Pou4f2 knock-in Cre mouse: A multifaceted genetic tool for vision researchers

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Purpose: A transgenic mouse that expresses Cre recombinase under control of the Pou4f2-promoter (also referred to as *Brn-3b* and *Brn-3.2*) was characterized. Pou4f2 expression has been reported in a subset of retinal ganglion cells (RGCs) in the retina, in the midbrain, and in the germline. In this study, we characterize the expression pattern of this Cre-recombinase line and report its utility in targeted deletion, temporal deletion, RGC depletion, and germline targeting, which can be regulated by the sex of the Cre-carrying mouse.

Methods: *Pou4f2*[Cre] was mapped by using a combination of PCR and sequencing of PCR products to better understand the construct and to locate where it was inserted within the *Pou4f2* locus. Cre expression patterns were examined by crossing *Pou4f2*[Cre/+] mice to Cre reporter mice. Immunohistochemistry was used to further define the pattern of Cre expression and Cre-mediated recombination within the retina, brain, and other tissues.

Results: An internal ribosome entry site (IRES)-Cre cassette was inserted into the *Pou4f2* gene disrupting normal gene function, as verified by the depletion of RGCs in mice homozygous for the insert. *Pou4f2*[Cre] expression was observed in the retina, brain, peripheral neurons, and male germ cells. Germline recombination was observed when the sire carried the Cre and the target for recombination. In all other breeding schemes, recombination was observed within subsets of cells within the retina, brain, intestines, heart, and gonads. In the retina, Cre efficiently targets recombination in neurons within the RGC layer (RGL), the inner nuclear layer (INL), and a small percentage of photoreceptors, activity that has not been previously reported. Unlike most other Cre lines active in the inner retina, recombination in Müller and other glia was not observed in mice carrying *Pou4f2*[Cre]. Within the visual centers of the brain, Cre targets recombination in about 15% of cells within the superchiasmatic nucleus, lateral geniculate nucleus, and superior colliculus.

Conclusions: *Pou4f2*[Cre] provides multiple uses for the vision researcher's genetic toolkit. First, *Pou4f2*[Cre] is a knock-in allele that can be used to eliminate *Pou4f2*, resulting in depletion of RGCs. Second, expression of Cre in male germ cells makes this strain an efficient germline activator of recombination, for example, to target LoxP-flanked sequences in the whole mouse. Third, *Pou4f2*[Cre] efficiently targets RGCs, amacrine cells, bipolar cells, horizontal cells, and a small number of photoreceptors within the retina, as well as the visual centers in the brain. Unlike other Cre recombinase lines that target retinal neurons, no recombination was observed in Müller or other retinal glia. These properties make this Cre recombinase line a useful tool for vision researchers.

Targeted manipulation of the genome has greatly improved our understanding of the mechanisms responsible for development; however, many such genetic manipulations result in severe abnormalities and high mortality rates. Understanding cell autonomy of defects often also necessitates a more targeted approach than eliminating or overexpressing a gene. Cre-lox technology, in which Cre recombinase excises or inverts the sequence flanked by LoxP sites, depending on the orientation of the sites, allows for targeted activation or inactivation of DNA sequences [1]. Using this technology has enabled researchers to target specific populations of cells or specific time points. Characterizing and disseminating various Cre transgenic lines allows researchers to refine experiments and ask better informed questions.

In this study, we characterize a mouse strain in which Cre recombinase has been inserted into the *Pou4f2* locus (also referred to as *Brn-3b* or *Brn-3.2*), replacing the *Pou4f2* gene. Previous work has shown that the POU domain transcription factor *Pou4f2* is expressed in a large subset of retinal ganglion cells (RGCs) and is essential for RGC differentiation [2-5], survival [6] and axon path-finding [7-9]. The study herein characterizes a *Pou4f2* knock-in Cre recombinase mouse line, *Pou4f2*[Cre], which expresses Cre recombinase in a subset of retinal neurons. In addition to targeting genes in the mouse retina, the *Pou4f2*[Cre] line can be used to generate mice in which the number of RGCs is depleted, when the transgene is homozygous [2,3]. When carried by male mice, along with a target allele, *Pou4f2*[Cre] efficiently targets recombination in

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the germline. Therefore, a single mouse strain can be used to target recombination in retinal neurons, to deplete RGCs, and to target germline recombination.

METHODS

Mouse care and housing: All protocols were performed in accordance with the University of Idaho Institutional Animal Care and Use Committee and with The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were fed ad libitum under a 12 h:12 h light-dark cycle.

Mouse strains: The following mouse strains were used in this study: *Pou4f2*[Cre] (courtesy of Dr. Vann Bennett; Duke University) [10], Ai9 reporter (The Jackson Laboratory, Bar Harbor, ME; stock number 007909) [11], Brainbow reporter (The Jackson Laboratories stock number 021,227) [12], and *Dscam* conditional mutant mice [10]. Mouse strains were maintained on a mixed C57Bl6/J, C3H/HeJ, and 129 background. All mice carried the wild-type allele of *Pde6b*.

Genotyping: Tissue biopsies (toe tips or tail tips) were taken from the mice and then prepared for PCR by first boiling the samples in 50 µl of 25 µM sodium hydroxide and 0.2 µm

EDTA for 15 min. Fifty microliters of Tris Cl (pH 5.0) was then added to the samples, neutralizing them. Each PCR reaction consisted of 7.5 µl of OneTaq Hot Start Master Mix (2X, New England Biolabs, Ipswich, MA) [13], 0.1 µl primers, 1 µl of DNA sample, and 6.4 µl of nuclease-free water, bringing the final volume to 15 µl. PCR conditions: (36 cycles; 94 °C 30 s, 57 °C 30 s, 72 °C 2 min). The following primers were used when genotyping: *Pou4f2* forward (#1, Figure 1A): 5′-GCT GAG GTC TGA AGC CAG AG-3′, *Pou4f2* reverse (#2, Figure 1A): 5′-CAG TCA GCT CCT CGC TTT CT-3′; Cre forward (#3, Figure 1B): 5′-GCA TTA CCG GTC GAT GCA ACG AGT G-3′, Cre reverse (#4, Figure 1B): 5′-GAG TGA ACG AAC CTG GTC GAA ATC A-3′; and neomycin forward (#6, Figure 1B): 5′-AGG ATC TCC TGT CAT CTC ACC TTG CTC CTG-3′, neomycin reverse (#5, Figure 1B): 5′-AAG AAC TCG TCA AGA AGG CGA TAG AAG GCG-3′.

