

Supplementary Information

Optimized CRISPR/Cas9-mediated *in vivo* genome engineering applicable to monitoring dynamics of endogenous proteins in the mouse neural tissues

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Supplemental Experimental Procedures

Expression vectors

pCAG-EGFP (Addgene #11150), pRho-DsRed (Addgene #11156), pCALNL-EGFP (Addgene #13770), and pRho-Cre (Addgene #13779) was reported previously^{1,2}. pCAG-tdTomato was constructed by digesting pCAG-EGFP with KpnI and NotI, and replacing the coding region of EGFP with that of tdTomato excised from pUB-tdTomato³ using KpnI and NotI. pCAG-mCherry was constructed by inserting the coding region of mCherry excised from pmCherry-N1 (Takara Bio, Shiga, Japan) with EcoRI and NotI into pCAGEN (Addgene #11160) digested with EcoRI and NotI. pCAG-mClover was constructed by inserting the coding region of monomeric Clover (mClover)⁴ with a 5' linker sequence (5'-GAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTGCGCCACC-3') synthesized by Thermo Fisher, into the EcoRI and NotI sites of pCAGEN. To construct pCAG-H2BGFP, the cDNA encoding a fusion protein between human histone H2B and EGFP⁵ was amplified by PCR using pFMHGW⁶ as a template, and primers (5'-AACTCGAGTGAGCCGCCACCATGCCAGAGCCAGCGAAGTCTGCTC-3' and 5'-GGAGTGCGGCCGCTTTACTTGTACAGCTCG-3'). The amplified PCR fragment was digested with XhoI and NotI, and cloned into pCAGEN digested with XhoI and NotI. pCAG-H2B-mCherry was constructed by digesting pCAG-H2B-EGFP with AgeI and NotI, and replacing the coding region of EGFP with that of mCherry excised from pCAG-mCherry using AgeI and NotI. To construct pRhodopsin promoter-Cas9/U6-gRNA, pX330 was digested with KpnI and AgeI, and the CBh promoter was replaced with the 2.2kb bovine rhodopsin promoter⁷ amplified by PCR using primers (5'-ATATGGTACCACGTTGTCAGAAGCGTTAGCGG-3' and 5'-AGCTACCGGTTCGAAGCTTGAGCTCGACTCTAG-3') and pRho-DsRed as a template, and digested with KpnI and AgeI. To construct pRhodopsin promoter-tdTomato, pRho-DsRed was digested with EcoRI and NotI, and the coding region of DsRed was replaced with that of tdTomato excised from pCAG-tdTomato. To construct pCALNL-tdTomato, pCALNL-EGFP was digested with EcoRI and NotI, and

the coding region of EGFP was replaced with that of tdTomato excised from pCAG-tdTomato. To construct pCAG-PSD-95-EGFP, the coding region of mouse *PSD-95* was amplified by RT-PCR using primers (5'-ATATCTCGAGCTGAGCCGCCACCATGGACTGTCTCTGTATAGTGACAAC-3' and 5'-AATTACCGGTGGATCCGAGTCTCTCTCGGGCTGGGACCCAGAT-3') and total RNA extracted from adult ICR mouse retina. Linker-EGFP sequence was generated by PCR using primers (5'-CTCGGATCCACCGGTAATTCCGCTGACGGCGGCGGAGGATCGGGTGGTAGTGGTGGTTCAGGAGGAGGATCGACCCAAGGAGGTACCGTGAGCAAGGGCGAGGAGCTGTT-3' and 5'-ATGCGCGGCCGCTTTACTTGTACAGCTC-3') and pCAG-EGFP as a template. These PCR fragments were digested with XhoI and AgeI, AgeI and NotI, respectively, and simultaneously cloned into pCAGEN digested with XhoI and NotI. Restriction sites in primer sequences were underlined.

Knock-in targeting vectors

Homology arms of knock-in targeting vectors were prepared by genomic PCR using mouse genomic DNA extracted from an adult ICR mouse liver as a template. The CRISPR target sequences were removed from the knock-in vectors by introducing silent mutations or deletions into the homology arms.

Rhodopsin targeting vector (EGFP): To construct a cloning plasmid vector, the pUC plasmid backbone was amplified by PCR using primers (5'-TGTATCTAAGCTTAATTAAGATCGCGTTTAAACGGCGCGCCGCTGCATTAATGATCGGCCA-3' and 5'-CCGCGGTACCGTCGACATTTAAATGGCCGGCCCAGGTGG-3') and pCAG-EGFP as a template. The coding region of EGFP was amplified by PCR using primers (5'-GTCGACGGTACCGCGGGCCCGG-3' and 5'-GATTATGAGCGGCCGCTTTACTTGTACAGCTCGTCCAT-3') and pCAG-EGFP as a template. SV40 polyA sequence was amplified by PCR using primers (5'-AAGCGGCCGCTCATAATCAGCCATACCACATTTGTA-3' and 5'-TAATTAAGCTTAGATACATTGATGAGTTTGGACA-3') and pEGFP-N1 (Takara Bio, Shiga, Japan) as a template. These PCR fragments were simultaneously ligated using the In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) to generate pUC-EGFP-pA. The 5' homology arm (1033 bp) was amplified by PCR using primers (5'-ACGTATTTAAATGGGACAGGGGAGGCATTGCACTC-3' and 5'-CGGTGGATCCCCGGCTGGAGCCACCTGGCTGGTC-3'). The 3' homology arm

1 (1114 bp) was amplified by PCR using primers
 2 (5'-ATATGCGGCCGCGCTGAAAGTAGGAGTCTCCTGTCCCCA-3' and
 3 5'-CGATGTTTAAACTCCATACTGGCCTGGAAGT-3'). The 5' homology arm
 4 digested with *Swa*I and *Bam*HI was ligated into pUC-EGFP-pA digested with *Swa*I
 5 and *Bam*HI to generate pUC-(Rho 5' 1033 bp)-EGFP-pA. Then the 3' homology arm
 6 digested with *Not*I and *Pme*I was ligated into pUC-(Rho 5' 1033 bp)-EGFP-pA
 7 digested with *Not*I and *Pme*I to generate Rhodopsin targeting vector (EGFP).
 8 Rhodopsin targeting vector (mCherry): Rhodopsin targeting vector (EGFP) was
 9 digested with *Age*I and *Not*I, and the coding region of EGFP was replaced with that of
 10 mCherry excised from pCAG-mCherry with *Age*I and *Not*I.
 11 Rhodopsin targeting vector (2A-EGFP-pA): The 5' homology arm (1031 bp) was
 12 amplified by PCR using primers
 13 (5'-ACGTATTTAAATGGGACAGGGGAGGCATTGCACTC-3' and
 14 5'-ATCTGGTACCGGCTGGAGCCACCTGGCTGGTC-3') and Rhodopsin targeting
 15 vector (EGFP) as a template. 2A-EGFP was generated by PCR using primers
 16 (5'-AGCCGGTACCAAGATCTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGT
 17 GGAGGAGAATCCCGGCCCTATGGTGAGCAAGGGCGAGGAGCT-3' and 5'-
 18 ATGAGCGGCCGCTTTACTTGTACAGCTCGTCCATG-3') and pCAG-EGFP as a
 19 template. These PCR fragments were digested with *Swa*I and *Kpn*I, *Kpn*I and *Not*I,
 20 respectively, and simultaneously ligated into pUC-EGFP-pA digested with *Swa*I and
 21 *Not*I to generate pUC-(Rho 5' 1033 bp)-2A-EGFP-pA. Then the 3' homology arm
 22 (1114 bp) was amplified by PCR using primers
 23 (5'-ATCTAAGCTTGCTGAAAGTAGGAGTCTCCTGTC-3' and
 24 5'-CGATGTTTAAACTCCATACTGGCCTGGAAGT-3') and Rhodopsin targeting
 25 vector (EGFP) as a template, digested with *Hind*III and *Pme*I, and ligated into
 26 pUC-(Rho 5' 1033 bp)-2A-EGFP-pA digested with *Hind*III and *Pme*I, to generate
 27 Rhodopsin targeting vector (2A-EGFP-pA).
 28 Rhodopsin targeting vector (2A-H2B-EGFP): The 5' homology arm (1031 bp) was
 29 amplified by PCR using primers
 30 (5'-ACGTATTTAAATGGGACAGGGGAGGCATTGCACTC-3' and
 31 5'-ATCTGGTACCGGCTGGAGCCACCTGGCTGGTC-3') and Rhodopsin targeting
 32 vector (EGFP) as a template. 2A-H2B-EGFP was generated by PCR using primers
 33 (5'-AGCCGGTACCAAGATCTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGT
 34 GGAGGAGAATCCCGGCCCTATGCCAGAGCCAGCGAAGTCTGC-3' and 5'-
 35 ATGAGCGGCCGCTTTACTTGTACAGCTCGTCCATG-3') and pCAG-H2B-EGFP as

1 a template. These PCR fragments were digested with *Swa*I and *Kpn*I, *Kpn*I and *Not*I,
 2 respectively, and simultaneously ligated into Rhodopsin targeting vector (EGFP)
 3 digested with *Swa*I and *Not*I to generate Rhodopsin targeting vector (2A-H2B-EGFP).
 4 Rhodopsin targeting vector (2A-H2B-EGFP) deletion constructs: To delete 5'- and
 5 3'-homology arms of Rhodopsin targeting vector (2A-H2B-EGFP), PCRs were
 6 performed using Rhodopsin targeting vector (2A-H2B-EGFP) as a template, and
 7 following primers. 5'_Fw_0.6kb:
 8 5'-ATATCTCGAGTCCAAGTCTGGGAATAGAATGGTC-3'. 5'_Fw_0.3kb:
 9 5'-ATATCTCGAGGACACATGGAATGCCACTTGAC-3'. 5'_Fw_0.2kb:
 10 5'-ATATCTCGAGCCAGGCTTAGTGAGGGGACATGC-3'. 3'_Rv_0.5kb:
 11 5'-ATCGAGATCTCAGAGAAAACCAAGGGCAGGTATGT-3'. 3'_Rv_0.2kb:
 12 5'-ATCGAGATCTGGTTGAGGATGGTCTTGGTGGAT-3'. 3'_Rv_0.1kb:
 13 5'-ATCGAGATCTAGAACACTGAGGGAGCCTGCATG-3'. Amplified PCR fragments
 14 were digested with *Xho*I and *Bgl*II, and ligated into the pUC plasmid backbone
 15 amplified by PCR using primers (5'-ATATAGATCTGCTGCATTAATGAATCGGCCAA-3'
 16 and 5'-CGATCTCGAGGTGGCACTTTTCGGGGAAATGTGC-3') and pCAG-EGFP as
 17 a template and digested with *Xho*I and *Bgl*II, or pBluescript II KS(-) (Agilent) digested
 18 with *Xho*I and *Bam*HI.
 19 Glul targeting vector (mClover): The 5' homology arm (1143 bp) was amplified by PCR
 20 using primers (5'-ATATCTCGAGTATTACTGCGGTGTGGGAGCAGAC-3' and
 21 5'-ATCTGGTACCTTCTTATACTGAAAGGGTTCGTCGCCT-3'). The 3' homology arm
 22 (978 bp) was amplified by PCR using primers
 23 (5'-ATATGCGGCCGCAGACTTCCAGTGATCCCTCTCCCAG-3' and
 24 5'-ATCGAGATCTCCATCAGTAACAGTGTTCAAGTTG-3'). Amplified PCR fragments
 25 were digested with *Xho*I and *Kpn*I, *Not*I and *Bgl*II, respectively. The coding sequence
 26 of mClover was excised from pCAG-mClover with *Kpn*I and *Not*I. These three
 27 fragments were simultaneously ligated into the pUC plasmid backbone amplified by
 28 PCR using primers (5'-ATATAGATCTGCTGCATTAATGAATCGGCCAA-3' and
 29 5'-CGATCTCGAGGTGGCACTTTTCGGGGAAATGTGC-3') and pCAG-EGFP as a
 30 template, and digested with *Xho*I and *Bgl*II, to generate Glul targeting vector
 31 (mClover).
 32 Arrestin targeting vector (EGFP): The genomic DNA including exon 16 of *arrestin* was
 33 amplified by PCR using primers
 34 (5'-CCACCTCGAGTAAGTCATCTGGAAGCTTCTCTGGG-3' and
 35 5'-AATGAGATCTTCTGGACACTATGGCTAGGAATGG-3'). The pUC plasmid

