TATTCGGCGGGCTGGATGAGAGTC (FW3) | GCCGTCGCCGATGGGGGTGTT (RV3) | 1673bp |
| Blas to outside *BRN3b* 3'HA | TCGACTAGAGCTTGCGGAACC (FW4) | AACCAGGCCATATACAGAACTCAA (RV4) | 1528bp |
| Outside *RCVRN* 5'HA to mCherry | AGCTTTGTTGAGCACCGACT(FW5) | GTTCTCCTCCACGTCTCCAG (RV5) | 1167bp |
| Neo to outside *RCVRN* 3'HA | TCGCCTTCTTGACGAGTTCT (FW6) | TGGATCTGGTCCTCTCCATC (RV6) | 1493bp |
| Outisde *VSX2* 5'HA to outside *VSX2* 3'HA | CCAAGTGGAGGAAGCGGGAGAAGT (FW1) | GCCCCAGCTCCTTATTCC (RV2) | 2627bp |
| Outisde *BRN3b* 5'HA to outside *BRN3b* 3'HA | TATTCGGCGGGCTGGATGAGAGTC (FW3) | AACCAGGCCATATACAGAACTCAA (RV4) | 2438bp |
| Outisde RCVRN 5'HA to outside *RCVRN* 3'HA | AGCTTTGTTGAGCACCGACT(FW5) | TGGATCTGGTCCTCTCCATC (RV6) | 2221bp |

**Supplemental Table S9: Description of real-time fluorescent imaging parameters in figure 3**

Zeiss LSM 710 Laser Scanning Confocal System
Image size 1024 pixel -1024 pixel

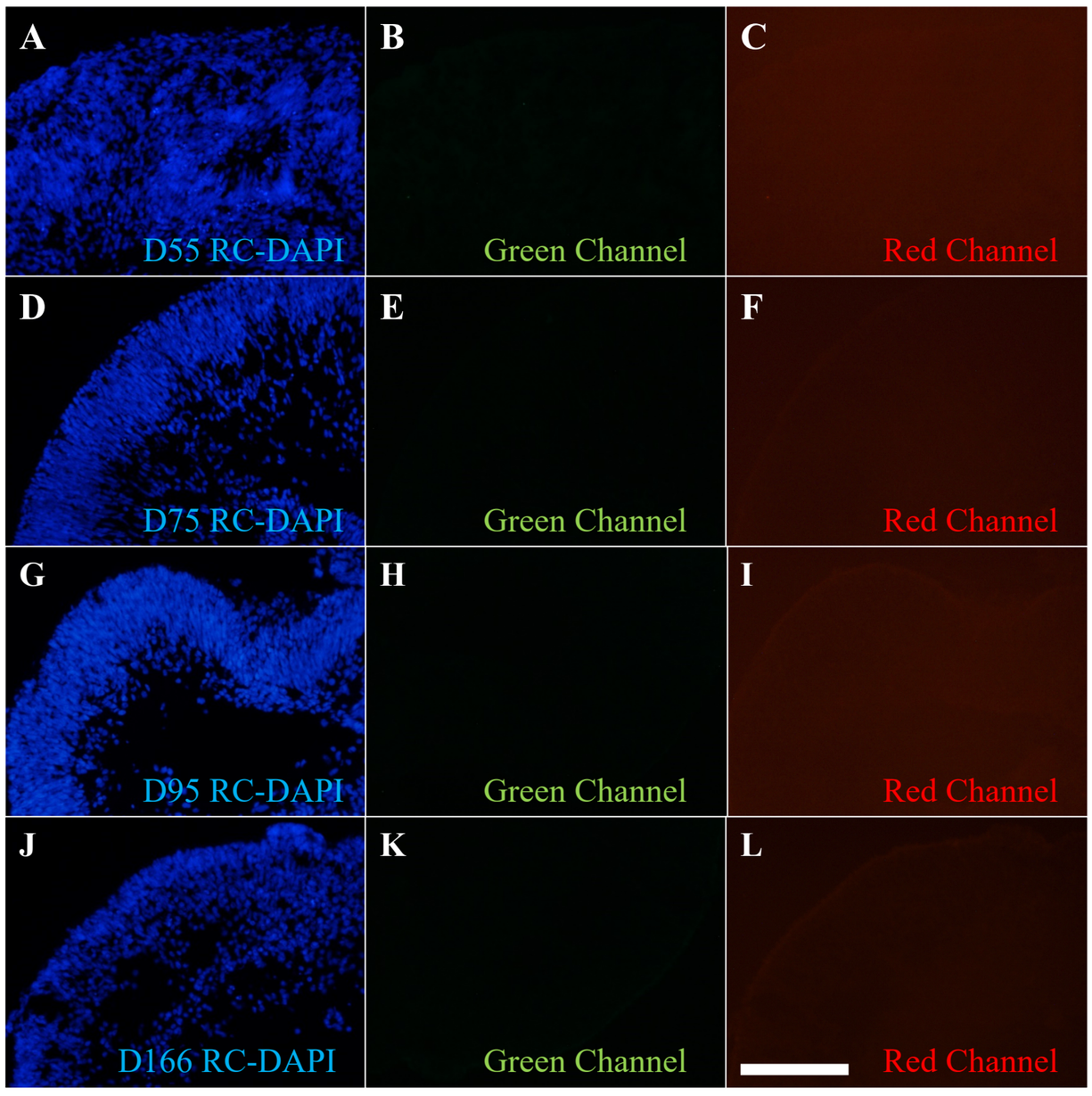
| | Cerulean | GFP | mCherry |
|---|---|---|---|
| Laser wavelength | 458 nm | 488 nm | 543 nm/633 nm |
| Laser transmissivity | 7.00% | 10.00% | 20.0%/22.0% |
| Detection wavelength | 436 nm-510 nm | 496 nm – 592 nm | 564 nm – 696 nm |