Tissue preparation: Mice were anesthetized with an intra-peritoneal injection of tribromoethanol (500 mg per kg body weight). Anesthetized mice were perfused with PBS (1X; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4; 1.8 mM KPO_4), pH 7.4. The eyes were then carefully removed using forceps and dissected in PBS to remove the cornea, iris, and lens. Eyes were fixed in 4% paraformaldehyde (PFA) solution for
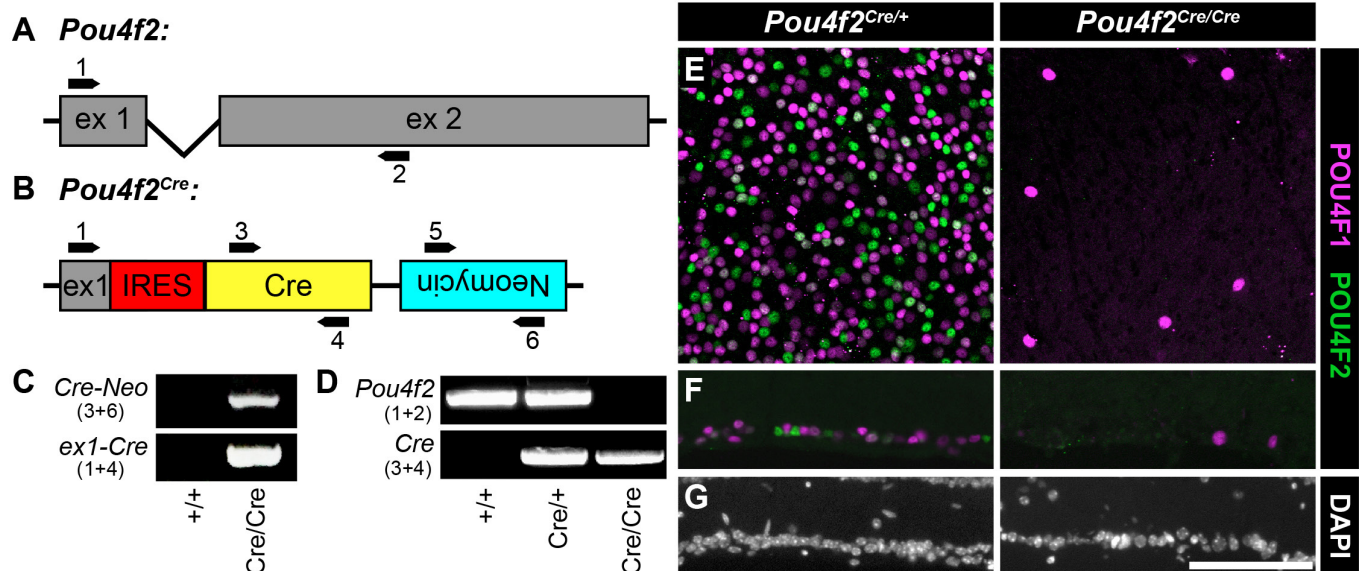

Figure 1. *Pou4f2*[Cre]. **A**: Diagram of the *Pou4f2* gene showing the exons, introns, and locations of the designed primers. **B**: Diagram showing the *Pou4f2*[Cre] construct and locations of the designed primers. The construct consisting of an internal ribosome entry site (IRES) Cre, fused to exon 1, and an inverted neomycin cassette. **C**: PCR product showing the DNA sequence unique to *Pou4f2*[Cre], compared to the wild-type. These bands were sequenced to confirm the authenticity of the bands. **D**: PCR was used to determine the zygosity of Cre. *Pou4f2*^{+/+} mice were *Pou4f2* positive and Cre negative. *Pou4f2*[Cre/+] mice were *Pou4f2* and Cre positive. *Pou4f2*[Cre/Cre] mice were *Pou4f2* negative and Cre positive. **E**: Retina whole mounts immunostained for POU4F1 (BRN3A) and POU4F2 (BRN3B). **F**: Retinal cryosections immunostained for POU4F1 and POU4F2. The *Pou4f2*[Cre/Cre] retinas display a large reduction in POU4F1-positive cells and no POU4F2 immunoreactivity. **G**: 4,6-diamidino-2-phenylindole (DAPI) staining for the panel. **F**: No significant difference was found between the POU4F1- and POU4F2-positive cells across the *Pou4f2*[Cre/+] and *Pou4f2*^{+/+} retinas (data not shown). Scale bar = 100 µm.

30 min at room temperature, and the retina was dissected from the eyecup. Fixed retinas were sunk in 30% sucrose overnight before freezing. Sucrose sunk tissues were placed in Tissue-Tek optimal cutting temperature (OCT) media (Sakura Finetek, Torrance, CA) and frozen by placing the block above liquid nitrogen to rapidly freeze the sample. Retinas were then sectioned at 10 µm and placed onto charged slides. Whole fixed retinas were washed thoroughly in PBS and then stained. All other tissues were fixed in 4% PFA overnight at 4 °C and washed thoroughly with PBS. Tissues for cryosectioning were handled in the same manner as the retinas. Brains that were used for the vibratome sections were placed in a 6% agarose block, sectioned at 150 µm thickness, and stained with 4,6-diamidino-2-phenylindole (DAPI) reagent at a dilution of 1:50,000 of 1 mg/ml stock.

Immunohistochemistry:

Sections—Tissues were blocked in a blocking solution (5% normal donkey serum, 0.1% Triton X-100, and PBS) for 30 min at room temperature. Blocking solution was used to dilute the primary antibodies. Sections were incubated in primary antibodies overnight at 4 °C. Sections were then washed in PBS at room temperature three times before secondary staining. Secondary antibodies were also diluted in blocking solution and incubated overnight at 4 °C. Sections were again washed three times in PBS at room temperature. DAPI was added to the second wash at a concentration of 1:50,000 from 1 mg /ml stock. Coverslips were applied using 80% glycerol as a mounting medium.

Whole retinas—Whole retinas were stained in a similar manner except the blocking solution used was composed of 5% normal donkey serum, 0.4% Triton X-100, and PBS, blocking was performed at 4 °C overnight. Both primary and secondary incubations lasted 2–4 days at 4 °C, and the final wash was performed in 0.4% Triton blocking solution overnight at 4 °C.

Antibodies: The following antibodies were used in this study: mouse anti-POU4F1/BRN3A (Millipore, Darmstadt, Germany; MAB1585; 1:100); goat anti-POU4F2/BRN3B (Santa Cruz Biotechnology, Santa Cruz, CA; sc-6026; 1:100); rabbit anti-melanopsin (generous gift from Ignacio Provencio; 1:10,000); rabbit anti-tyrosine hydroxylase (Millipore; AB152; 1:500); mouse anti-AP2α (Developmental Studies Hybridoma Bank, Trevor J. Williams; 3B5; 1:50); rabbit anti-calbindin (Swant, Marly, Switzerland; CB-38a; 1:1,000); goat anti-choline acetyltansferase (Millipore; AB144P; 1:250); rabbit anti-bNOS (Sigma, St. Louis, MO; N7280; 1:5,000); mouse anti-PKCα (Santa Cruz Biotechnology, sc-17769; 1:500); rabbit anti-cone arrestin (Millipore; AB15282; 1:10,000);

mouse anti-glutamine synthetase (Millipore; MAB302; 1:1,000); mouse anti-calsenilin (Millipore; 05–756; 1:1,000); mouse anti-PAX6 (developed by Kawakami, Developmental Studies Hybridoma; 1:500); and rabbit anti-Cre (Millipore; 69,050-3; 1:500). All secondary antibodies were used at 1:1,000 (Jackson ImmunoResearch, West Grove, PA).