1 backbone was amplified by PCR using primers
 2 (5'-TAGTGTCCAGAAGATCTCATTAATGAATCGGCCAACG-3' and
 3 5'-AGATGACTTACTCGAGGTGGCACTTTTCGGGGAAATGTGC-3') and
 4 pCAG-EGFP as a template. These two PCR fragments were ligated using the
 5 In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) to generate pUC-arrestin
 6 genomic DNA. Then two PCRs were performed using primers
 7 (5'-TCAGTATTTTCACCTGTATCTTTCAGATTTTGGCGAG-3' and
 8 5'-GAAACTGCTTGTAGTTGAAGCCCCCGGTAGACTCTCA-3') and pUC-arrestin
 9 genomic DNA as a template, and primers
 10 (5'-AGGTGAAAATACTGAAGGTAAGAAAGATGAGGATGCTGGCCAGGATGAGGGA
 11 TCCACCGGTAATTCCGCTGACG-3' and
 12 5'-AACTACAAGCAGTTTCTAGGCATCTAGGCTAAGTCTGCGGCCGCTTTACTTGTA
 13 CAGC-3') and pCAG-PSD-95-EGFP as a template, respectively. The two PCR
 14 fragments were ligated using the In-Fusion HD Cloning Kit to generate Arrestin
 15 targeting vector (EGFP) that contains the 899 bp 5' homology arm and the 997 bp 3'
 16 homology arm.
 17 PSD-95 targeting vector (mClover): The genomic DNA including exon 20 of *PSD-95*
 18 was amplified by PCR using primers
 19 (5'-ATATGTCGACTGCATCCTTGATGTCTCAGCCAATG-3' and
 20 5'-AGTCAAGCTTTCTGTCTGCCTCGGTCTGGTAAGTC-3'). The PCR fragment was
 21 digested with Sall and HindIII, and ligated into the pUC plasmid backbone prepared
 22 from pUC-EGFP-pA digested with Sall and HindIII, to generate pUC-PSD-95 genomic
 23 DNA1. Then PCR was performed using primers
 24 (5'-AAATGCTAAGCGGCCGCTGGACTCACCCCTGCCTCCAC-3'
 25 -3' and 5'-AATTACCGGTGGATCCGAGTCTCTCTCGGGCTGGGAC-3') and
 26 pUC-PSD-95 genomic DNA1 as a template. The PCR fragment was ligated with
 27 linker-mClover prepared by PCR using primers
 28 (5'-GATCCACCGGTAATTCCGCTGACGGCGGCGGAGGATCGGGTGGTAGTGGTG
 29 GTTCAGGAGGAGGATCGACCCAAGGAGGTACCGTGAGCAAGGGCGAGGAGCT
 30 G-3' and 5'-GCGGCCGCTTAGCATTAGGTGA-3') and pCAG-mClover as a template,
 31 using the In-Fusion HD Cloning Kit, to generate PSD-95 targeting vector (mClover)
 32 that contains the 1037 bp 5' homology arm and the 966 bp 3' homology arm.
 33 PSD-95 targeting vector (EGFP): The genomic DNA including exon 20 of *PSD-95* was
 34 amplified by PCR using primers
 35 (5'-CCTGGTCGACAATGCAGCCTGCCAGGAAGGAGGTA-3' and

1 5'-CAGCAAGCTTAGCAAGTGGCTTATCTGTCAAGTT-3'). The pUC plasmid
 2 backbone was amplified by PCR using primers
 3 (5'-ACTTGCTAAGCTTGCTGCATTAATGAATCGGCCAACG-3' and
 4 5'-AGGCTGCATTGTGCGACCAGGTGGCACTTTTCGGGGAAATG-3') and
 5 pCAG-EGFP as a template. These two PCR fragments were ligated using the
 6 In-Fusion HD Cloning Kit to generate pUC-PSD-95 genomic DNA2. Then PCR was
 7 performed using primers
 8 (5'-TACAAGTAAAGCGGCCGCTGGACTCACCTGCCTCCAC-3' and
 9 5'-AATTACCGGTGGATCCGAGTCTCTCTCGGGCTGGGAC-3') and pUC-PSD-95
 10 genomic DNA2 as a template. The PCR fragment was ligated with linker-EGFP
 11 excised from pCAG-PSD-95-EGFP with BamHI and NotI, using the In-Fusion HD
 12 Cloning Kit to generate PSD-95 targeting vector (EGFP) that contains the 496 bp 5'
 13 homology arm and the 2039 bp 3' homology arm.
 14 Syp targeting vector (mClover): The genomic DNA including exon 6 of *Syp* was
 15 amplified by PCR using primers (5'-
 16 ATATGTCGACTTGAGACAGGATCTACTTATGTGAC-3' and
 17 5'-AGTCAAGCTTCACACACAGGGTCTCCAAGCAGCCT-3'). The PCR fragment
 18 was digested with Sall and HindIII, and ligated into the pUC plasmid backbone
 19 prepared from pUC-EGFP-pA digested with Sall and HindIII, to generate pUC-Syp
 20 genomic DNA1. Then PCR was performed using primers
 21 (5'-AAATGCTAAGCGGCCGCTAATCTGGTGAGTGACAACTG-3' and 5'-
 22 AATTACCGGTGGATCCGGAGAAGGAGGTGGGCGCACCC-3') and pUC-Syp
 23 genomic DNA1 as a template. The PCR fragment was ligated with linker-mClover
 24 prepared by PCR using primers
 25 (5'-GATCCACCGGTAATTCCGCTGACGGCGGCGGAGGATCGGGTGGTAGTGGTG
 26 GTTCAGGAGGAGGATCGACCCAAGGAGGTACCGTGAGCAAGGGCGAGGAGCT
 27 G-3' and 5'-GCGGCCGCTTAGCATTAGGTGA-3') and pCAG-mClover as a template,
 28 using the In-Fusion HD Cloning Kit, to generate Syp targeting vector (mClover) that
 29 contains the 894 bp 5' homology arm and the 964 bp 3' homology arm.
 30 Syp targeting vector (EGFP): The genomic DNA including exon 6 of *Syp* was
 31 amplified by PCR using primers
 32 (5'-CCTGGTCGACATGCCCATTCCTGTTCACTCAGCC-3' and
 33 5'-CAGCAAGCTTACCTTTCTTCTGGACTCCATGGACA-3'). The pUC plasmid
 34 backbone was amplified by PCR using primers
 35 (5'-GAAAGGTAAGCTTGCTGCATTAATGAATCGGCCAACG-3' and

5'-GAATGGGCATGTCGACCAGGTGGCACTTTTCGGGGAAATG-3') and pCAG-EGFP as a template. These two PCR fragments were ligated using the In-Fusion HD Cloning Kit to generate pUC-Syp genomic DNA 2. Then PCR was performed using primers (5'-TACAAGTAAAGCGGCCGC TAATCTGGTGAGTGACAACCTG-3' and 5'-AATTACCGGTGGATCCGGAGAAGGAGGTGGGCGCACCT-3') and pUC-Syp genomic DNA 2 as a template. The PCR fragment was ligated with linker-EGFP excised from pCAG-PSD-95-EGFP with BamHI and NotI, using the In-Fusion HD Cloning Kit to generate Syp targeting vector (EGFP) that contains the 405 bp 5' homology arm and the 2275 bp 3' homology arm. Restriction sites in primer sequences were underlined, and homology overlaps in primer sequences for In-Fusion cloning were shown in italics.

Single-strand annealing (SSA) assay

Activities of CRISPR constructs were evaluated in HEK293T cells using a single-strand annealing assay as described previously⁸. The reporter plasmid (pCAG-split Luc2) encoding two split inactive parts of the luciferase gene with overlapped sequences was constructed as follows. Luc2 (1-1131) was amplified by PCR using primers (5'-ATGCGAATTCTGAGCCGCCACCATGGAAGATGCCAA-3' and 5'-CTCCTACTCGAGGTAACGCAACTAAGTCATAGTCCGCCTAGAAGCATTGCGG TGGACGATGGAGGGTACCCTAGTCCAAGTCCACCACCTTAGC-3') and pGL4.10 (Promega) as a template. Luc2 (331-1635) was amplified by PCR using primers (5'-CGTTACCTCGAGTAGGAGCGCGAGCTGCTGAACAGC-3' and 5'-ATATGCGGCCGCTTACACGGCGATCTTGCCGCCCTTCTTG-3') and pGL4.10 as a template. These PCR fragments were digested with EcoRI and XhoI, and XhoI and NotI, respectively, and simultaneously cloned into pCAGEN digested with EcoRI and NotI. Mouse genomic sequences containing CRISPR target sites were amplified by PCR using following primers.

Rhodopsin; 5'- ATATGGTACCAGATGACGACGCCTCTGCCACC-3' and 5'- ATATCTCGAGCAGGAGACTCCTACTTTCAGCC-3'.

Glul; 5'-ATATGGTACCTGCCGGTGTTGCCAACCGCGGTGC-3' and 5'- ATGCCTCGAGTGGTGTGTAGAGATTAAGAACCTG-3'.

Arrestin; 5'- ATATCTCGAGTCCTCAGCTTCTGAGAGTAAGCACTG-3' and 5'- ATATGGTACCTGACACATGATAAGAGGACCCAGAG-3'.

1 *PSD-95*; 5'- ATCGCTCGAGAGAGATCAATAAGCGGATCACAGA-3' and 5'-
2 ATGCGGTACCCACAGTTAGACCTTCCACTCATG-3'.
3 *Syp*; 5'- ATCGCTCGAGGTGTTTGGCTTCCTGAACCTGGTG-3' and 5'-
4 ATGCGGTACCTCCTCCACCTACTGGCGTTACCTCC-3'. Adult ICR mouse genomic
5 DNA was used as a PCR template. Amplified PCR fragments were digested with KpnI
6 and XhoI, and inserted between Luc2 (1-1131) and Luc2 (331-1635) of pCAG-split
7 Luc2 digested with KpnI and XhoI. HEK293T cells were maintained in DMEM
8 (Thermo Fisher, 11965) supplemented with 10% FCS (Equitech-Bio) and antibiotics
9 (100 u/ml penicillin and 100 µg/ml streptomycin, Nacalai Tesque, Kyoto, Japan). Cells
10 were plated onto 24-well dishes at a density of 1x10⁵ cells per well. Eighteen hours
11 later, the cells were co-transfected with 50 ng of the pCAG-split Luc2 reporter
12 construct and 100 ng of the CRISPR construct using polyethylenimine (Polysciences,
13 24765-2). Twenty-four hours after transfection, the cells were harvested, and
14 luciferase activity was measured using Luciferase Assay System (Promega, E1500)
15 and Lumat LB 9507 (Berthold Technologies). Restriction sites in primer sequences
16 were underlined.