**Description of real-time fluorescent imaging parameters in supplemental movie**

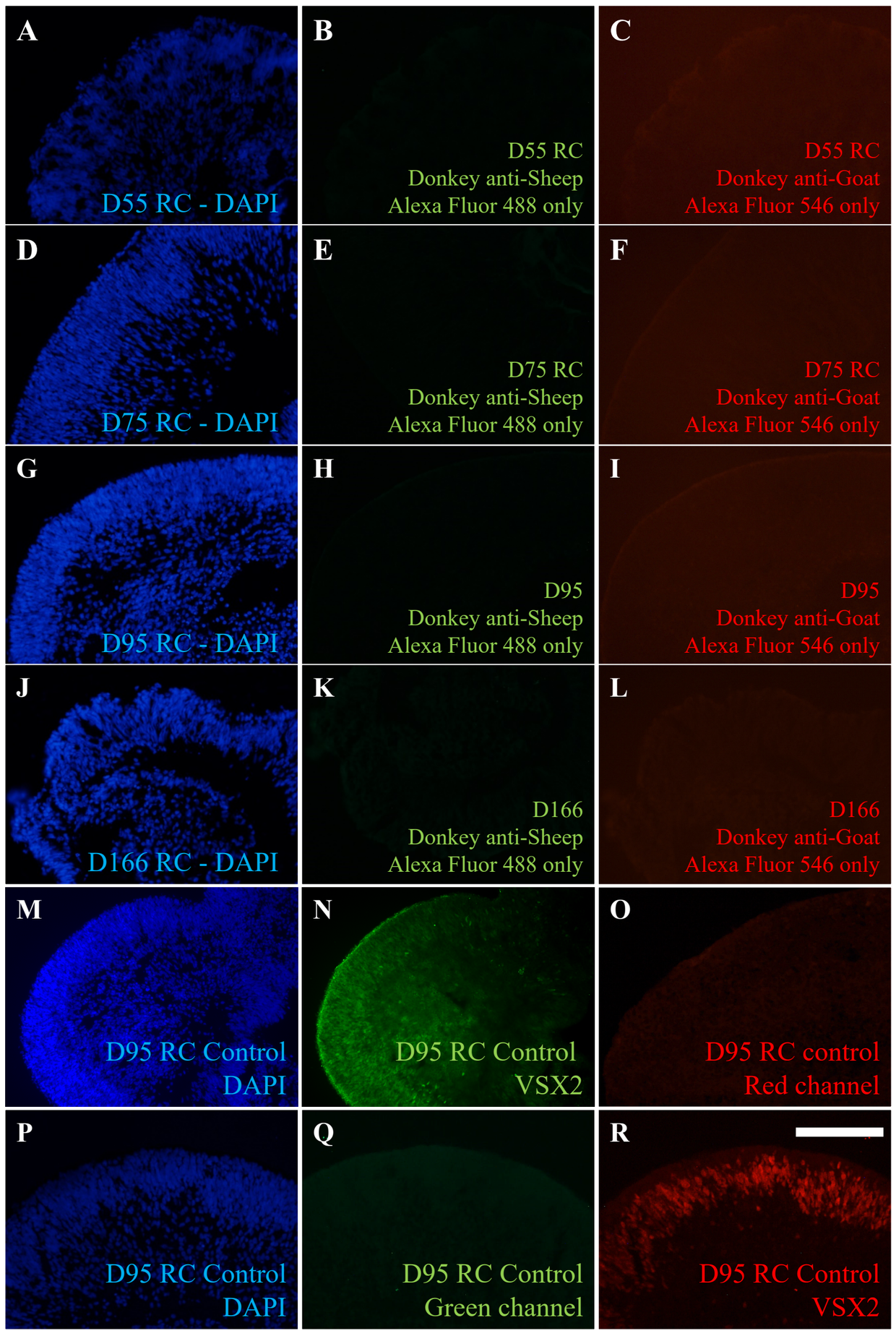
Olympus FV3000Laser Scanning Confocal System
Image size 1024 pixel -1024 pixel
Stack of 105 z-axis optical section each of 0.990 µM thickness

| | Cerulean | GFP | mCherry |
|---|---|---|---|
| Emission wavelength | 475 nm | 520 nm | 610 nm |
| Laser wavelength | 405 nm | 488 nm | 561 nm |
| Laser transmissivity | 8.00% | 9.80% | 10.90% |
| Detection wavelength | 422 nm – 482 nm | 496 nm – 548 nm | 571 nm – 671 nm |