Fluorescent microscopy: Sections and whole retinas were imaged using an Olympus (Center Valley, PA) or Nikon (Melville, NY) spinning disk confocal microscope or an Olympus FluoView confocal microscope. Adobe Photoshop (CS6; San Jose, CA) was used to crop and rotate the images. If brightness or contrast was altered, it was done uniformly across the entire image.

RESULTS

Pou4f2^{Cre} can be used to deplete RGCs: The *Pou4f2*^{Cre} transgenic line used in this study was previously generated but never characterized [10]. As the method used to generate the mice was not known, numerous primers were designed specific to the native *Pou4f2* sequence and Cre recombinase to determine the site of Cre insertion within the *Pou4f2* locus (Figure 1A,B and not shown). Additionally, primers designed specifically for neomycin were created to determine whether this cassette still remained within the transgene (Figure 1B). Through the use of PCR and sequencing of the PCR products, we successfully mapped the location of *Pou4f2*^{Cre} (Figure 1C and not shown). The inserted transgene consists of an internal ribosome entry site (IRES) Cre fused to exon 1 of the native *Pou4f2* sequence and an inverted neomycin cassette (Figure 1B). Homozygous carriers of the transgene can be differentiated from heterozygotes by the lack of the *Pou4f2* gene, assayed with PCR (Figure 1D). Elimination of POU4F2 (BRN3B) immunoreactivity in the retina and a reduction in RGCs, assayed with an antibody to the homologous protein POU4F1 (BRN3A), was observed in mice homozygous for Cre recombinase, consistent with previous investigations of *Pou4f2* function [2,3] (Figure 1E–G).

Location of Pou4f2^{Cre} recombination is dependent upon breeding scheme: To test the efficiency and location of *Pou4f2*^{Cre} recombination activity, *Pou4f2*^{Cre} mice were crossed to a mouse strain carrying a sensitive and ubiquitously expressed Cre reporter, Ai9 [11]. The Ai9 reporter (Figure 2A) is driven by the CAG promoter, which is followed by a LoxP-flanked transcription termination cassette and red fluorescent protein (RFP). Upon Cre-mediated recombination, the transcription termination cassette is removed, allowing for the transcription of RFP. This results in the production of RFP in tissues that have expressed Cre recombinase.
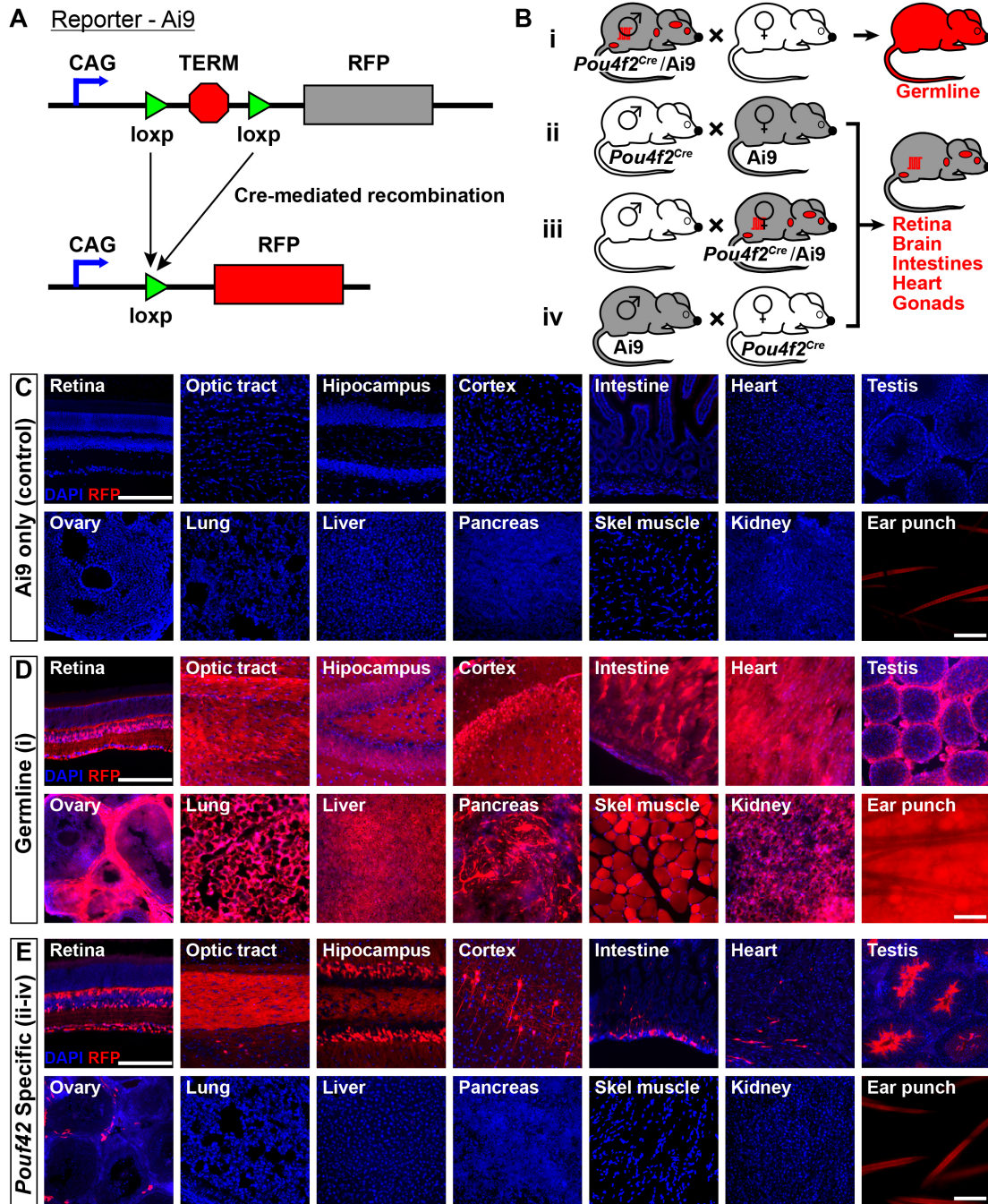
Figure 2. *Pou4f2*Cre recombination patterns. **A**: Diagram of the Ai9 Cre reporter construct. This fluorescent reporter is driven by the CAG promoter and is designed to transcribe red fluorescent protein (RFP) after Cre-mediated recombination removes the transcription termination cassette. **B**: The cartoon diagram showing varying Cre-mediated recombination patterns, which were dependent upon the parent carrying *Pou4f2*Cre and the reporter. **Bi**: Ubiquitous production of RFP in offspring when *Pou4f2*Cre and Ai9 are carried by the sire. **Bii–iv**: *Pou4f2*Cre specific recombination was observed in offspring when the sire carries *Pou4f2*Cre and the dam carries Ai9 (ii), *Pou4f2*Cre and Ai9 are carried by the dam (iii), or Ai9 is carried by the sire and the dam carries *Pou4f2*Cre (iv). **C–E**: Images of cryosections and tissue biopsies (ear) that are counterstained with 4,6-diamidino-2-phenylindole (DAPI). **C**: No RFP was detected in mice that carried Ai9 only. **D**: Offspring with germline recombination. RFP was detected throughout all tissues. **E**: Offspring with *Pou4f2*Cre-specific recombination. RFP was detected in the retina, brain, intestine, heart, and gonads. Scale bars = 100 µm (retina-kidney, scale bar in the retina image; ear punch, scale bar in the image).