18 Genotyping

19 Single-cell genotyping was performed by nested PCR using PrimeSTAR GLX DNA
20 polymerase (Takara Bio, Shiga, Japan) or KOD FX neo DNA polymerase (Toyobo,
21 Osaka, Japan). FACS-purified single cells in 15 µl of 50 µg/ml proteinase K were
22 incubated for 1 h at 50°C, followed by 4 min at 99°C. For single-cell genotyping of
23 retinal cells transfected with Rhodopsin targeting vector (EGFP), the first PCR was
24 carried out in a reaction volume of 50 µl with four primers: Primers
25 (5'-CATCTATAACCCGGTCATCTACATC-3' and
26 5'-GTGCAGATGAACTTCAGGGTCAGCTTG-3') to amplify the EGFP knock-in allele
27 (1610 bp) and primers (5'-TCATGTTTGAGACCTTCAACACC-3' and
28 5'-TCCTGCTCGAAGTCTAGAGCAAC-3') to amplify *Actb* (313 bp) as a positive
29 control. Reaction conditions were: 1 cycle of 98°C for 3 min, 30 cycles of 98°C for 10
30 sec, 58°C for 30 sec and 68°C for 2 min. Then, using 0.5 µl of the first PCR products
31 as a template, the second PCR was carried out in a reaction volume of 25 µl with
32 primers (5'-CATCTATAACCCGGTCATCTACATC-3' and
33 5'-GTGCAGATGAACTTCAGGGTCAGCTTG-3') to amplify the EGFP knock-in allele
34 (1168 bp) or primers (5'-ATGTACGTAGCCATCCAGGCTGT-3' and
35 5'-AGCTGTGGTGGTGAAGCTGTAG-3') to amplify *Actb* (219 bp). Reaction

conditions were: 1 cycle of 98°C for 3min, 30 cycles of 98°C for 10 sec, 60°C for 15
 sec and 68°C for 1 min. For single-cell genotyping of retinal cells transfected with Glul
 targeting vector (mClover), the first PCR was carried out in a reaction volume of 50 µl
 with primers (5'-GCAGGACTAGTGAAGTCAAGGAAAGCAGG-3' and
 5'-CTGAACTTGTGGCCGTTTACGTC-3') to amplify the mClover knock-in allele
 (1393 bp) and the primers to amplify *Actb*. Then, using 0.5 µl of the first PCR products
 as a template, the second PCR was carried out in a reaction volume of 25 µl with
 primers (5'-TGCTGTGTCTTGAACCTCCTTCA-3' and
 5'-CGTCCAGCTCGACCAGGATGGGCAC-3') to amplify the mClover knock-in allele
 (1278 bp). For single-cell genotyping of retinal cells transfected with Arrestin targeting
 vector (EGFP), the first PCR was carried out in a reaction volume of 50 µl with primers
 (5'-ACAACCATCTGTAATAGAATCCGATGC-3' and
 5'-GTGCAGATGAACTTCAGGGTCAGCTTG-3') to amplify the EGFP knock-in allele
 (1249 bp) and the primers to amplify *Actb*. Then, using 0.5 µl of the first PCR products
 as a template, the second PCR was carried out in a reaction volume of 25 µl with
 primers (5'-GTACAGCTGCAGTGTATTACACATA-3' and
 5'-GACACGCTGAACTTGTGGCCGTTTACG-3') to amplify the EGFP knock-in allele
 (1202 bp).
 For genotyping of retinal and brain cells transfected with PSD-95 targeting vector
 (EGFP), PSD95 targeting vector (mClover), Syp targeting vector (EGFP), or Syp
 targeting vector (mClover), genomic DNAs were purified from the harvested mouse
 retinas or brains using QIAamp DNA mini kit (Qiagen). Nested PCRs were performed
 to detect the knock-in alleles. The first PCRs were carried out in a reaction volume of
 25 µl: 1 cycle of 98°C for 4 min, 20 cycles of 98°C for 10 sec, 60°C for 15 sec and
 68°C for 1 min. Using 0.5 µl of the first PCR products as a template, the second PCRs
 were carried out in a reaction volume of 25 µl: 1 cycle of 98°C for 3 min, 27 cycles of
 98°C for 10 sec, 60°C for 10 sec and 68°C for 40 sec.
 For genotyping of retinal cells transfected with PSD95 targeting vector (EGFP), the
 first PCR was performed with primers (5'-ATCTGTCACTTTCC CTTTGGCCAAC-3'
 and 5'-GTGCAGATGAACTTCAGGGTCAGCTTG-3', product size 1409 bp) and the
 second PCR was performed with primers (5'-ATATTCTCTGTCTCTTCCTGGCACC-3'
 and 5'-GACACGCTGAACTTGTGGCCGTTTACG-3', product size 1308 bp). For
 genotyping of retinal cells transfected with PSD95 targeting vector (mClover), the first
 PCR was performed with primers (5'-ATCTGTCACTTTCC CTTTGGCCAAC-3' and
 5'-CTGAACTTGTGGCCGTTTACGTC-3', product size 1329 bp) and the second PCR

1 was performed with primers (5'-ATATTCTCTGTCTCTTCCTGGCACC-3' and
 2 5'-CGTCCAGCTCGACCAGGATGGGCAC-3', product size 1260 bp). For genotyping
 3 of retinal cells transfected with Syp targeting vector (EGFP), the first PCR was
 4 performed with primers (5'-CACCACTTGAGAAGCAGAAGCATGTAG-3' and
 5 5'-GTGCAGATGAACTTCAGGGTCAGCTTG-3', product size 1534 bp) and the
 6 second PCR was performed with primers
 7 (5'-TATATATGCATCAATGAGTGATGTCTG-3' and
 8 5'-GACACGCTGAACTTGTGGCCGTTTACG-3', product size 1297 bp).
 9 For genotyping of retinal cells transfected with Syp targeting vector (mClover), the first
 10 PCR was performed with primers (5'-CACCACTTGAGAAGCAGAAGCATGTAG-3'
 11 and 5'-CTGAACTTGTGGCCGTTTACGTC-3', product size 1441 bp) and the second
 12 PCR was performed with primers (5'-TATATATGCATCAATGAGTGATGTCTG-3' and
 13 5'-CGTCCAGCTCGACCAGGATGGGCAC-3', product size 1266 bp).
 14 Electroporated pCAG-mCherry was detected by PCR using primers
 15 (5'-TAACATGGCCATCATCAAGGAGTTC-3' and
 16 5'-AGCCCATGGTCTTCTTCTGCATTAC-3', product size 419 bp), and the following
 17 condition: 1 cycle of 98°C for 4 min, 35 cycles of 98°C for 10 sec, 60°C for 15 sec and
 18 68°C for 30 sec. Electroporated pCAG-tdTomato was detected by PCR using primers
 19 (5'-CGATTACAAGAAGCTGTCCTTCC-3' and
 20 5'-GCCATGTAGATGGTCTTGAAGCTC-3', product size 309 bp), and the following
 21 condition: 1 cycle of 98°C for 4 min, 35 cycles of 98°C for 10 sec, 62°C for 15 sec and
 22 68°C for 30 sec. *Actb* was detected by genomic PCR using primers
 23 (5'-ATGTACGTAGCCATCCAGGCTGT-3' and
 24 5'-AGCTGTGGTGGTGAAGCTGTAG-3', product size 219 bp), and the following
 25 condition: 1 cycle of 98°C for 4 min, 35 cycles of 98°C for 10 sec, 60°C for 15 sec and
 26 68°C for 30 sec. Electroporated PSD-95 targeting vector (EGFP), Syp targeting vector
 27 (EGFP), and PSD-95 targeting vector (mClover) were detected with primer sets
 28 (5'-AATGCAGCCTGCCAGGAAGGAGGTA-3' and
 29 5'-GACACGCTGAACTTGTGGCCGTTTACG-3', product size 670 bp,
 30 5'-ATGCCCATTCTGTTCACTCAGCC-3' and
 31 5'-GACACGCTGAACTTGTGGCCGTTTACG-3', product size 578 bp,
 32 5'-TGCATCCTTGATGTCTCAGCCAATG-3' and
 33 5'-CGTCCAGCTCGACCAGGATGGGCAC-3', product size 1320 bp, respectively)
 34 and the following condition: 1 cycle of 98°C for 4 min, 38 cycles of 98°C for 10 sec,
 35 65°C for 10 sec and 68°C for 30 sec.

RT-PCR

Total RNAs were extracted from mouse brains using RNeasy Mini kit (Qiagen), and reverse-transcribed using SuperScript®III Reverse Transcriptase (Thermo Fisher) according to the manufacturer's instruction. To detect the expression of *PSD-95-mClover* fusion gene, RT-PCR was carried using KOD FX Neo DNA polymerase, primers (5'-ACAACAGCCACCTCTACGGGACC-3' and 5'-CGTCCAGCTCGACCAGGATGGGCAC-3', product size 504 bp), and the following condition: 1 cycle of 98°C for 3 min, 40 cycles of 98°C for 10 sec, 68°C for 40 sec. The expression of *tdTomato* from pCAG-tdTomato was detected by RT-PCR with primers (5'-CGATTACAAGAAGCTGTCCTTCC-3' and 5'-GCCATGTAGATGGTCTTGAAGCTC-3', product size 309 bp) and the following condition: 1 cycle of 98°C for 3 min, 35 cycles of 98°C for 10 sec, 62°C for 15 sec, 68°C for 30 sec. The expression of *Actb* was detected by RT-PCR with primers (5'-ATGTACGTAGCCATCCAGGCTGT-3' and 5'-AGCTGTGGTGGTGAAGCTGTAG-3', product size 219 bp) and the following condition: 1 cycle of 98°C for 3 min, 30 cycles of 98°C for 10 sec, 60°C for 15 sec, 68°C for 30 sec.

Quantitative RT-PCR was performed on a CFX96 Touch Real-time PCR Detection System (Bio-Rad) using KOD SYBR®qPCR Mix (Toyobo, Osaka, Japan) and the primers described above. All qRT-PCR samples were analyzed in triplicate. The expression level of *PSD-95-mClover* fusion gene for each sample was normalized to the expression of *tdTomato* from pCAG-tdTomato co-electroporated with the CRISPR construct and the targeting vector as a transfection control.

Dark and light adaptation

For preparation of dark-adapted retinas, mice were kept in the dark for 6 hours, and eyes were enucleated under dim red light using Darkroom Safelight (Paterson Photographic Ltd. West Midlands, UK), and fixed in 4% PFA. For preparation of light-adapted retinas, dark-adapted mice were kept under room light (1500 lux) for 30 min, and then eyes were enucleated and fixed.

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Figure S1.

Tagging endogenous rhodopsin with EGFP in the mouse retina.