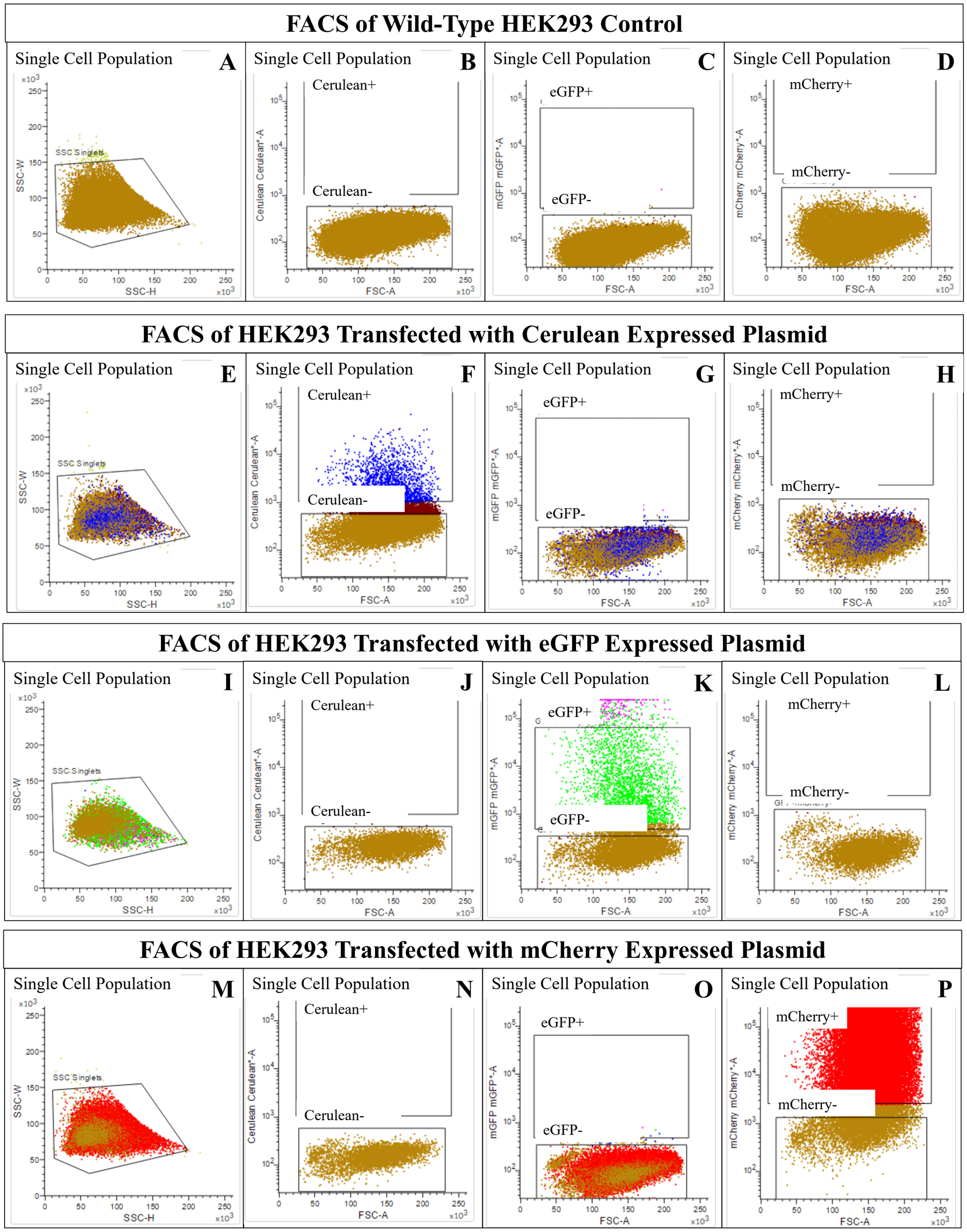
# Supplemental Figure S1



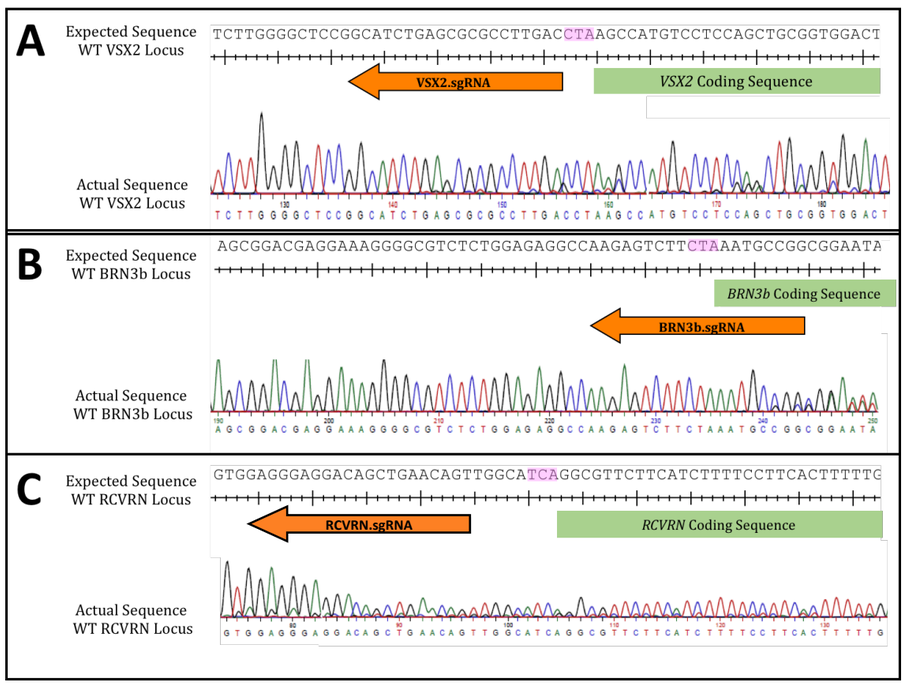
# Supplemental Figure S2



# Supplemental Figure S3

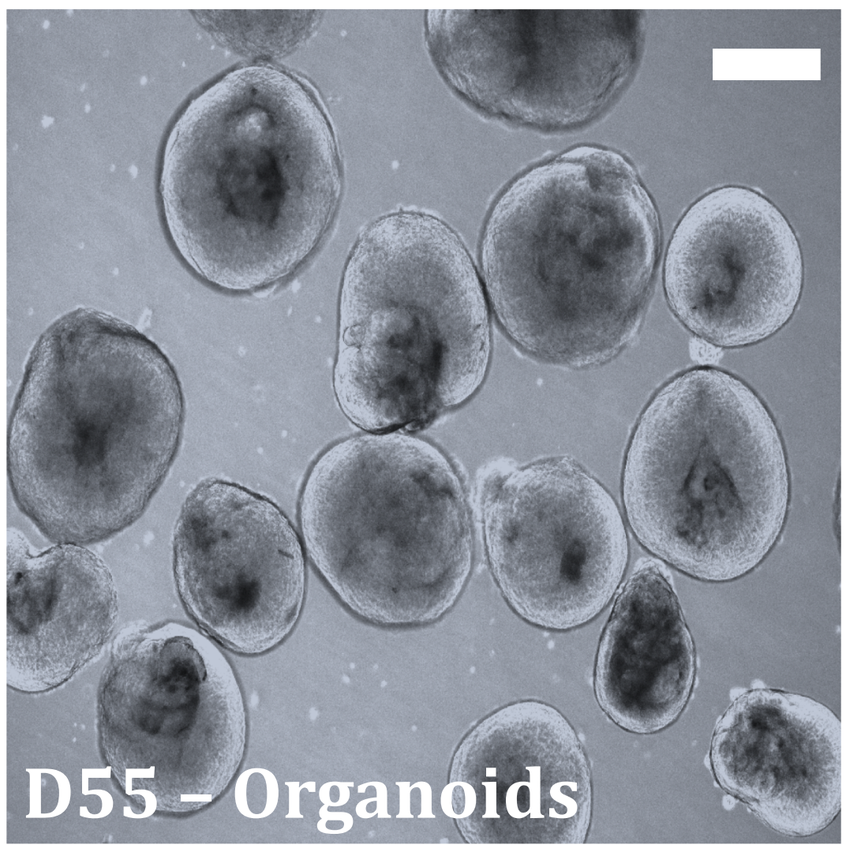


# Supplemental Figure S4

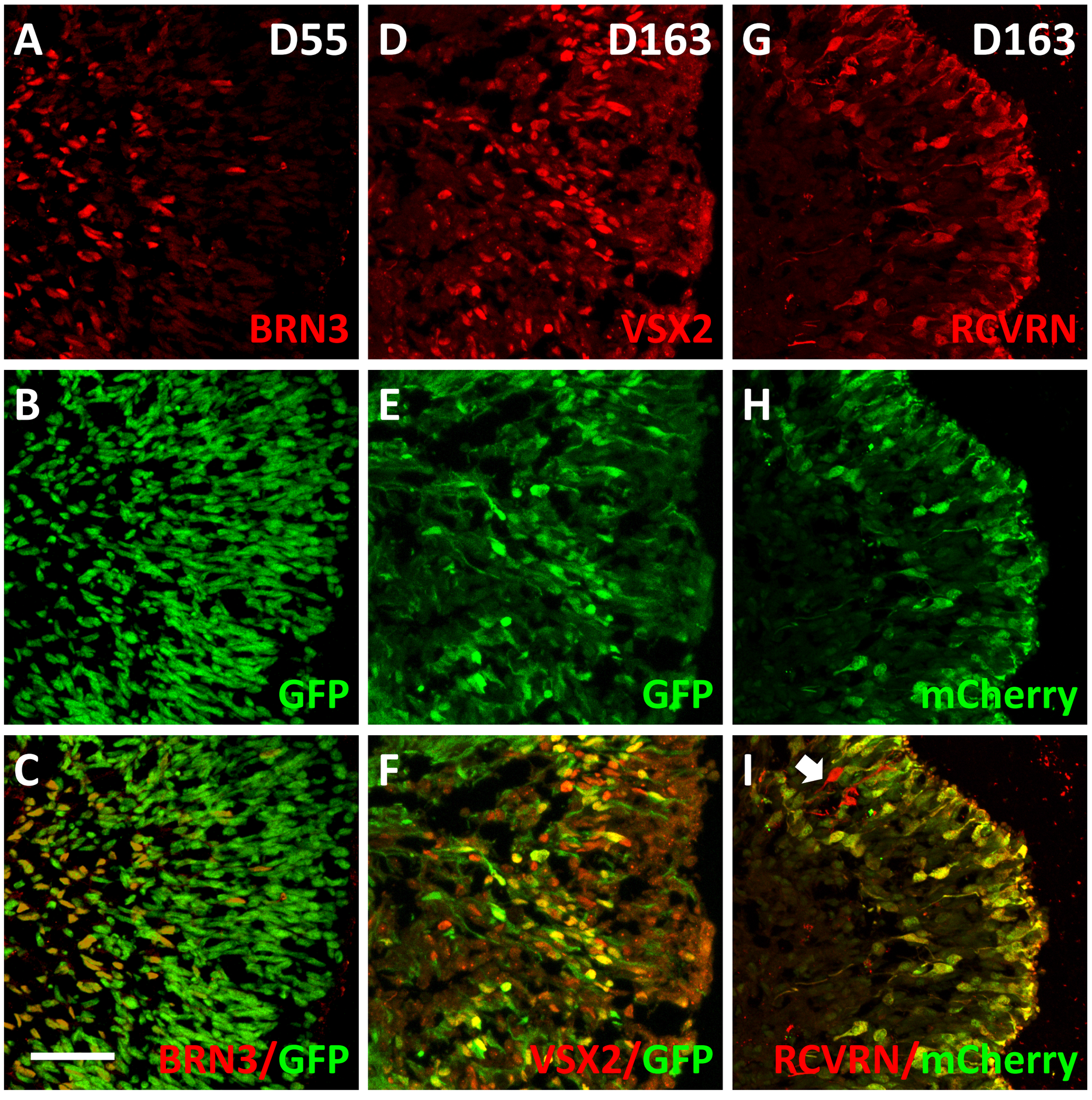