Cre activity was determined by visualization of RFP in cryosections and tissue biopsies of mouse tissues (Figure 2C–E). The pattern of recombination was dependent upon which parent carried the Cre and the target of Cre. When the sire carried *Pou4f2*^{Cre} and Ai9, germline recombination occurred (Figure 2B,D). Germline recombination of the reporter was observed in offspring regardless of whether they inherited *Pou4f2*^{Cre}, verified with PCR (not shown), suggesting that recombination was targeted in the germline. RFP was observed within the testis of sexually mature mice that carried *Pou4f2*^{Cre} and the Ai9 reporter (Figure 2E), consistent with these results and previous reports that *Pou4f2* is expressed within germ cells [14,15]. When the sire carried *Pou4f2*^{Cre} and the dam carried the reporter, recombination was limited to subsets of cells within the retina, brain, intestines, heart, and gonads (Figure 2Bii,E). When the dam carried *Pou4f2*^{Cre}, recombination was limited to subsets of cells within the retina, brain, intestines, heart, and gonads, regardless of which parent carried the reporter allele (Figure 2Biii–iv,E). No visualization of the Ai9 reporter was detected in the mice that lacked *Pou4f2*^{Cre} (Figure 2C).

Pou4f2^{Cre}*-mediated recombination within the retina:* To test the specificity of *Pou4f2*^{Cre} expression within the retina, *Pou4f2*^{Cre/+} Ai9 retinal sections from crosses generating specific recombination (Figure 2Bii–iv,E) were immunostained with a panel of cell-type specific markers (Figure 3, Figure 4, and Table 1). Reporter activity was limited to neurons but was also detected in neuron populations not known to express *Pou4f2*. Recombination was observed in RGCs (Figure 3A,B), amacrine cells (ACs; Figure 3C–E and Figure 4A–B), bipolar cells (BCs; Figure 4C), horizontal cells (HCs; Figure 3D), and a very small number of photoreceptors (Figure 4D). No Cre activity was detected within the microglia, astrocytes, or blood vessels (not shown). Müller glia were also not normally targeted by Cre (Figure 4E,E''). However, one of the 34 mice observed in this study contained columns of cells that produced RFP, suggesting that a small number of retinal progenitor cells had expressed Cre (not shown). For the majority of markers used in this study, only a portion of the cells produced RFP, with the exception of POU4F2, where all counted cells produced RFP (Table 1). *Pou4f2*^{Cre/+} mice were also crossed with mice carrying the Brainbow reporter (Figure 4F). The Brainbow reporter is a transgene driven by the *Thy1* promoter that encodes a series of fluorescent proteins, each flanked by LoxP sites [16]. This genetic construct allows for differential expression and visualization of fluorescent proteins. Cre expression within the retina was again limited to neurons within the retina but only those that express *Thy1*: RGCs, ACs, and HCs (Figure 4F).

Pou4f2^{Cre/+} Ai9 retinal sections were taken from crosses that generated a specific recombination to map out the developmental time course at which *Pou4f2*^{Cre} targeted recombination in different cell populations (Figure 2Bii–iv,E). The proportion of cells producing RFP and positive for specific markers were quantified at P0, P10, P20, and P40 (Figure 5). The proportion of cells positive for RFP and PAX6, ChAT, and calsenilin increased incrementally from P0 to P40, while POU4F2-positive cells were all targeted starting from P0, the earliest time point sampled (Figure 5A–I). Immunohistochemistry was then used to detect the location of the Cre protein (Figure 5J–M). The Cre protein was observed within the neurites and nuclei of cells in which recombination occurred (Figure 5J), while staining was absent in retinal sections taken from mice that lacked Cre (Figure 5K). The Cre protein was observed within the nuclei and neurites of subsets of ganglion cells and horizontal cells, confirmed by costaining with POU4F2 and PAX6 (not shown) but was absent from the nuclei of amacrine and bipolar cells at P10 (Figure 5L,L'). The Cre protein was observed within the nuclei of cells in the RGL and the INL by P40, consistent with reporter activation (Figure 5M,M'). Late activity of *Pou4f2*^{Cre} was also effective in targeting constructs other than the highly sensitive Ai9 and Brainbow reporters. A conditional allele of Down syndrome cell adhesion molecule (*Dscam*) was used to demonstrate this activity, which is evidenced by the accumulation of DSCAM protein with the cells bodies of targeted cells [17]. *Dscam* targeting was observed in ganglion cells and amacrine cells early during development and later (about P45) in bipolar cells (Figure 5N).