(A) Structures of the mouse *rhodopsin* locus and the knock-in targeting vector to produce a rhodopsin-EGFP fusion protein. The gRNA targeting sequence is underlined and the PAM sequence is shown in red. The predicted Cas9-gRNA cutting position is indicated with a scissor symbol. (B) Low magnification views of the retinal section shown in **Fig.1D**. Mouse retinas were co-electroporated at P0 with three plasmids: Rhodopsin targeting vector (EGFP), CAG-mCherry, and CBh-Cas9/U6-gRNA(Rho), and harvested at P21. Retinas were sectioned and stained with anti-GFP (green) and anti-RFP (red) antibodies. Cell nuclei were visualized with DAPI (blue). GFP signal was detected only in rod photoreceptors. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

Figure S2.

Effect of the length of the homology arms of the donor DNA on knock-in efficiency at the mouse *rhodopsin* locus.

(A) Structures of the knock-in targeting vectors to produce a rhodopsin-2A-H2B-EGFP fusion protein, having various lengths of 5' and 3' homology arms. Each targeting vector has the 2kb (pUC)- or 3kb (pBluescript II (pBS))-plasmid backbone. (B) Knock-in efficiency of the targeting vectors. Mouse retinas were co-electroporated at P0 with three plasmids: Rhodopsin targeting vector (2A-H2B-EGFP), CAG-H2B-mCherry, and CBh-Cas9/U6-gRNA(Rho). Retinas were harvested at P21, dissociated into single cells, and subjected to FACS analysis to determine the numbers of EGFP- and mCherry-positive cells. Three independently electroporated retinas were analyzed, and relative knock-in efficiency (the number of EGFP/mCherry double-positive cells among the total number of mCherry-positive cells) for each targeting vector was shown. Data represent mean $\pm$ SD (n=3).

Figure S3.

Characterization of the rhodopsin promoter introduced into developing mouse retina by electroporation.

(A, B) P0 mouse retinas were electroporated with Rhodopsin promoter-tdTomato and CAG-EGFP. Retinas were harvested at P2, P5, and P8, and sectioned and stained with anti-GFP (green), anti-RFP (red), and anti-phospho-Histone H3 (white, A) or anti-Ki67 (white, B) antibodies. Cell nuclei were visualized with DAPI (blue). Retinal

cells expressing tdTomato cells were negative for phospho-Histon H3 and Ki67. **(C)** P0 mouse retinas were electroporated with Rhodopsin promoter-Cre, CAG-EGFP, and a Cre-dependent fluorescent reporter, CALNL-tdTomato. Retinas were harvested at P10 when retinogenesis is complete, and sectioned and stained with anti-GFP (green), and anti-RFP (red) antibodies. Cell nuclei were visualized with DAPI (blue). tdTomato-positive signal was detected only in rod photoreceptors in the ONL, indicating that the rhodopsin promoter is not leaky in multipotent retinal progenitors. NBL, neuroblastic layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

Figure S4.

Tagging endogenous glutamine synthetase with mClover in the mouse retina.

(A) Structures of the mouse *glutamine synthetase* (*Glul*) locus and the knock-in targeting vector to produce a Glul-EGFP fusion protein. The gRNA targeting sequence is underlined and the PAM sequence is shown in red. The predicted Cas9-gRNA cutting position is indicated with a scissor symbol. **(B)** Isolation of mClover-positive cells by FACS for single-cell genotyping. P0 mouse retinas were electroporated with Glul targeting vector (mClover), CAG-H2B-mCherry, and CBh-Cas9/U6-gRNA (*Glul*). Retinas were harvested at P21, dissociated into single cells by enzymatic digestion, and mClover/H2B-mCherry double-positive cells were purified by FACS. **(C)** Collected mClover/H2B-mCherry double-positive single cells (Cell G1-G4) in **(B)** were subjected to genomic PCR with the PCR primers shown in **(A)**. A mClover-negative single cell (Cell WT) was used as a negative control.

Figure S5.

Tagging endogenous arrestin with EGFP in the mouse retina.

(A) Structures of the mouse *arrestin* locus and the knock-in targeting vector to produce an arrestin-EGFP fusion protein. The predicted Cas9-gRNA cutting position is indicated with a scissor symbol. **(B)** Isolation of EGFP-positive cells by FACS for single-cell genotyping. P0 mouse retinas were electroporated with Arrestin targeting vector (EGFP), CAG-H2B-mCherry, and CBh-Cas9/U6-gRNA (*arrestin*). Retinas were harvested at P21, dissociated into single cells by enzymatic digestion, and EGFP/H2B-mCherry double-positive cells were purified by FACS. **(C)** Collected EGFP/H2B-mCherry double-positive single cells (Cell A1-A4) in **(B)** were subjected to genomic PCR with the PCR primers shown in **(A)**. A EGFP-negative single cell (Cell

WT) was used as a negative control. (D) Western blot analysis of retinas electroporated with the indicated plasmids at P0 and harvested at P21. Membranes were probed with anti-GFP, anti-arrestin, and anti-RFP antibodies. An arrestin-EGFP fusion protein (~74 kDa) was detected only in the presence of CBh-Cas9/U6-gRNA (arrestin).

Figure S6.

Tagging endogenous synaptophysin with EGFP in the mouse retina.

(A) Structures of the mouse *synaptophysin* (*Syp*) locus and the knock-in targeting vector to produce a Syp-EGFP fusion protein. The gRNA targeting sequences are underlined and the PAM sequences are shown in red. The predicted Cas9-gRNA cutting positions are indicated with scissor symbols. These two gRNAs were used together to increase the knock-in efficiency. (B) P0 mouse retinas were electroporated with Syp targeting vector (EGFP), CAG-mCherry, and CBh-Cas9 with or without gRNAs for *Syp*, and harvested at P21. DNAs were extracted from the intact retinas, and subjected to PCR analysis. PCR primer sets were used to detect the knock-in allele, the targeting vector, *mCherry*, and endogenous mouse *Actb*, respectively. Lane 1: DNA prepared from the retinas electroporated without CBh-Cas9/U6-gRNA (*Syp*). Lane2: DNA prepared from the retinas electroporated with CBh-Cas9/U6-gRNA (*Syp*). Lane3: mouse *Syp* targeting vector (EGFP) plasmid DNA.

Figure S7.

Tagging endogenous PSD-95 with EGFP in the mouse retina.

(A) Structures of the mouse *PSD-95* locus and the knock-in targeting vector to produce a PSD-95-EGFP fusion protein. The gRNA targeting sequences are underlined and the PAM sequences are shown in red. The predicted Cas9-gRNA cutting positions are indicated with scissor symbols. These two gRNAs were used together to increase the knock-in efficiency. (B) P0 mouse retinas were electroporated with PSD-95 targeting vector (EGFP), CAG-mCherry, and CBh-Cas9 with or without gRNAs for *PSD-95*, and harvested at P21. DNAs were extracted from the intact retinas, and subjected to PCR analysis. PCR primer sets were used to detect the knock-in allele, the targeting vector, *mCherry*, and endogenous mouse *Actb*, respectively. Lane 1: DNA prepared from the retinas electroporated without CBh-Cas9/U6-gRNA(PSD-95). Lane2: DNA prepared from the retinas electroporated with CBh-Cas9/U6-gRNA(PSD-95). Lane3: mouse PSD-95 targeting vector (EGFP)

1 plasmid DNA.

3 **Figure S8.**

4 **Use of two gRNAs increases knock-in efficiency in the mouse retina**

5 (A) Structures of the mouse *synaptophysin* (*Syp*) locus and the knock-in targeting
6 vector to produce a Syp-mClover fusion protein. Two gRNA targeting sequences
7 (gRNA_A and gRNA_B) are underlined and the PAM sequences are shown in red.
8 The predicted Cas9-gRNA cutting positions are indicated with scissor symbols. (B)
9 Estimated knock-in efficiency at the mouse *Syp* locus. P0 mouse retinas were
10 electroporated with Syp targeting vector (mClover), CAG-H2B-mCherry, and
11 CBh-Cas9 with single gRNA or two gRNAs for *Syp*, or without gRNA. Retinas were
12 harvested at P18, sectioned and stained with anti-GFP and anti-RFP antibodies. Cell
13 nuclei were visualized with DAPI. Sections were made from two independently
14 electroporated retinas for each DNA sample, and ten sections per retina were
15 analyzed. Knock-in efficiency (the number of mClover-positive cells per the number of
16 mCherry-positive cells) was shown. Data represent mean +/- SD (n=20).

18 **Figure S9.**

19 **Tagging endogenous PSD-95 with mClover in the mouse brain.**

20 (A) Structures of the mouse *PSD-95* locus and the knock-in targeting vector to
21 produce a PSD-95-mClover fusion protein. The gRNA targeting sequences are
22 underlined and the PAM sequences are shown in red. The predicted Cas9-gRNA
23 cutting positions are indicated with scissor symbols. These two gRNAs were used
24 together to increase the knock-in efficiency. (B) RT-PCR analysis of electroporated
25 mouse brains. E14.5 mouse brains were electroporated with mouse PSD-95 targeting
26 vector (mClover), CAG-tdTomato, and CBh-Cas9 with or without gRNAs for *PSD-95*,
27 and harvested at P65. Total RNAs were extracted from the brains, and subjected to
28 RT-PCR analysis. PCR primer sets were used to detect cDNAs encoding
29 *PSD-95-mClover*, *tdTomato*, and endogenous mouse *Actb*, respectively. Lane 1: RNA
30 prepared from the brains electroporated without CBh-Cas9/U6-gRNA (*PSD-95*).
31 Lanes 2, 3: RNA prepared from the brains electroporated with CBh-Cas9/U6-gRNA
32 (*PSD-95*). Lane 4: mouse PSD-95 targeting vector (mClover) plasmid DNA. (C)
33 Genomic PCR analysis of electroporated mouse brains. E14.5 mouse brains were
34 electroporated with mouse PSD-95 targeting vector (mClover), CAG-tdTomato, and
35 CBh-Cas9 with or without gRNAs for PSD-95, and harvested at P65. DNAs were

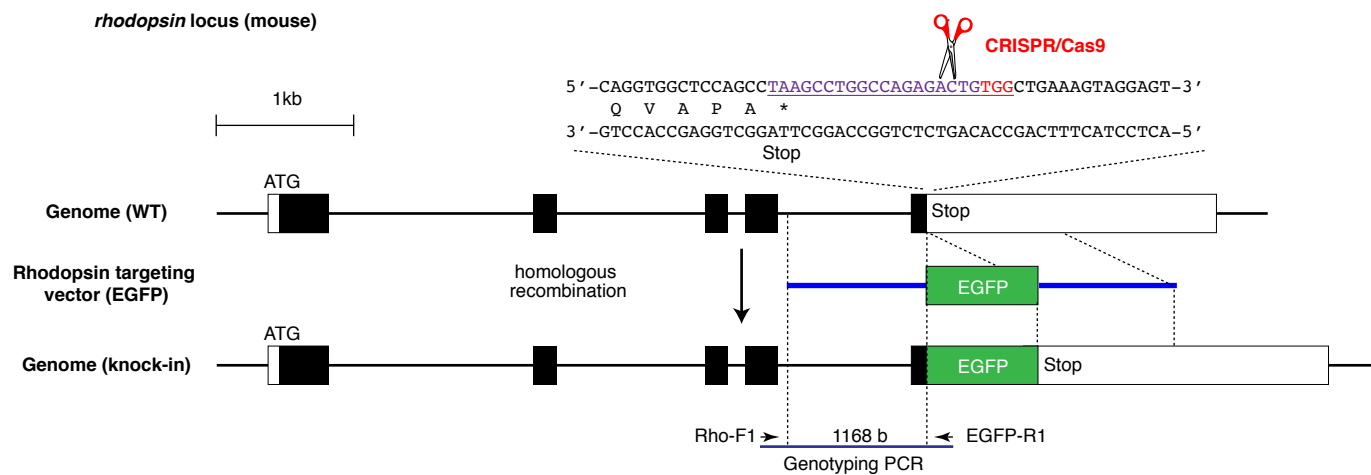
1 extracted from the brains, and subjected to PCR analysis. PCR primer sets were used
2 to detect the knock-in allele, the targeting vector, *tdTomato*, and endogenous mouse
3 *Actb*, respectively. Lane 1: DNA prepared from the brains electroporated without
4 CBh-Cas9/U6-gRNA (PSD-95). Lanes 2, 3: DNA prepared from the brains
5 electroporated with CBh-Cas9/U6-gRNA (PSD-95). Lane 4: mouse PSD-95 targeting
6 vector (mClover) plasmid DNA.