# Supplemental Figure S5

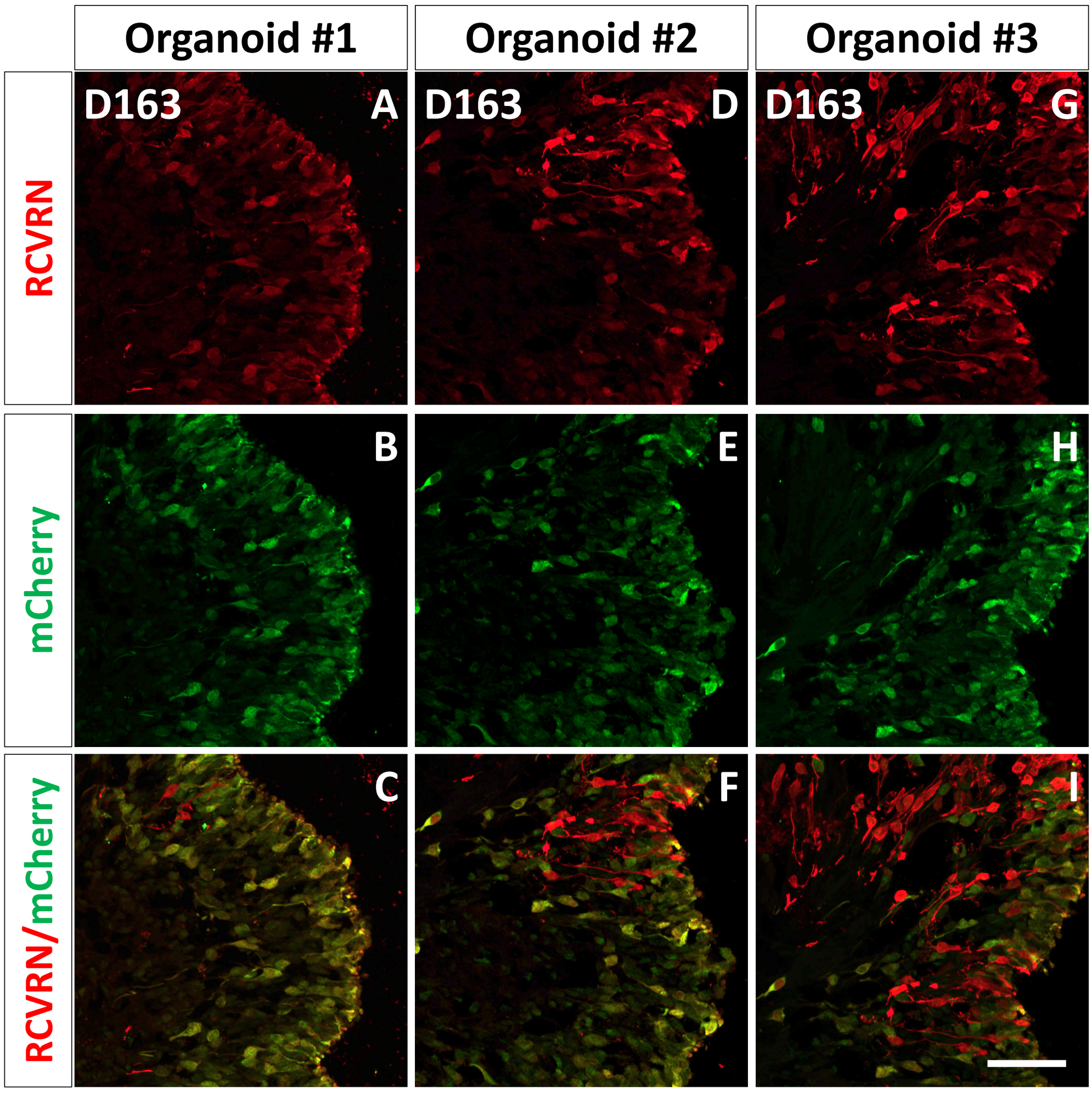
# Supplemental Figure S6



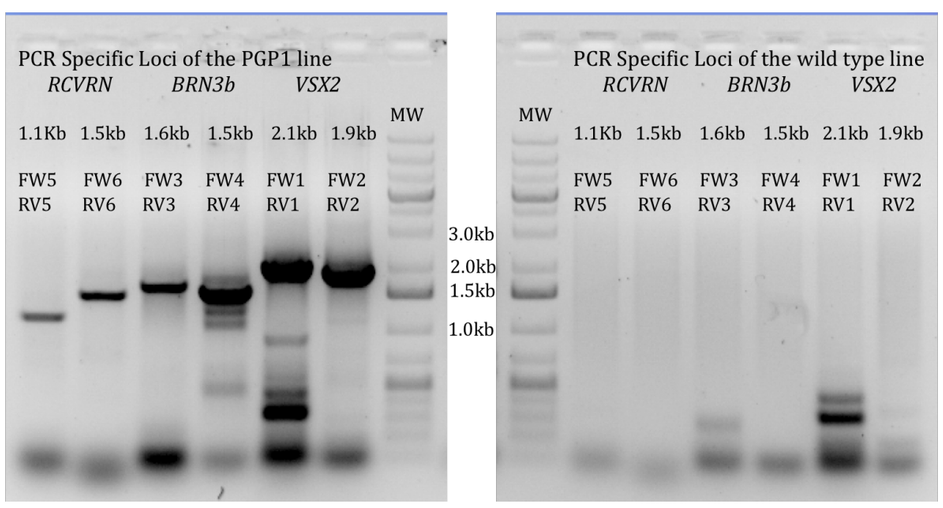
# Supplemental Figure S7



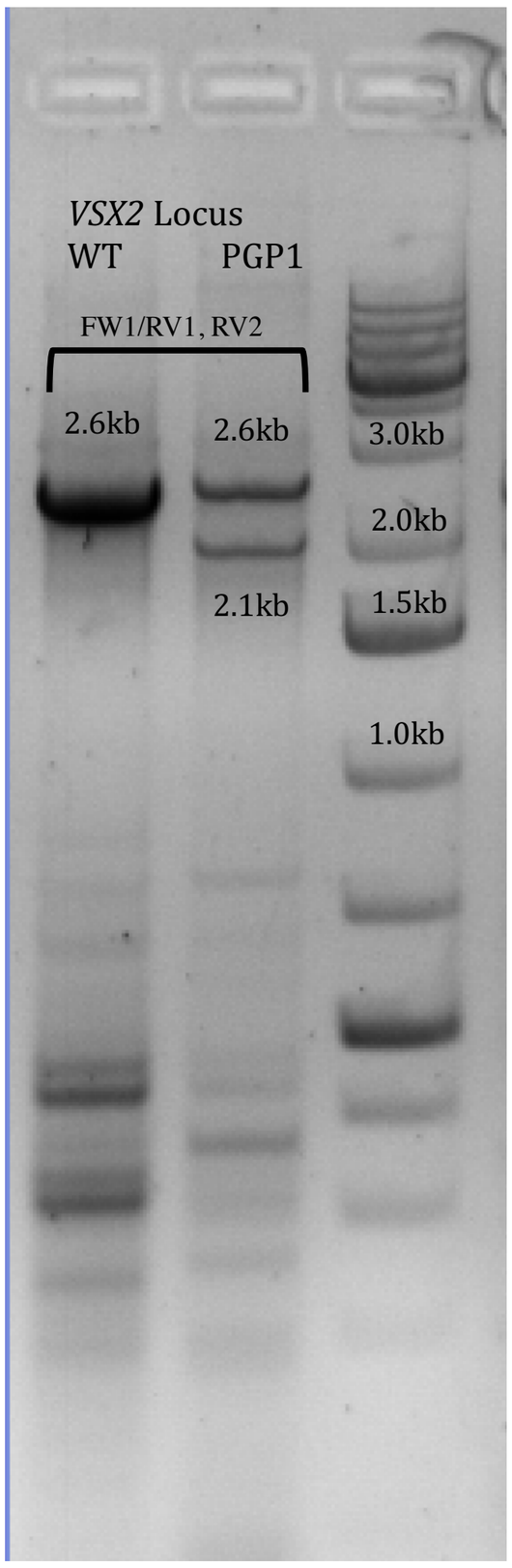
# Supplemental Figure S8

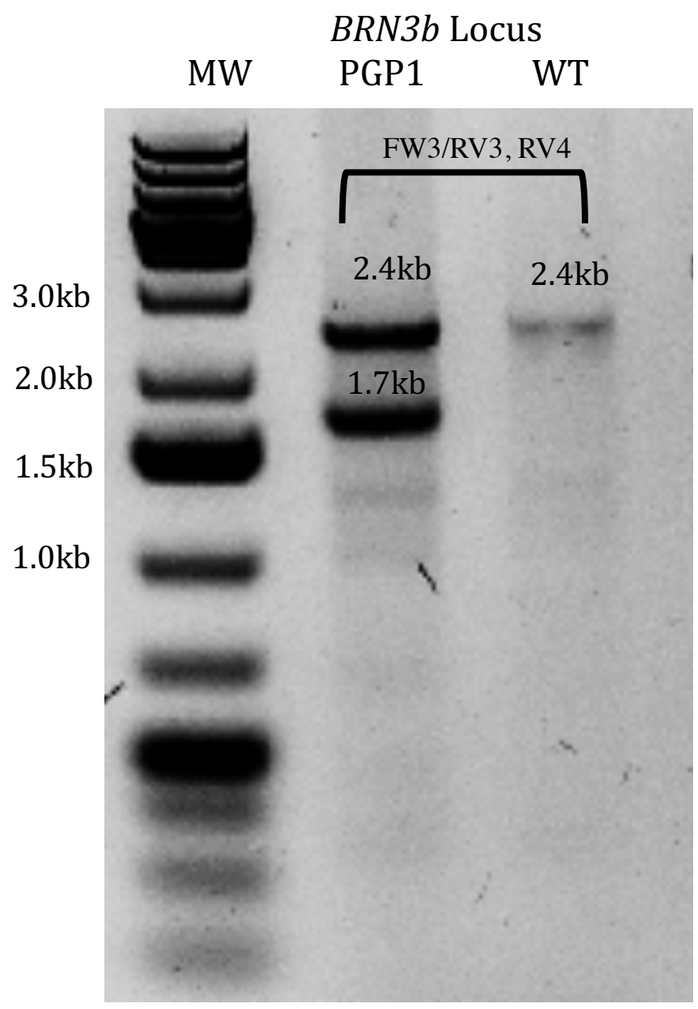