Pou4f2^{Cre} *activity within the brain:* *Pou4f2*^{Cre/+} Ai9 brains from crosses that generated a specific recombination were imaged to test the specificity of *Pou4f2*^{Cre} expression within the brain (Figure 2Bii–iv,E). RFP was observed in many regions of the brain (Figure 6). The expression of RFP was observed in retinal ganglion cell axons projecting throughout the brain but was also found within neural cell bodies and their neurites in other regions of the brain, including the visual centers, consistent with previous studies [9,18]. The percentage of cells targeted by *Pou4f2*^{Cre} within three visual centers was determined: the suprachiasmatic nucleus (SCN), the lateral geniculate nucleus (LGN), and the superior colliculus (SC; Figure 7). This was accomplished by staining vibratome sections of the brain with DAPI and counting the total number of cells positive for RFP. Our results indicate that 18% of cells within the SCN had expressed Cre, 11% in the LGN, and 17% in the SC (Figure 7D-G).
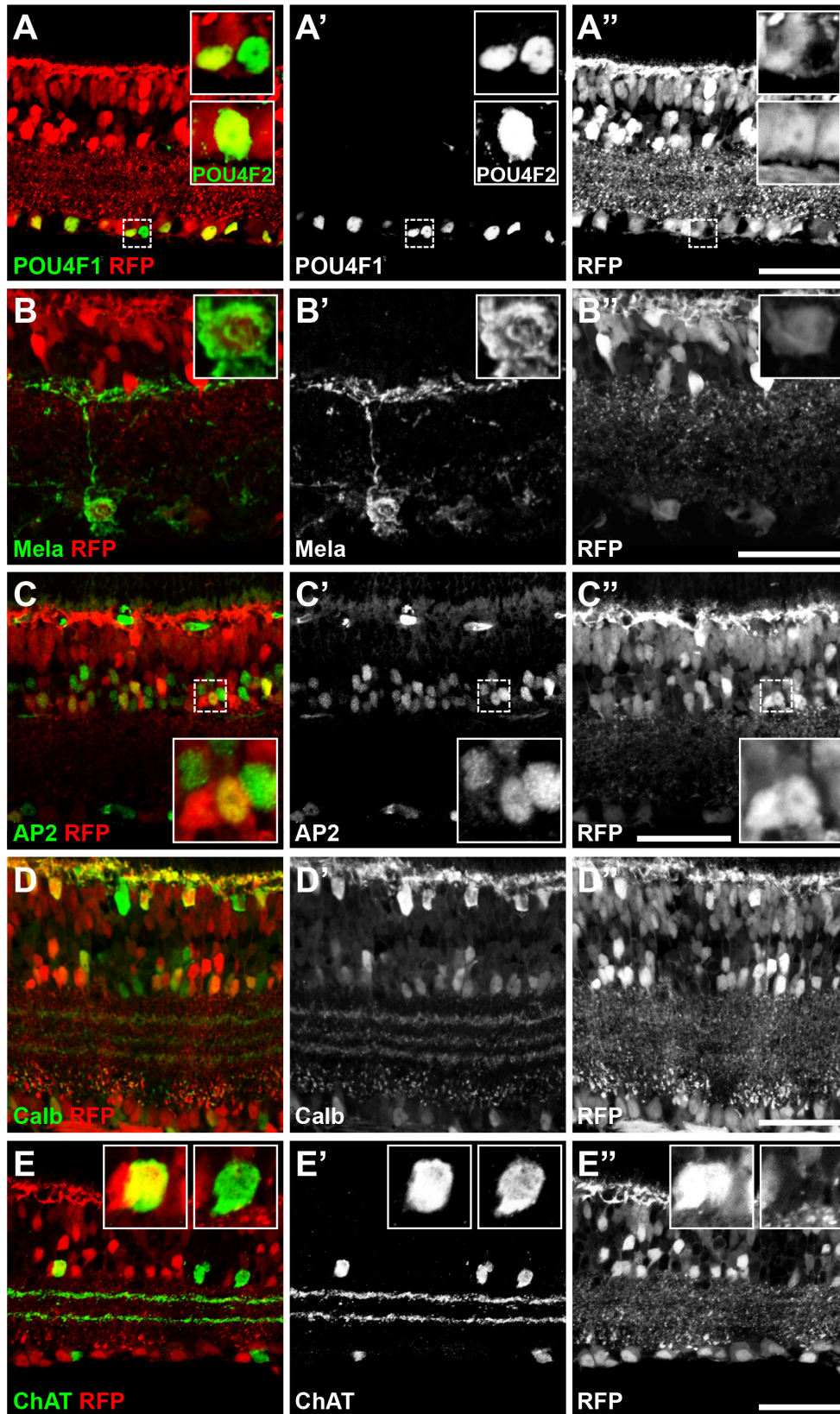
Figure 3. *Pou4f2*^Cre^-mediated recombination within the retina. Cre-mediated recombination within the retina was assayed in *Pou4f2*^Cre/+^ Ai9 mice by immunostaining retinal cryosections with a panel of markers. Channels are split out for better visualization. Markers are described in further detail in Table 1. Abbreviations: mela = melanopsin, Calb = calbindin. Scale bars = 50 μm. Insets = 16 μm boxes.
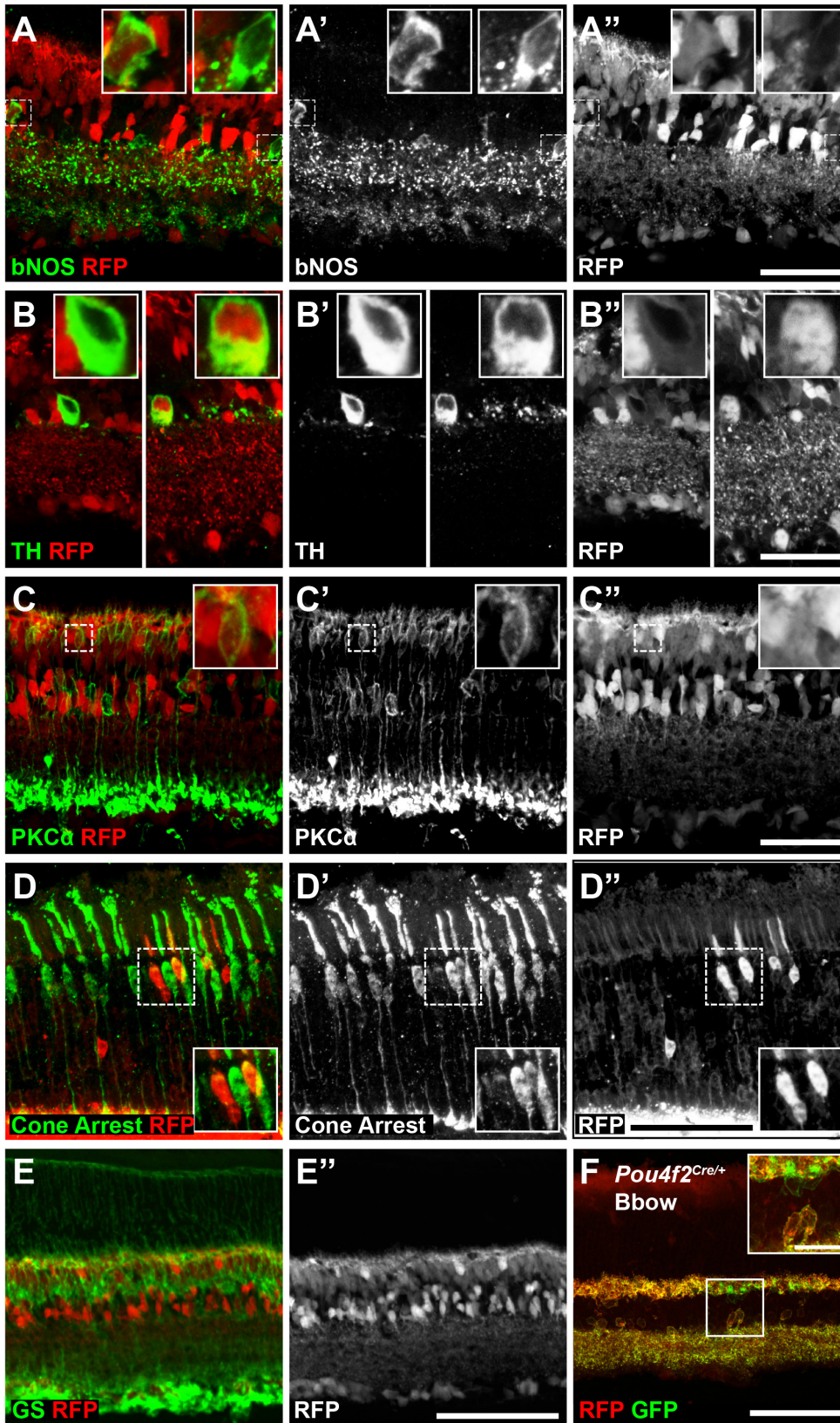
Figure 4. *Pou4f2*^{Cre}-mediated recombination within the retina continued. Recombination within the retina was assayed in *Pou4f2*^{Cre/+} Ai9 mice by immunostaining retinal cryosections with a panel of markers. Channels are split out for better visualization. Markers are described in further detail in Table 1. Abbreviations: TH = tyrosine hydroxylase, PKC = protein kinase C, GS = glutamine synthetase. Scale bars = 50 µm. Insets A–C = 16 µm boxes, Insets in D and F = 24 µm boxes.