7
8 **Figure S10.**

9 **Full-length images of the agarose gels and immunoblots presented in Figure 6.**

10 **(A)** Full-length images of the immunoblots shown in **Fig.6D**. **(B)** Full-length images of
11 the immunoblots shown in **Fig.6G**. **(C)** Full-length images of the agarose gels shown
12 in **Fig.6H**.

A



B

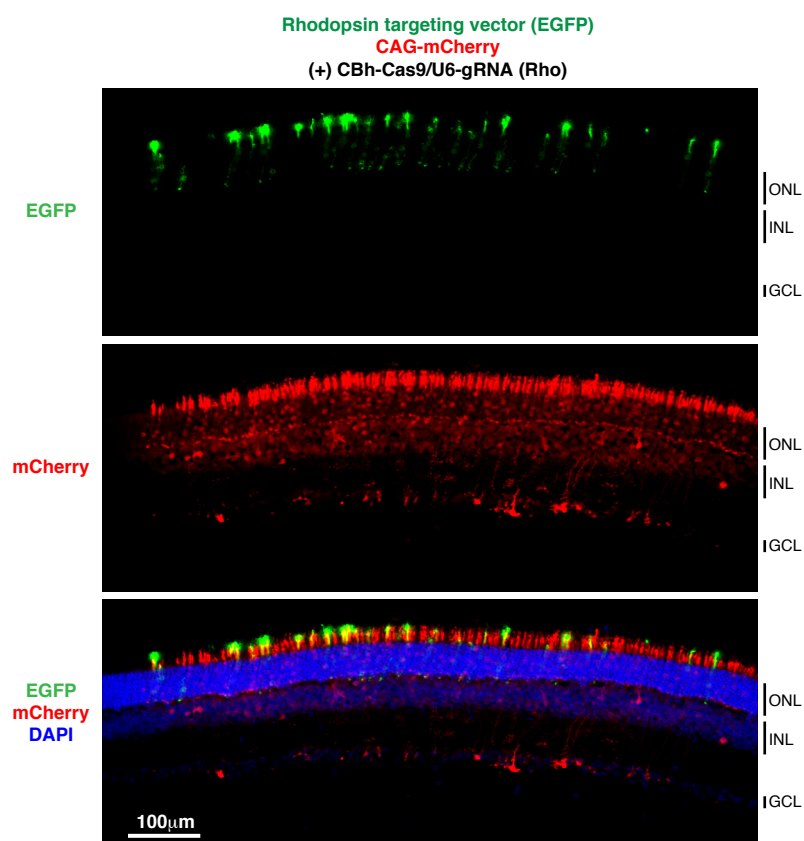
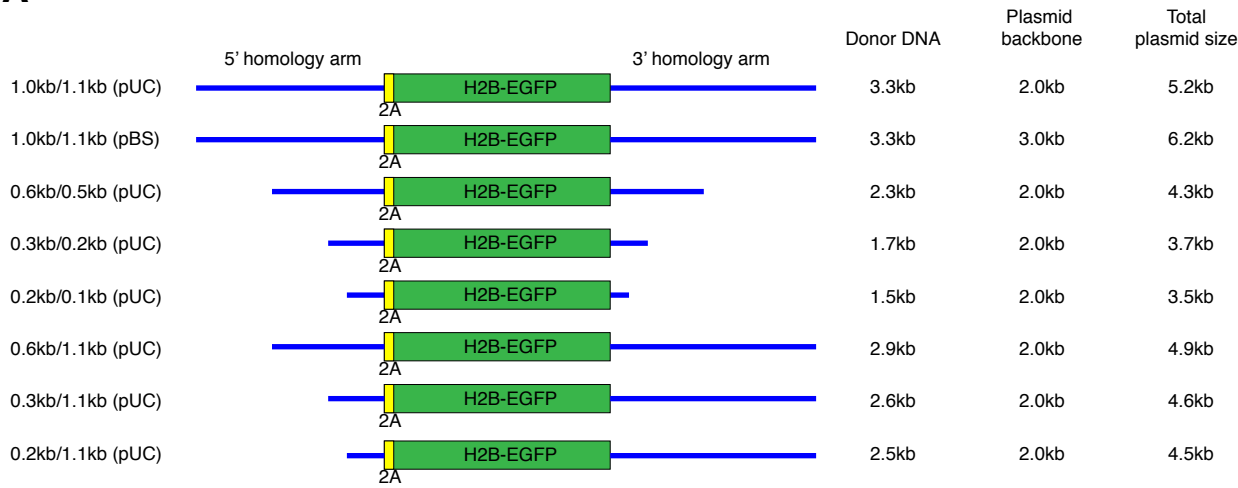


Fig.S1

A



B

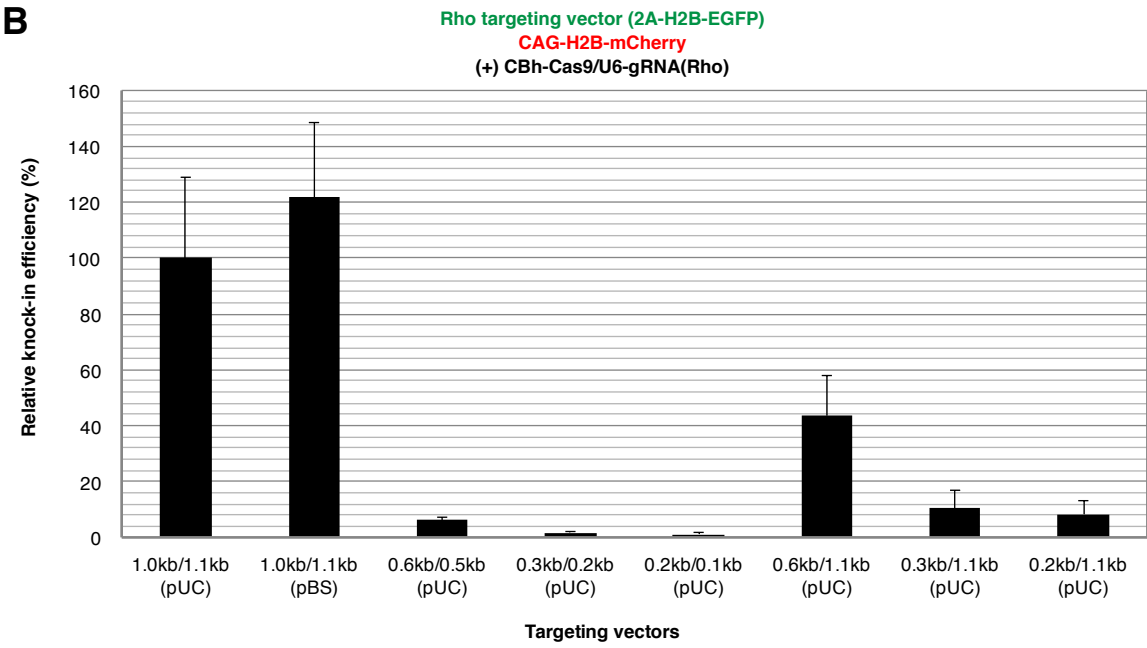
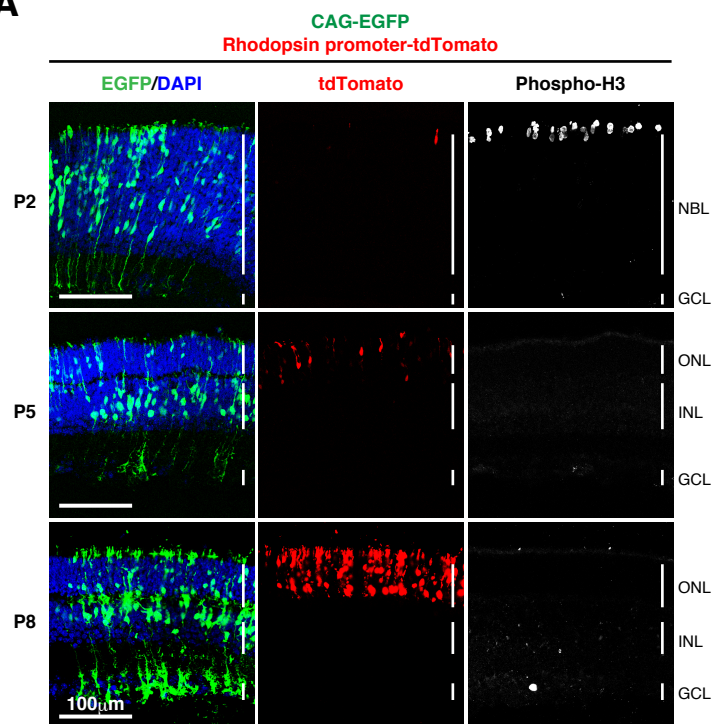
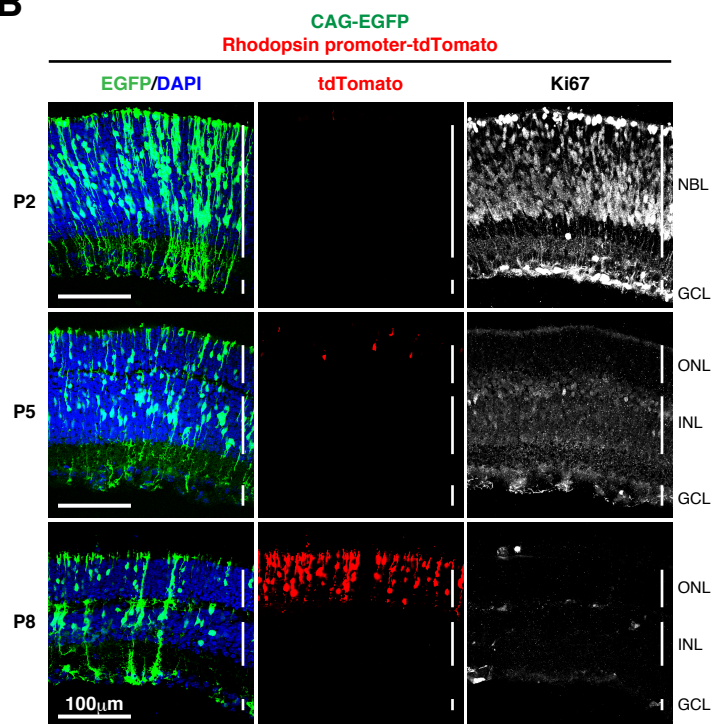
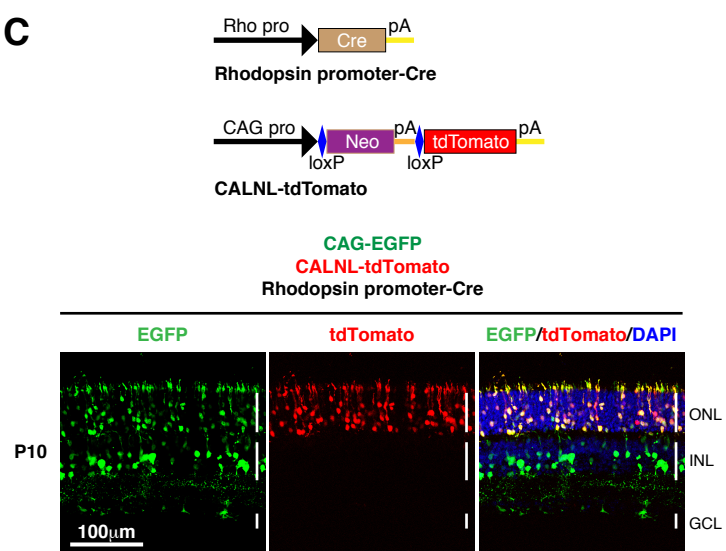
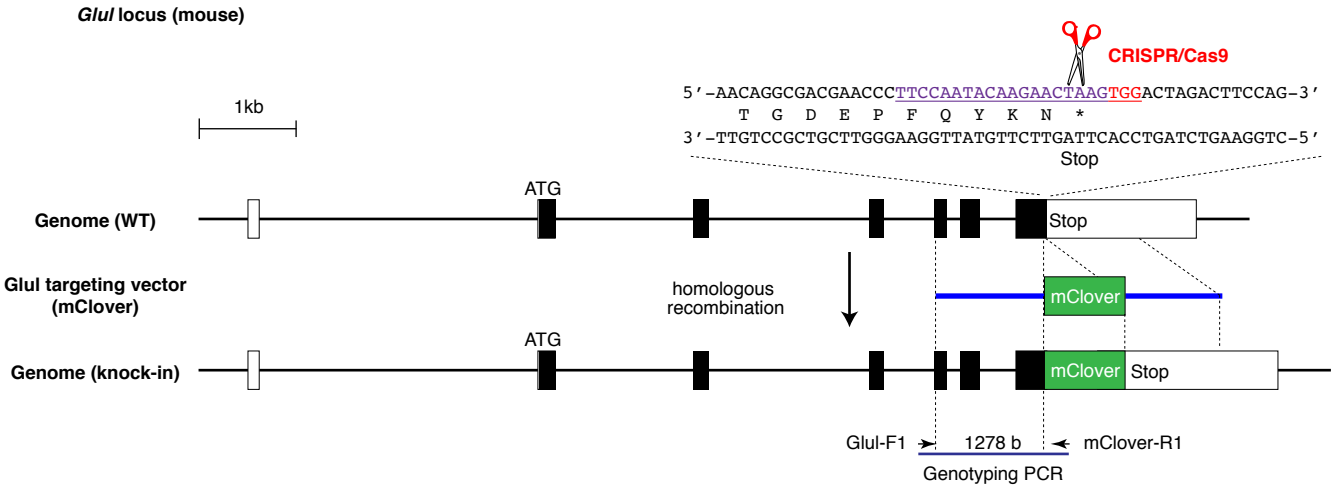