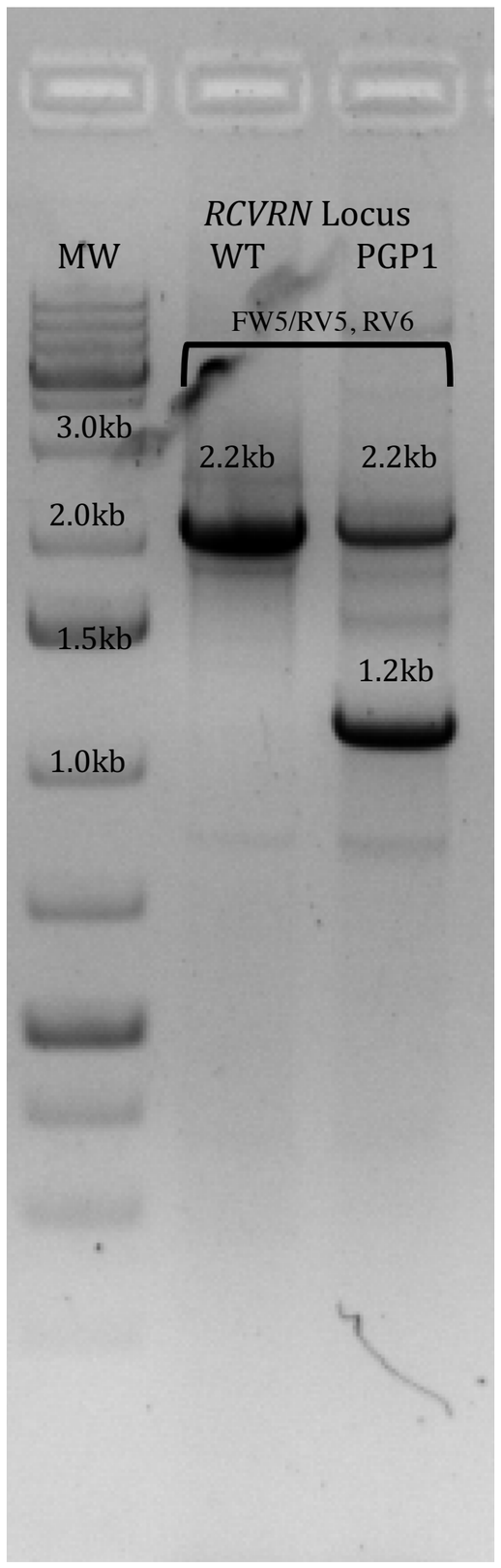
# Supplemental Figure S9



# Supplemental Figure S10



## SUPPLEMENTAL FIGURE LEGENDS

**Supplemental Figure S1: Confirmation that Fluorescent Protein Expression in PGP1-Derived Retinal Cup Organoids Does Not Survive Fixation and Frozen Sectioning.** Sections of PGP1 hiPSC-derived retinal organoids were prepared after fixation in 4% paraformaldehyde, overnight incubation in 30% sucrose at 4ºC, and embedding in OCT compound. Organoid sections from D55 (A-C), D75 (D-F), D95 (G-I), and D166 (J-L) of differentiation were visualized following DAPI staining on the blue DAPI filter (A, D, G, J), the green FITC filter (B, E, H, K) and the red Texas Red filter (C, F, I, L). No green or red signals consistent with eGFP or mCherry were detected. Magnification bar 100 μm, applies to all images.