DISCUSSION

This study characterized a *Pou4f2*^{Cre} transgenic mouse line, in which Cre recombinase is driven by the *Pou4f2* promoter. Our results show that this transgenic mouse can be used (1) to deplete RGC populations when homozygous for the *Pou4f2*^{Cre}, (2) that the pattern of Cre recombination is dependent upon which parent carries *Pou4f2*^{Cre} and the target of recombination, making it useful to target germline recombination, (3) that Cre specific activity within the retina is limited to the neurons (RGCs, ACs, BCs, HCs, and a small number of photoreceptors), and (4) that the timing of *Pou4f2*^{Cre} recombination is subclass specific. These properties make this line a useful multipurpose genetic reagent for vision researchers.

The *Pou4f2*^{Cre} knock-in transgenic mouse can be used to generate RGC-specific depletion, consistent with previous literature demonstrating that deletion of *Pou4f2* results in the depletion of about 70% of the RGCs [2,3]. Previous studies have also shown that the remaining RGCs within *Pou4f2* knockout mice maintain normal RGC diversity and a grossly normal morphology [19]. Using the *Pou4f2*^{Cre} transgenic mouse line to drastically reduce RGC populations allows questions to be asked about how retinas develop without normal populations of RGCs and the effects major depletion of RGCs has on the developing retina.

The *Pou4f2*^{Cre} line can also be used to target recombination in the germline, depending on which parent carries the *Pou4f2*^{Cre} and the target for recombination. Germline recombination occurs when the sire carried the *Pou4f2*^{Cre} gene and the target for recombination yet was always specific when the dam carried *Pou4f2*^{Cre}. This was an interesting observation consistent with the expression of *Pou4f2* in the male and female germline and suggests that the *Pou4f2*^{Cre} transgenic mouse line could be used to understand the genetic underpinnings of varying germline development between sexes [14,15]. In crosses, we have established using floxed alleles of the target genes that the recombination rate is complete, resulting in 100% transmission of recombined alleles, which increases the efficiency of producing mice that carry mutations that would otherwise prevent or reduce breeding efficiency.

The activity of *Pou4f2*^{Cre} recombination was observed in multiple populations of cells in the retina. Here we mapped patterns of recombination using two reporter lines, Ai9 and Brainbow. The *Pou4f2*^{Cre} line targets RGCs during early stages of retinal development and would be useful for studying processes such as RGC axon guidance. At later time points, recombination is observed within neurons in the retinal ganglion cell layer, the inner nuclear layer, and a small number of photoreceptors. The Brainbow reporter

TABLE 1. RETINAL CELL TYPES EXPRESSING *POU4F2*CRE.		
Marker	**Population**	**Cre+ (%)**
POU4F2 (BRN3B)	RGCs	100
POU4F1 (BRN3A)	RGCs	86
Melanopsin	RGCs	79
AP2α	ACs	44
Calbindin	ACs	60
	HCs	100
ChAT	Cholinergic ACs	97
bNOS	bNOS ACs	38
TH	Dopaminergic ACs	50
PKCα	Rod BCs	0
Cone Arrestin	Cones	2
GS	Müller Glia	0*
IBA-1	Microglia	0
GFAP	Astroctyes	0
GS iso B4	Blood Vessels	0