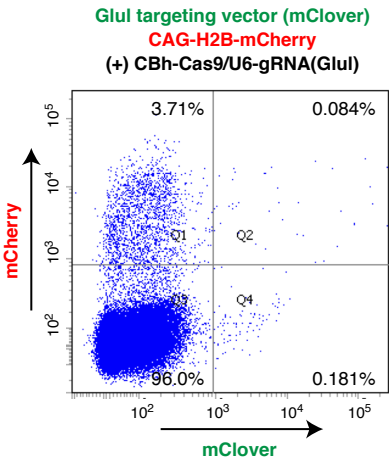
Fig.S2

A**B****C**

A



B



C

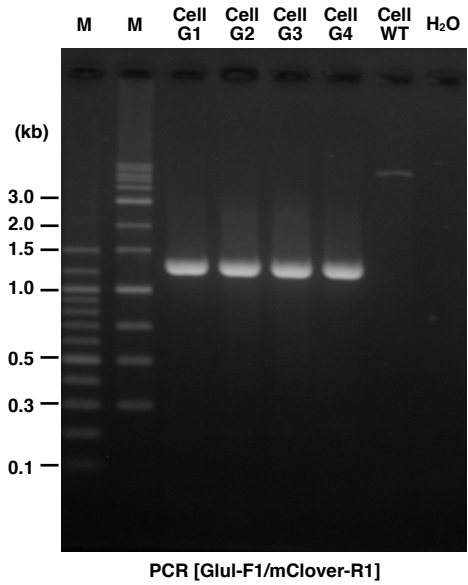


Fig.S4

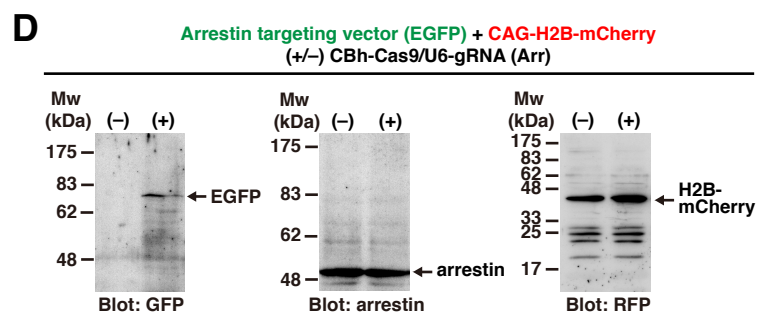
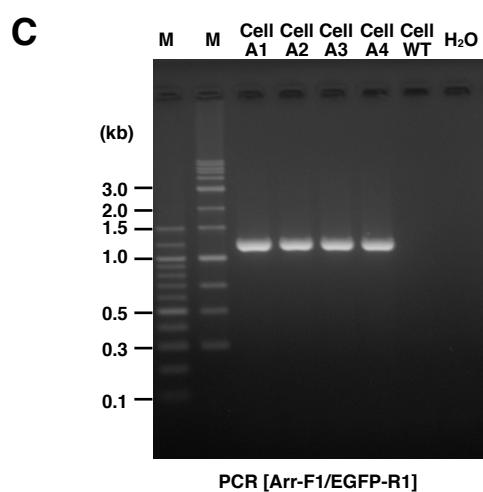
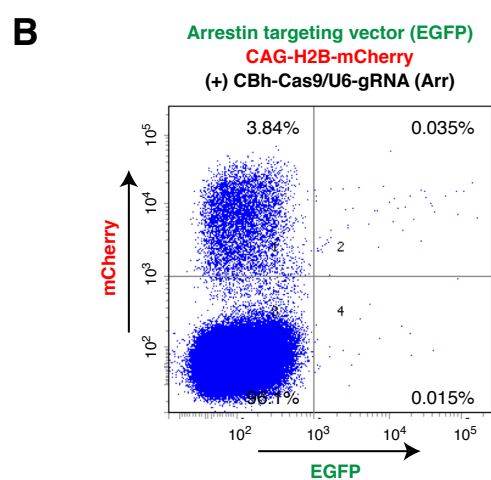
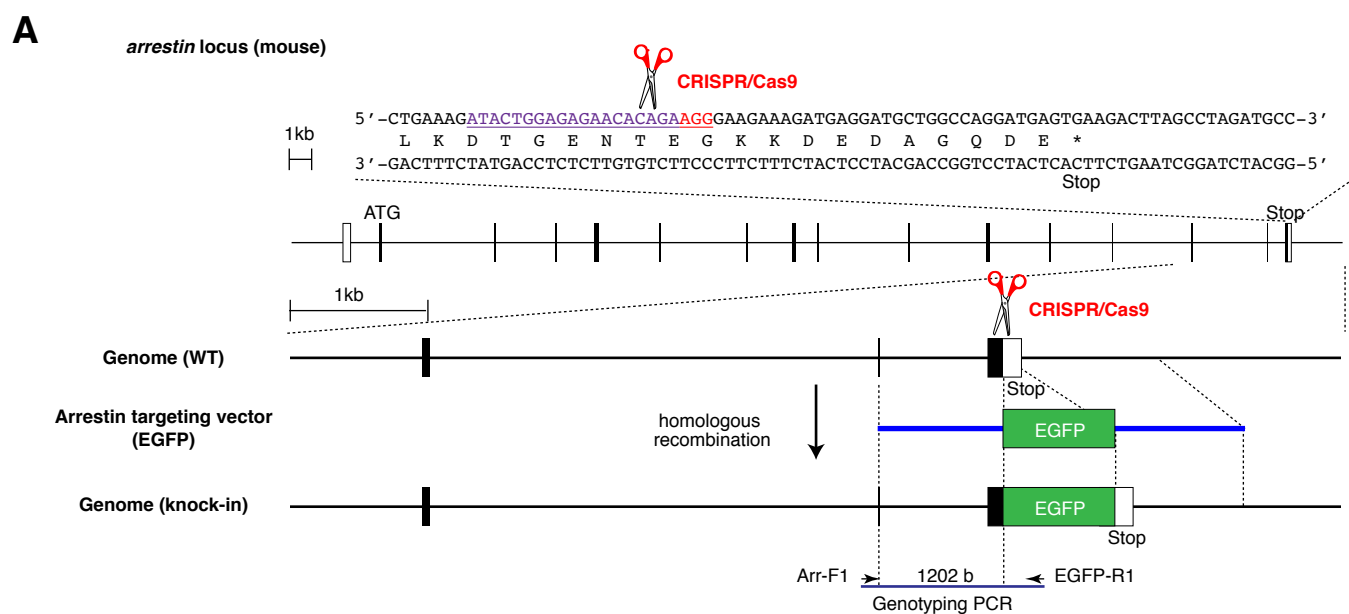