**Supplemental Figure S2: Additional Controls for PGP1-Derived Retinal Cup Organoids Prepared for Immunohistochemistry.** To ensure the signals from immunofluorescent staining experiments are specific for the intended antigens, the retina cup organoids were stained for DAPI and secondary antibody only (A-L). The staining for DAPI, and the secondary antibodies Donkey anti-sheep Alexa Fluor 488, and Donkey anti-sheep Alexa Fluor 546 were done for sections from organoids at D55 (A-C), D75 (D-F), D95 (G-I), and D166 (J-L) of differentiation. To ensure that the DAPI signal did not represent the VSX2-Cerulean signal, D95 organoid sections were stained with DAPI, and a sheep anti-VSX2 primary antibody and secondary anti-sheep Alexa Fluor 488 antibody (M-O), or a secondary anti-sheep Alexa Fluor 546 antibody (P-R). Note that only a subset of the DAPI signals in (M) are VSX2 positive (N). Likewise, only a subset of the DAPI signals in (P) are VSX2 positive (R). Images in the first column (A, D, G, J, M, P) were photographed with a DAPI filter, while images in the middle (B, E, H, K, N, Q) were photographed with a FITC filter and the last column (C, F, I, L, O, R) were photographed with a Texas Red filter. Magnification Bar. 50 μm, applies to all images.

**Supplemental Figure S3: Establishing FACS Gates Using Transiently Transfected HEK293 Cells.** Wild-type HEK293 cells (A-D) or HEK293 cells transiently transfected with expression plasmids for Cerulean (E-H), eGFP (I-L), or mCherry (M-P) were dissociated into single cell populations (A, E, I, M) and Gates were established for each fluorescent protein based on parameters that would lead to capturing the appropriate fluorescent protein expressing cells without capturing any wild-type cells. We confirmed that the captured cells in the Cerulean positive gate (F) the eGFP positive gate (K) and the mCherry positive gate (P) expressed the appropriate fluorescent protein when sorted and cultured.

**Supplemental Figure S4: Lack of Indel Mutations in the Non-Targeted Alleles of PGP1.** Sequence analysis of the WT alleles of VSX2 (A), BRN3b (B) and RCVRN (C) failed to detect any Cas9-mediated indel mutations in PGP1. Orange arrows represent the sgRNA sequence, the endogenous stop codons are shaded in pink and coding sequences are represented by green rectangles.

**Supplemental Figure S5**: **Cerulean Positive Retina Progenitors Appear Before eGFP or mCherry Positive Cells During PGP1 Retinal Organoid Differentiation**. After 20 days of differentiation, retinal domains (A) first express Cerulean (blue) (B), but not eGFP (C) or mCherry (D). The composite of the bright field and VSX2/Cerulean (E). Magnification bar 20 μm, applies to all images.

**Supplemental Figure S6: Brightfield View of Three Dimension Retinal Organoids at D55 of differentiation.** Free-floating organoids have variable size, but all maintain a three dimensional shape with characteristic spherical structure with a distinct thick exterior and hollower interior. Scale bar 40 μm.

**Supplemental Figure S7: Double Labeling Immunohistochemical Showed a Co-localization of the Fluorescent Reporter with Its Targeted Gene.** IHC analysis showed a co-localization of BRN3 (labels BRN3a, BRN3b and BRN3c) and GFP on D55 (Fig. S7 A-C), and VSX2 with GFP (this antibody also detects Cerulean) on D163 of differentiation (Fig. S7 D-F). D163 organoids showed a co-localization RCVRN with mCherry (Fig. S7 G-I). White arrow indicates RCRVN expressing cells that do not express mCherry. Scale bar in C is 50 μm and applies to all the images.

**Supplemental Figure S8: Double Labeling Immunohistochemistry of RCVRN with mCherry Show Organoid-Organoid Variability.** IHC of PGP1-derived organoid at D163 of differentiation showed all mCherry (green) -expressing cells co-localized with RCVRN (red). Colocalization is indicated by yellow. However, some RCVRN-expressing cells failed to express mCherry (red cells) and the RCVRN+/mCherry- proportion of cells exhibited variability between organoids. Scale bar in I is 50 μm and applies to all the images.

**Supplemental Figure S9: Original PCR Gels Supporting Figure 1.** The original ethidium bromide stained PCR gels used to support figure 1. PCR reactions using genomic DNA as template with the primers indicated above each lane. The expected band sizes for each targeted allele are shown above each lane. The template DNA for the gel on the left came from the PGP1 line while the template for the gel on the right was from a WT hiPSC clone. MW indicates a DNA size ladder run on each gel.

**Supplemental Figure S10: Original PCR Gels Supporting Figure 2.** The original ethidium bromide stained PCR gel that was cropped for clarity in figure 2. The PGP1 line and wild-type hiPSCs (WT) provided the genomic DNA template for a three primer PCR strategy to detect the

wild-type and targeted alleles for the *VSX2, BRN3b* and *RCVRN* loci. The primers used for each reaction are indicated above the relevant lanes. MW indicates DNA size ladders on the gel.