Immunohistochemistry was performed on *Pou4f2*^{Cre/+} Ai9 retinas to determine which cells types within the retina express *Pou4f2*^{Cre}. This table summarizes Figure 3 and Figure 4 and contains markers not shown (IBA-1, GFAP, GS isolection-B4). *1 out of 34 mice contained sparsely labeled Müller glia, where columns of cells expressed tdTomato. Abbreviations: ACs—amacrine cells, BCs—rod bipolar cells, HCs—horizontal cells, RGCs—retinal ganglion cells, TH—tyrosine hydroxylase, PKC—protein kinase C, GS—glutamine synthetase.
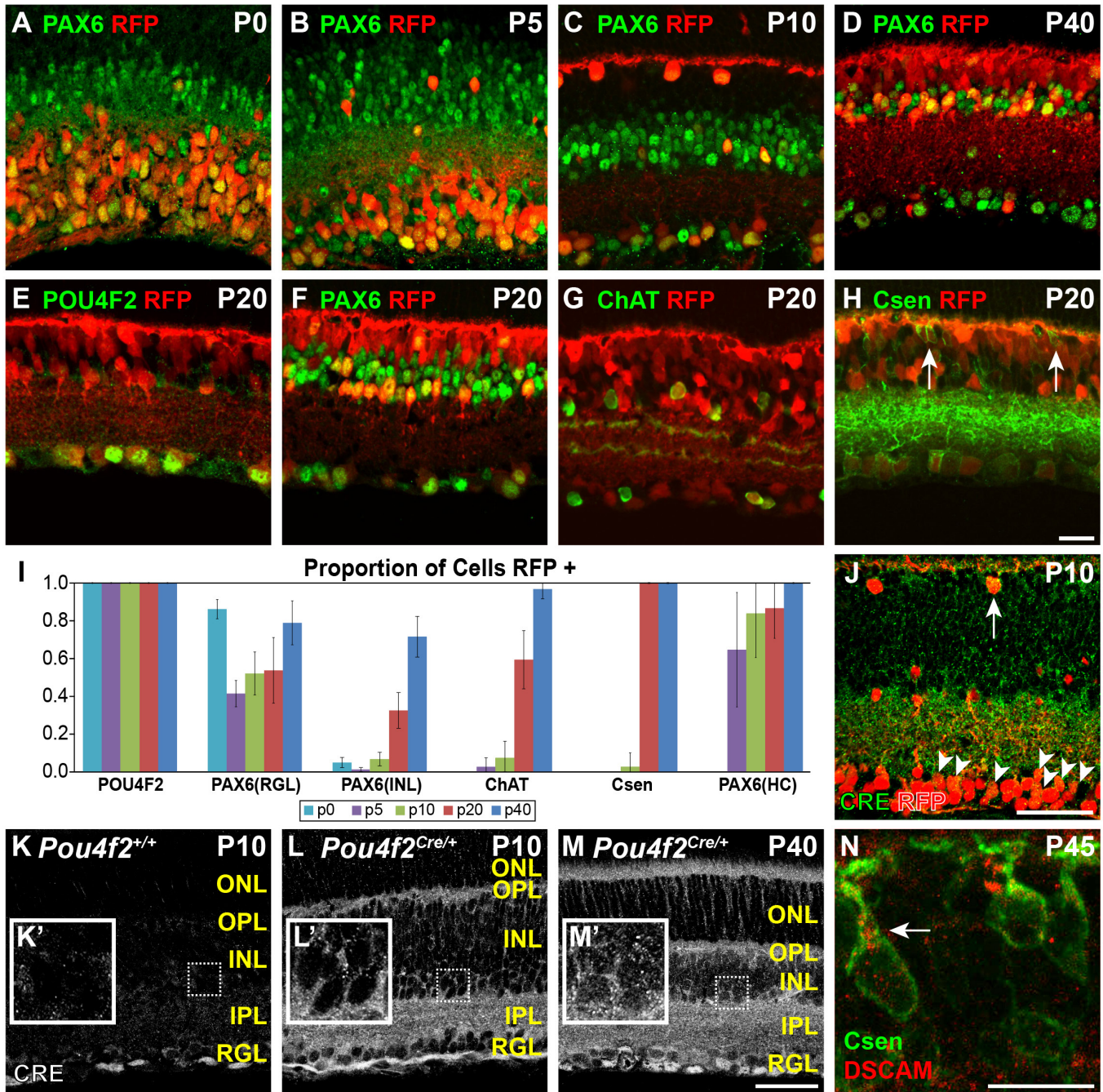
Figure 5. Developmental time course of *Pou4f2*[Cre] activity within the retina. A developmental time course of Cre targeting within the retina was performed by immunostaining the retinal cryosections with neural markers (PAX6, POU4F2, ChAT, and Calsenilin). The proportions of reporter-positive cells for each of the markers at P0, P5, P10, P20, and P40 were quantified. Progression in the proportion of cells targeted by *Pou4f2*[Cre] increased in all of the markers over developmental time, except the POU4F2-positive cells, which were 100% targeted starting from P0. **A–D**: Developmental time course of PAX6 except P20. **E–H**: Representative images of all the markers at P20. **I**: Graph illustrating the proportion of cells positive for the Ai9 reporter over time. PAX6-positive cells were subdivided into cells found within the retinal ganglion layer (RGL), the inner nuclear layer (INL), and horizontal cells (HCs) in the outer INL. Calsenilin (Csen) was used to label type 4 bipolar cells, and bipolar cells were the only quantified cell type reported for this stain. **J–M**: Cre protein staining. **J**: Cre was detected within the cell bodies and neurites of cells in the RGL (arrowheads) and horizontal cells (arrow). **K**: Cre negative control. The Cre protein was not observed. **L**: Cre was detected within the nuclei and neurites of cells within the RGL but was absent from most nuclei within the INL at P10 (**L'**). **M**: Cre was detected within the nuclei of cells in the RGL and the INL, consistent with reporter activation. **M'**: Inset showing Cre within cells of the INL. **N**: *Pou4f2*[Cre] effectively targets a conditional allele of *Dscam* at P45 in bipolar cells, evidenced by accumulation of the DSCAM protein in the cell body. Abbreviations: Csen = calsenilin. Scale bar in H = 20 µm. Scale bars in J–L = 50 µm. Scale bar in M = 20 µm. Insets in J–K = 25 µm box.
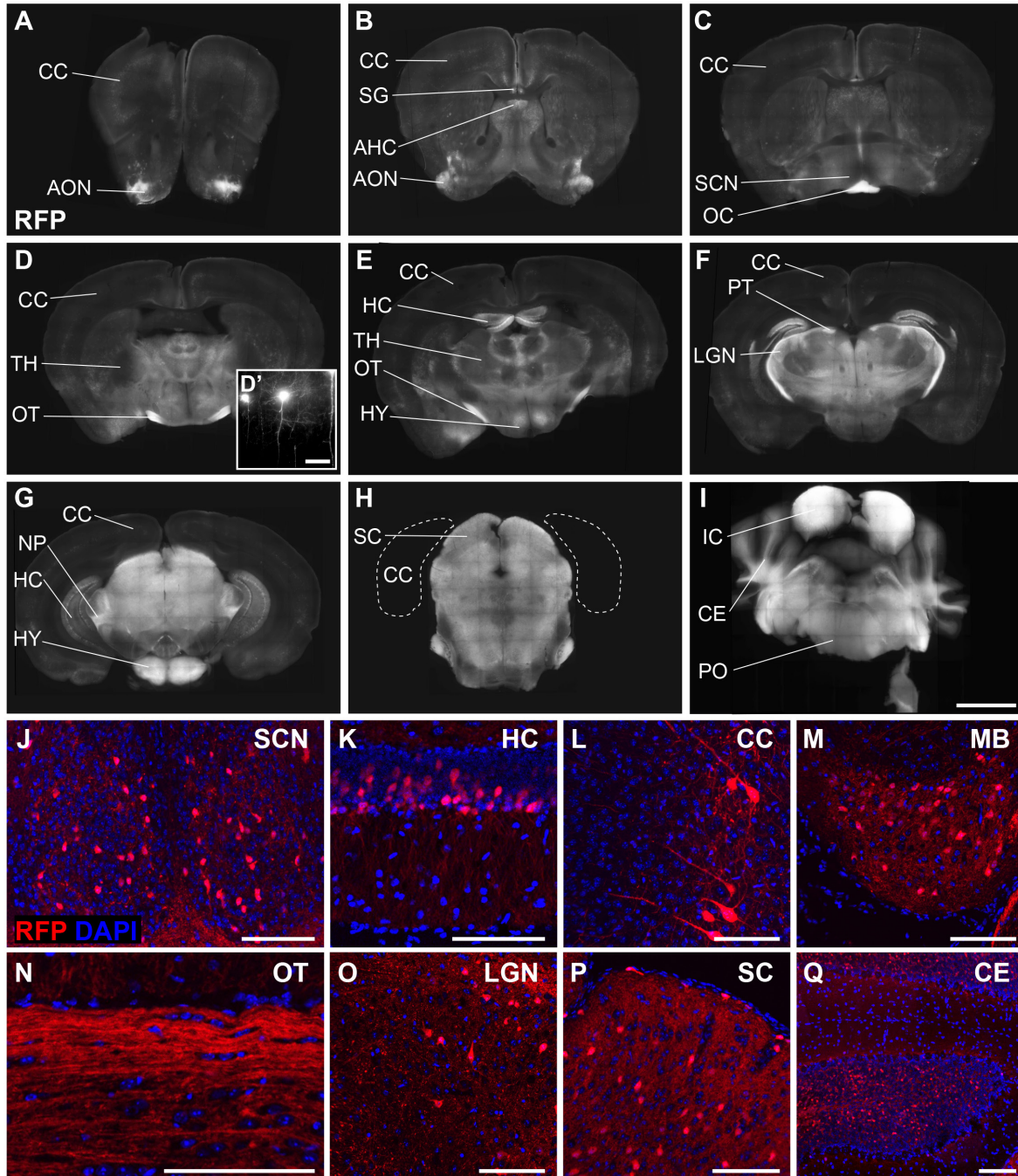
Figure 6. *Pou4f2*^Cre^-mediated recombination within the brain. Cre-mediated recombination within the brain was assayed in the vibratome and cryosections of *Pou4f2*^Cre/+^ Ai9 mice. **A–I**: In the vibratome sections, reporter activity was detected throughout many regions of the brain. **D'**: High-magnification image showing the pyramidal cells targeted by Cre. **J–Q**: In the cryosections, reporter activity was found within retina ganglion cell axons projecting throughout the brain but was also found within neural cell bodies and neurites within the brain, within many different regions. Abbreviations: AHC = anterior hippocampus, AON = anterior olfactory nucleus, CC = cerebral cortex, CE = cerebellum, HC = hippocampus, HY = hypothalamus, IC = inferior colliculus, LGN = lateral geniculate nucleus, MB = mammillary body, NP = nucleus peduncularis, OC = optic chiasm, OT = optic tract, PO = pons, PT = pretectum, SC = superior colliculus, SCN = suprachiasmatic nucleus, SG = supracallosal gyrus, TH = thalamus. Scale bar in D' = 50 µm. Scale bar in I = 2 mm. Scale bars in J–Q = 100 µm.
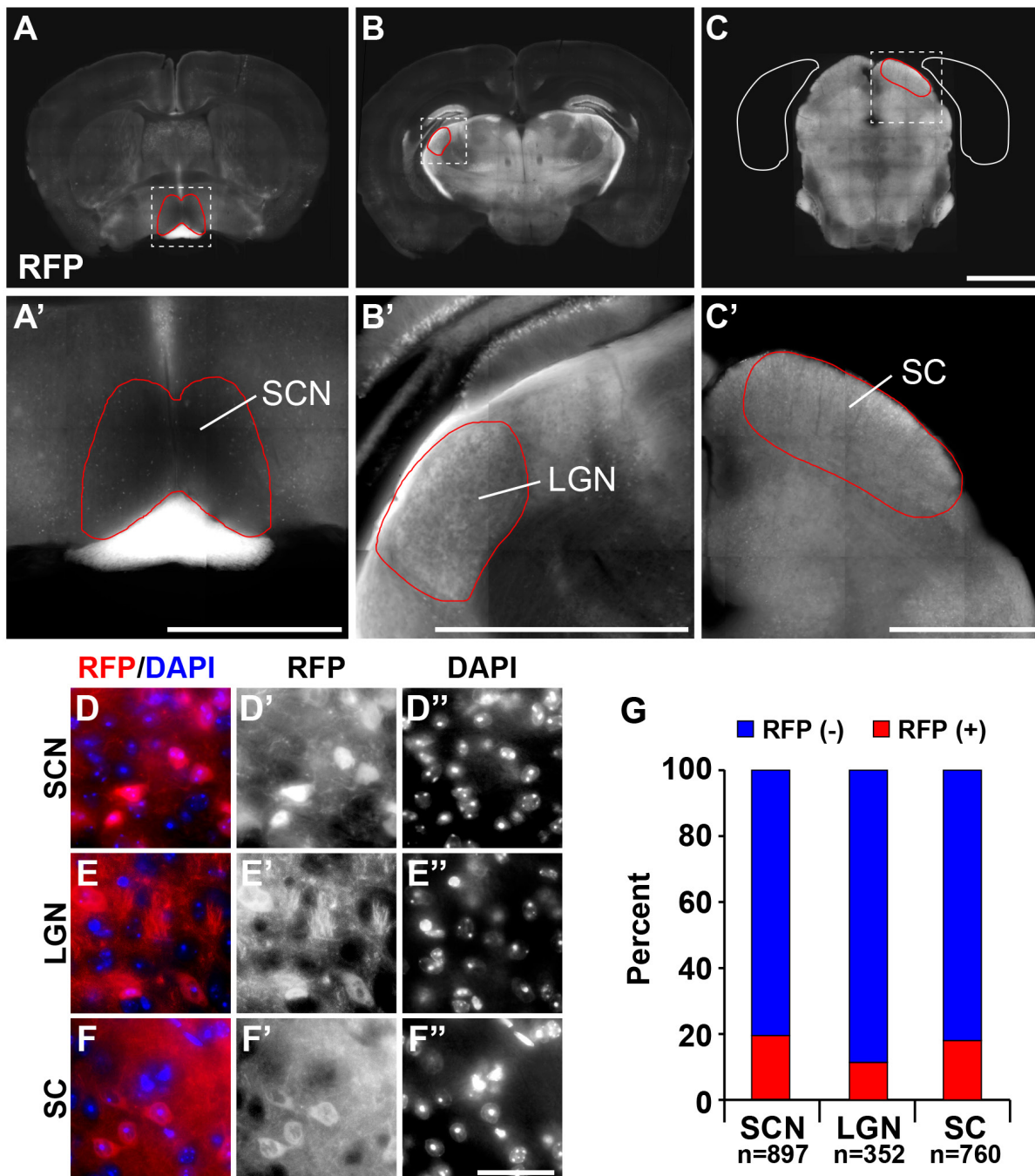
Figure 7. *Pou4f2*Cre-mediated recombination within visual centers of the brain. The percentage of neurons in which recombination was targeted by Cre was quantified within three visual centers of the brain (SCN, LGN and SC) in the vibratome sections of the *Pou4f2*Cre/+ Ai9 brains stained with 4,6-diamidino-2-phenylindole (DAPI). **A–C**: Images showing the location of the visual centers. **D–F**: High-magnification images used to quantify the number of cells positive for Cre-mediated recombination. **G**: Graph illustrating the percentage of cells targeted by Cre (n = 1 animal). Abbreviations: SCN = suprachiasmatic, LGN = lateral geniculate nucleus, SC = superior colliculus. Scale bar in C = 2 mm. Scale bars in A'–C'= 1 mm. Scale bar in F'' = 40 μm.