Fig.S5

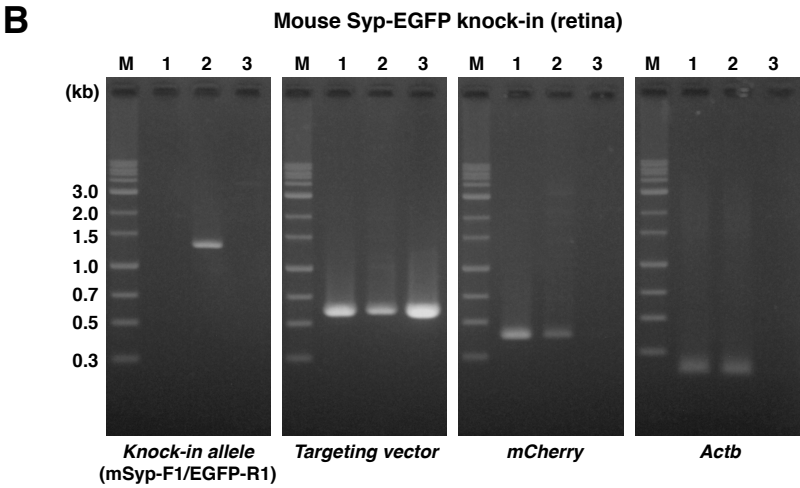
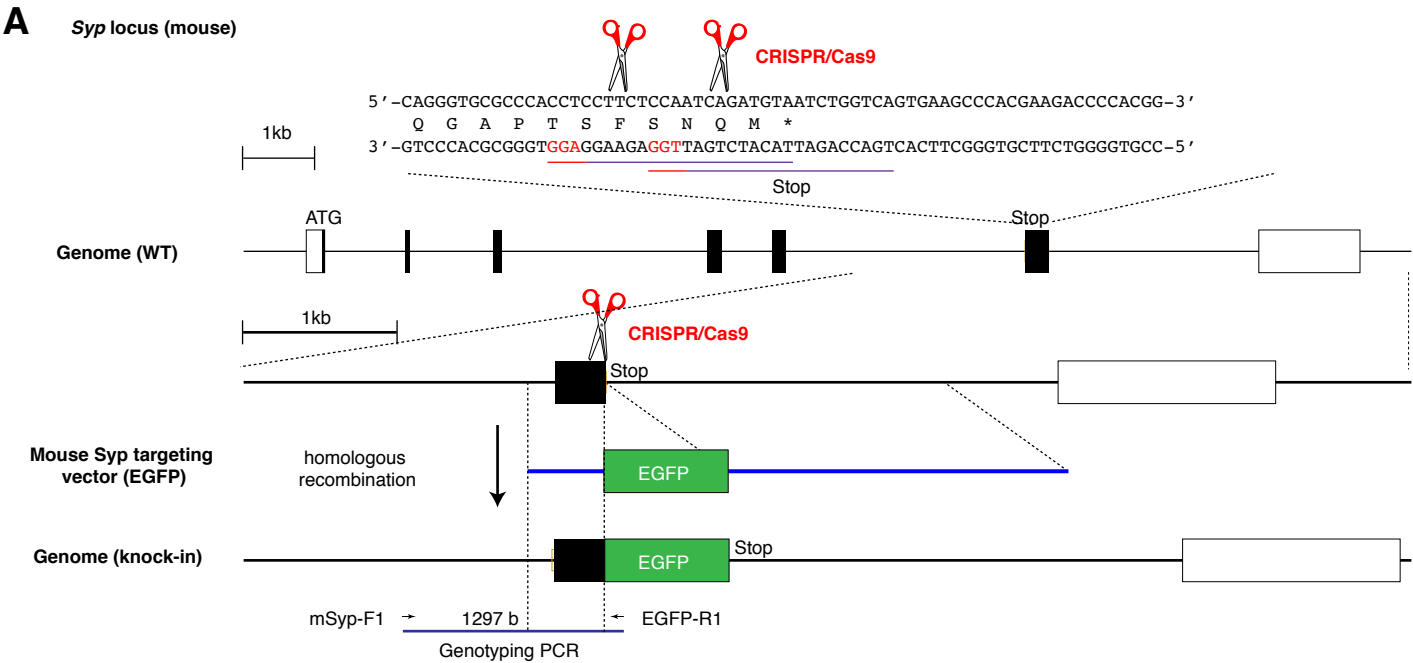


Fig.S6

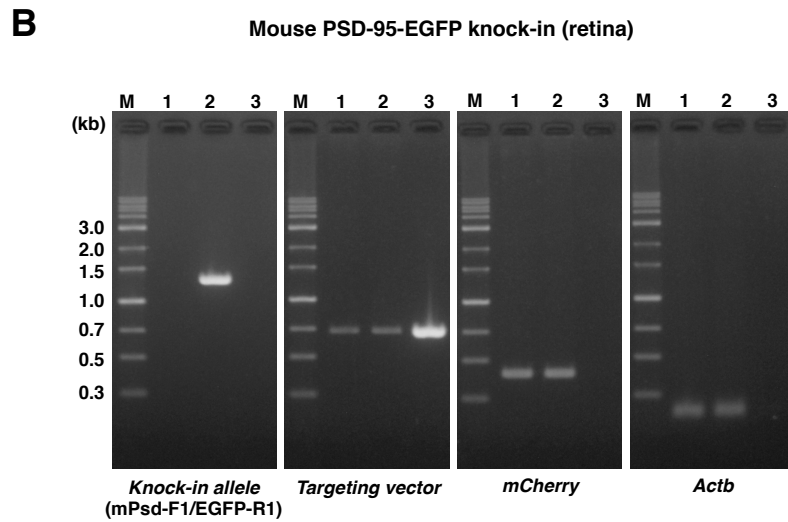
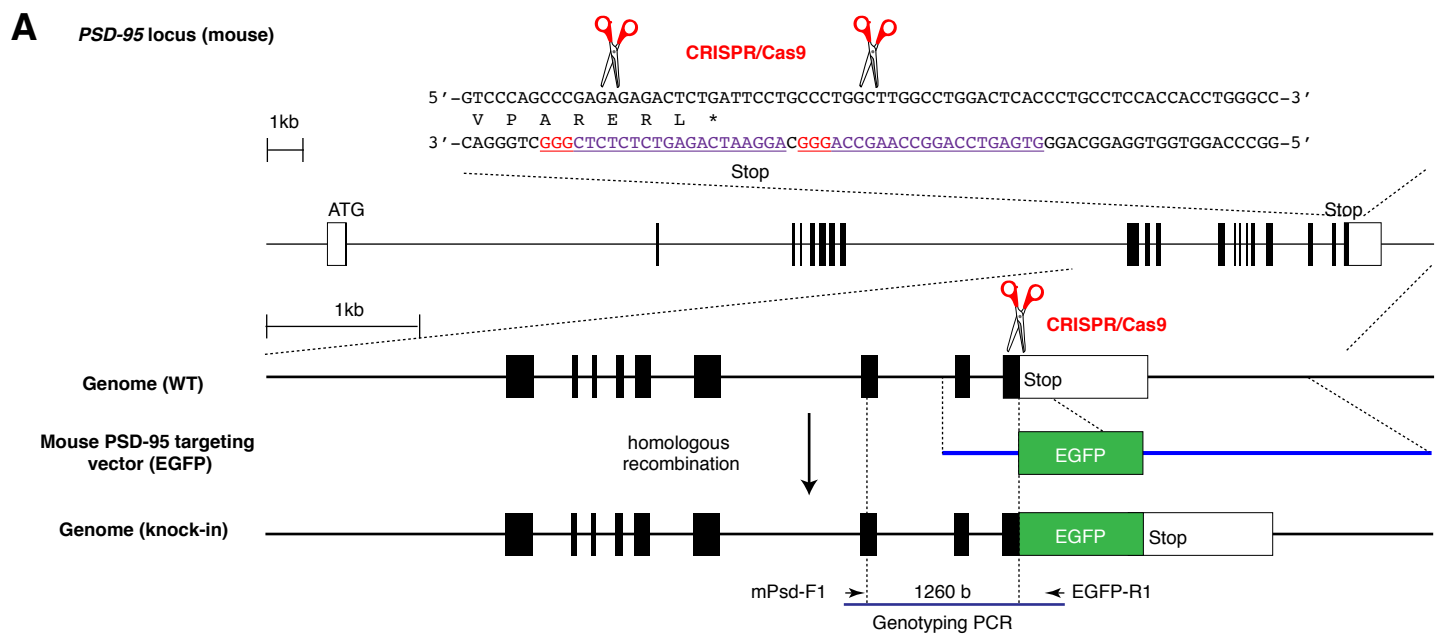