was observed only in RGCs, ACs, and HCs, consistent with activity of the *Thy1* promoter. The expression of Cre from the *Pou4f2* locus differs from immunohistological studies in which RGCs are the primary cell type of the retina expressing *Pou4f2*[Cre] and related proteins [9]. The IRES sequence added to the construct is highly GC-rich, and such sequences are likely to alter local DNA methylation patterns. We speculate that this is why the expression pattern of Cre also differs from a recently reported *Pou4f2* Dre/Cre line [18]. In this line, Dre-mediated recombination replaces the *Pou4f2* gene with Cre. The Cre driven from the *Pou4f2* locus in this transgenic mouse is observed in the entire animal, which is different compared to the expression pattern of *Pou4f2* as detected in situ [18]. These results suggest that expression driven from the *Pou4f2* locus is highly sensitive to small changes, such as those made in engineering the recombinant lines and could account for the expression of Cre in non-RGC populations in the retina reported in this study. Alternatively, this could reflect low expression of *Pou4f2* in these cell types (i.e., ACs).

We also report that the timing of *Pou4f2*[Cre]-mediated recombination within the retina is also highly dependent upon the cell type in which the target of recombination is located. By taking advantage of the timing of this recombination pattern, investigators will have a reproducible method to target amacrine and bipolar cells after the completion of developmental processes such as developmental cell death and dendrite outgrowth to complement the use of Cre:ER lines. Sparse recombination was also observed in the neurons of the enteric nervous system, photoreceptors, and some other regions of the brain, allowing for genetic manipulations to be targeted in a small number of cells.

In conclusion, this study characterizes a transgenic *Pou4f2* knock-in Cre recombinase transgenic mouse line. This line can be used to deplete RGC number, to target germ-line recombination, and to target recombination in retinal neurons while sparing Müller glia and most photoreceptors.

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