Fig.S7

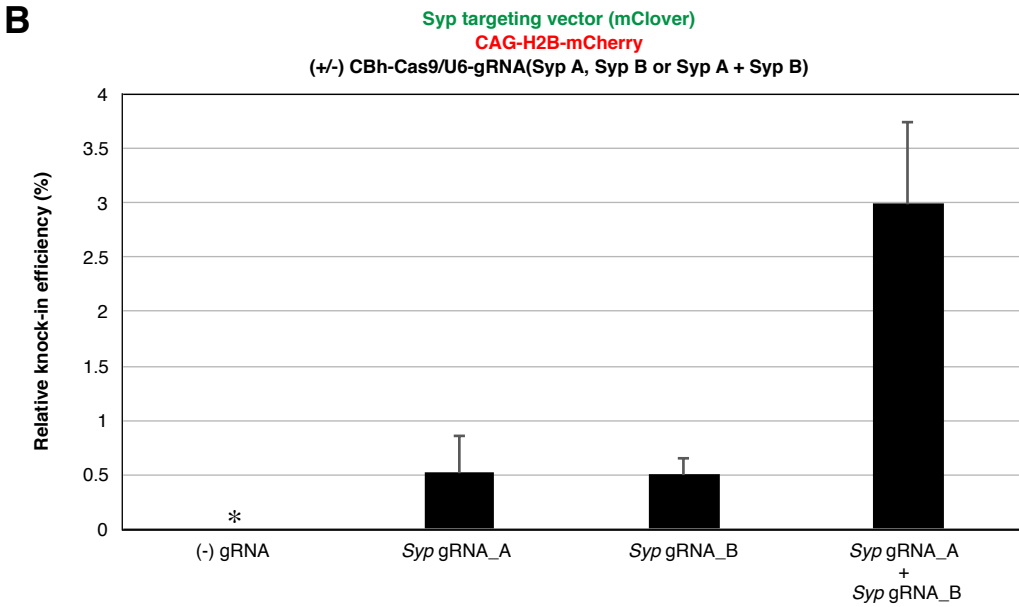
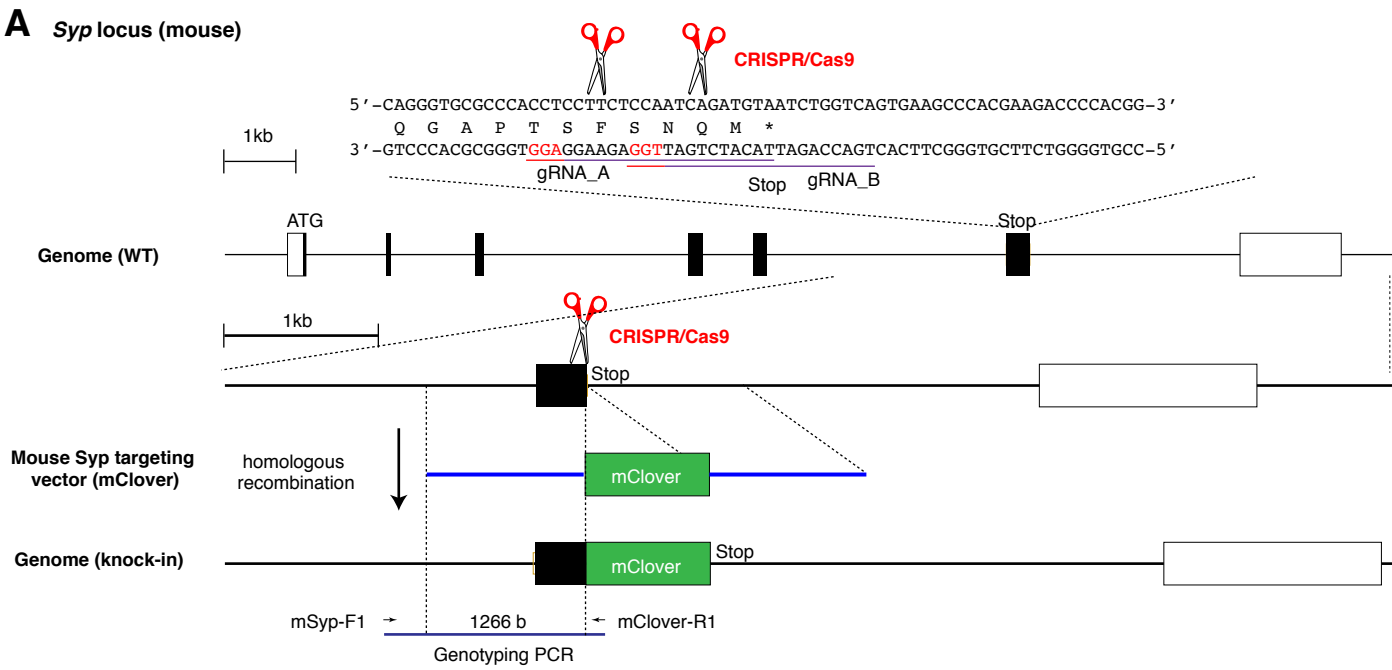
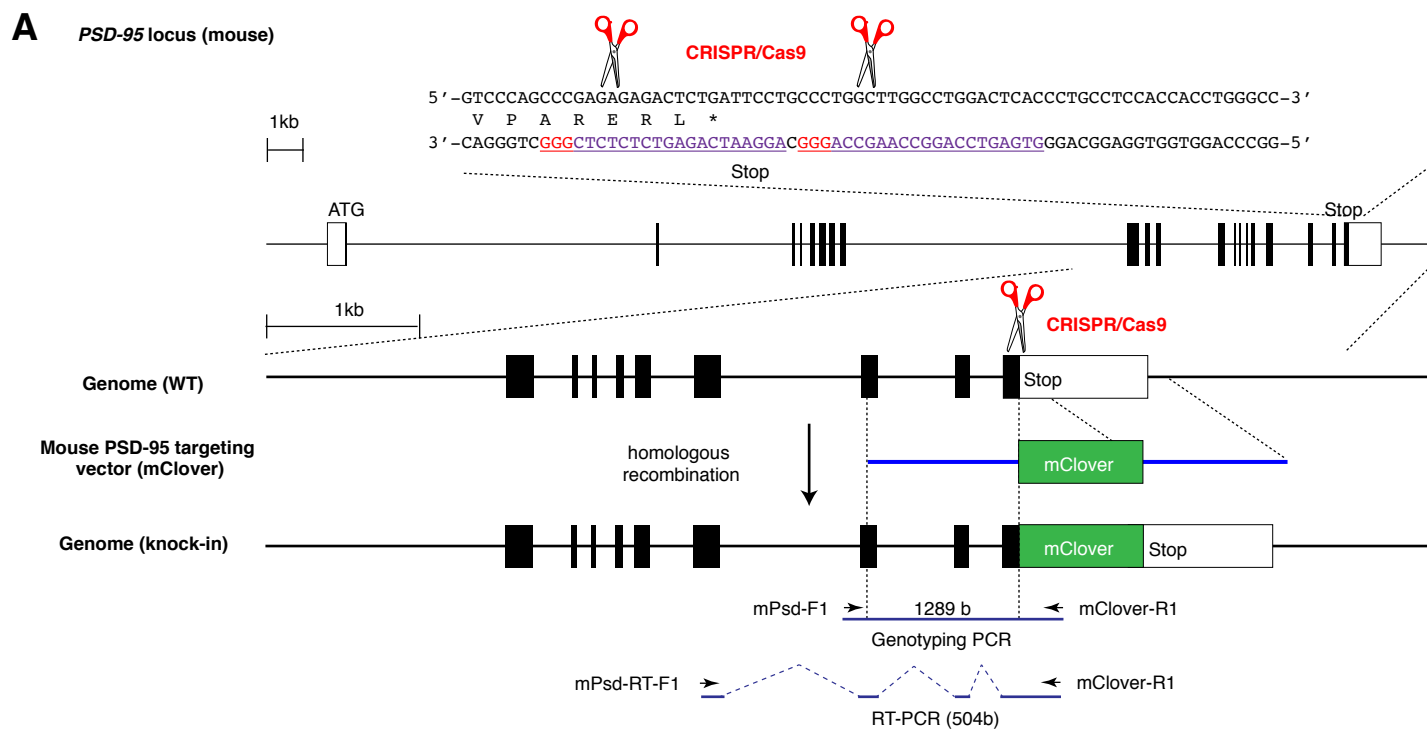
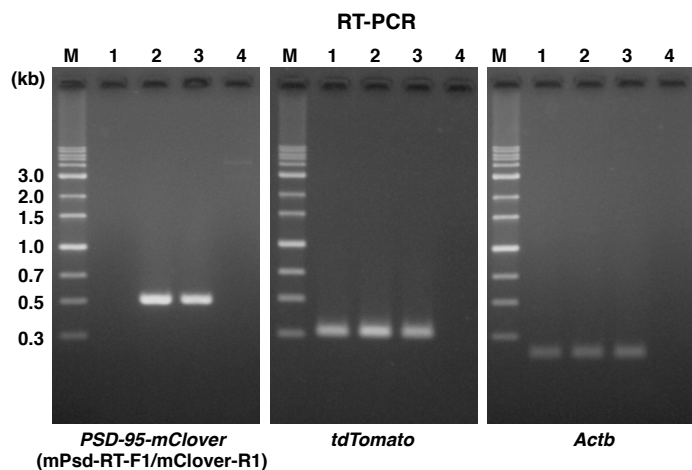


Fig.S8



B Mouse *PSD-95*-mClover knock-in (brain)



C Mouse *PSD-95*-mClover knock-in (brain)

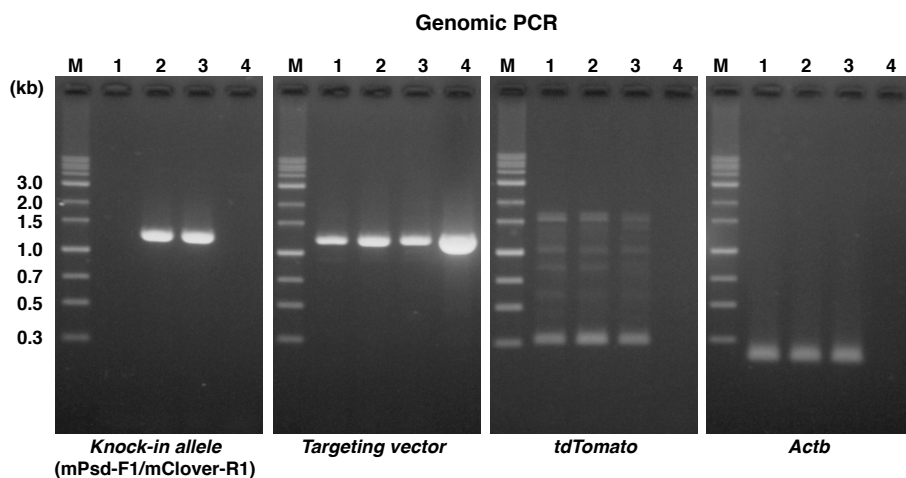
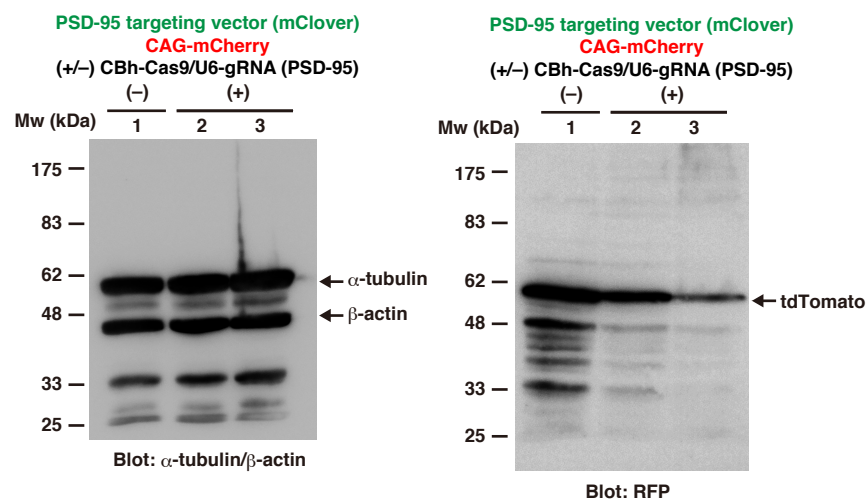
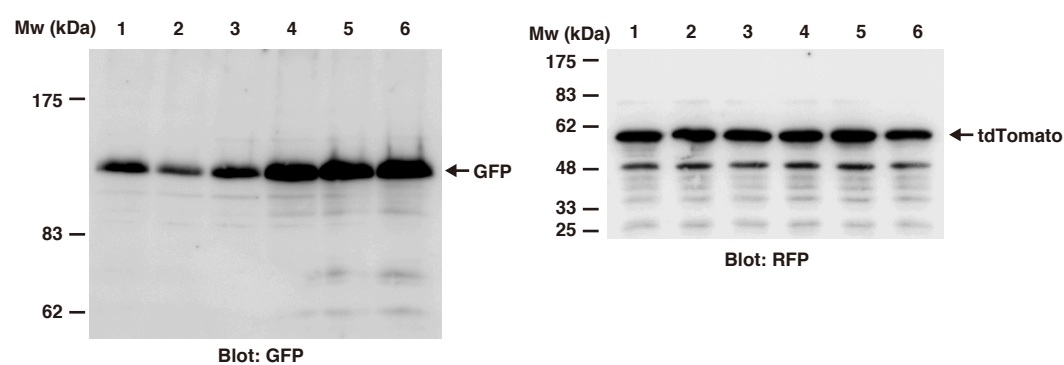


Fig.S9

A



B



C

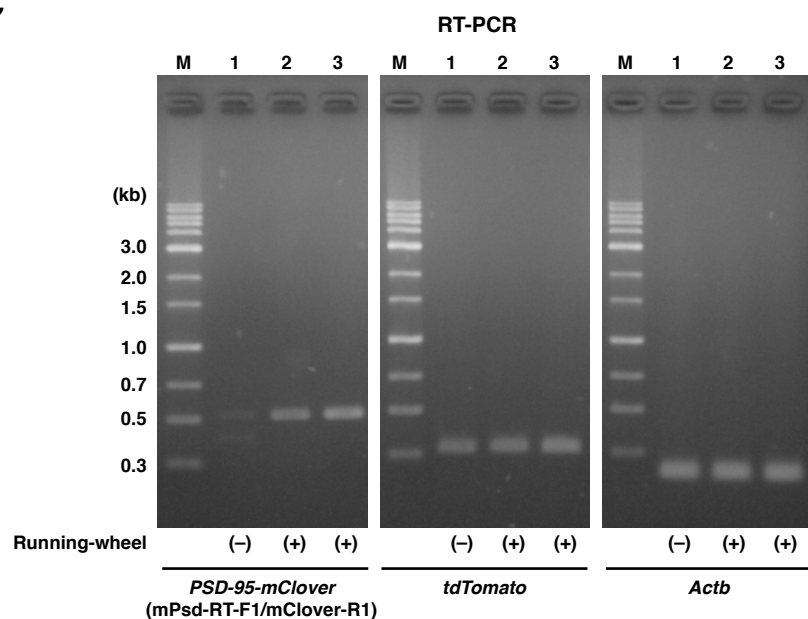


Fig.S10