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A regulatory sequence from the *retinoid X receptor γ gene* directs expression to horizontal cells and photoreceptors in the embryonic chicken retina

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Purpose: Combining techniques of episomal vector gene-specific Cre expression and genomic integration using the piggyBac transposon system enables studies of gene expression–specific cell lineage tracing in the chicken retina. In this work, we aimed to target the retinal horizontal cell progenitors.
Methods: A 208 bp gene regulatory sequence from the chicken *retinoid X receptor γ gene* (RXRγ208) was used to drive Cre expression. RXRγ is expressed in progenitors and photoreceptors during development. The vector was combined with a piggyBac "donor" vector containing a floxed STOP sequence followed by enhanced green fluorescent protein (EGFP), as well as a piggyBac helper vector for efficient integration into the host cell genome. The vectors were introduced into the embryonic chicken retina with in ovo electroporation. Tissue electroporation targets specific developmental time points and in specific structures.
Results: Cells that drove Cre expression from the regulatory RXRγ208 sequence excised the floxed STOP-sequence and expressed GFP. The approach generated a stable lineage with robust expression of GFP in retinal cells that have activated transcription from the RXRγ208 sequence. Furthermore, GFP was expressed in cells that express horizontal or photoreceptor markers when electroporation was performed between developmental stages 22 and 28. Electroporation of a stage 12 optic cup gave multiple cell types in accordance with *RXRγ gene* expression in the early retina.
Conclusions: In this study, we describe an easy, cost-effective, and time-efficient method for testing regulatory sequences in general. More specifically, our results open up the possibility for further studies of the *RXRγ-gene* regulatory network governing the formation of photoreceptor and horizontal cells. In addition, the method presents approaches to target the expression of effector genes, such as regulators of cell fate or cell cycle progression, to these cells and their progenitor.

The formation of specific cell types is dependent on interactions between various gene regulatory factors and DNA elements, and they cooperatively produce cell type– or tissue-specific expression of one or more key differentiation genes [1]. Reporter genes under the control of a regulatory gene element that is part of such a cell type–specific gene regulator network (GRN) have been used when the relations between specific genes and cell types are studied. Transgenic or knock-in mice that express LacZ or enhanced green fluorescent protein (EGFP) under the control of specific regulatory sequences have often been used to study cell type [2,3] or cell lineage formation [4]. Tissue electroporation is an effective way to introduce reporter constructs at a specific developmental time point or in a specific structure [5-10]. Electroporation in combination with a transposon system that integrates the reporter gene into the host cell genome enables establishment of tissue-specific cell lineages with a defined initiation time [11]. Furthermore, to achieve cell-specific and robust reporter gene expression, the transposon vector system can be combined with the Cre-LoxP recombination technique. Three essential components are needed for this to work: 1) An enhancer trap vector (trap vector) that drives expression of Cre recombinase from a gene- or cell type–specific regulatory element [12]. 2) A "donor" reporter gene construct with a transposon cassette that contains a strong ubiquitously active promoter, such as CAG [13], followed by a "floxed" STOP sequence [14]. 3) An episomal "helper" transposase vector that is ubiquitously expressed and catalyzes the integration of the "donor" reporter construct into the genome of electroporated cells. Only cells that drive specific Cre expression will remove the STOP sequence from the integrated reporter, establishing a lineage with robust and stable reporter gene expression that is defined by the gene or cell-type specificity.

In this work, we focused on chicken retinal horizontal cells (HCs) and their immediate progenitors. We aimed to develop a method for targeting the HCs to label them with a reporter and study their lineage. We also aimed to develop a method for directing gene expression to these cells. The HCs are of interest because their regulation of the cell cycle

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deviates from that of other retinal cells [15-17], and HCs are candidates for being the "cell of origin" for retinoblastoma [18]. Chicken HCs express the homeodomain transcription factors Prox1 and Pax6, whereas the LIM/homeodomain transcription factors Lim1 (Lhx1) and Isl1 are expressed mutually in half of the HC population [19-21]. The generation of HCs and cone photoreceptors (PRs) overlaps, and cell lineage analysis in the zebrafish, mouse, and chicken suggests that they are derived from the same progenitor [22-24]. Otx2 and members of the *retinoid X receptor (RXR) gene* family are important for PR development and are expressed by the suggested shared progenitor cells [25-27]. In the chicken retina, HCs are generated between embryonic day (E) 3 and 8 in a central to peripheral wave-like manner [20,28]. The first PRs exit the cell cycle at about the same time as the HCs [28]; however, the opsins first appear several days later at E14–16 [29].

RXRγ expression has been identified in the retina of several species, such as the chicken, mouse, cow, human, frog, and zebrafish [26,27,30-33]. In the mouse, *RXRγ* is expressed in cones, transiently downregulated during S-opsin onset, and then reexpressed again [31]. In the *RXRγ*-null mice retina, S-opsin expression is upregulated, whereas M-opsin expression remains unchanged [31]. The signaling molecule retinoic acid (RA), important for embryonic eye development, acts via interactions with nuclear receptors, such as RXRγ [34]. In zebrafish, treatment with RA promotes differentiation of rods and L-opsin cones whereas RA inhibits the differentiation of S-opsin cones [32]. Rat retinal cultures treated with RA also show an increase in rods [35].

We analyzed gene regulatory sequences for several genes that regulate HC formation and development. Regions were PCR amplified and inserted into a trap vector that was electroporated into the chicken retina for reporter expression analysis. We tested three trap vectors, HSP68-LacZ [36], ptkEGFP [37], and Stagia3 [12], that have previously been used for similar purposes. The Stagia3 vector was selected based on undetectable reporter gene expression in the absence of an enhancer element. An Otx2 regulatory sequence that has been shown to drive cell-specific expression in a similar system was tested [25]. Several different cell types showed expression, and a similar pattern was achieved with a 300 bp element taken from the *PAX6 gene* (Pax6.300). A 208 bp sequence from the *RXRγ gene* (RXRγ208) gave specific GFP expression in cells located in the outer nuclear layer (ONL) and in the outer portion of the inner nuclear layer (INL) that expressed markers for PRs and HCs. The results support an ontogenetic relationship and a common GRN for PRs and HCs by identifying a regulatory sequence in the *RXRγ gene* that singles out and directs expression to these cell types. These findings highlight a new area of study of the GRN that drives formation of these cell types. In addition, the results present approaches to target expression of effector genes, such as regulators of cell fate or cell cycle progression, to these cells and their common progenitor.

METHODS

Animals: Fertilized White Leghorn chicken eggs were obtained from Ova Production (Vittinge, Sweden). Eggs were incubated at 37 °C in a humidified incubator (Grumbach, Asslar, Germany). Embryos were staged according to Hamburger and Hamilton's method [38]. Animal experiments were performed according to the guidelines given by the Association for Research in Vision and Ophthalmology and were approved by the local animal ethics committee in Uppsala, Sweden.

DNA constructs and PCR: The following trap vectors were used: Stagia3 [12] (kindly provided by C. L. Cepko and M. M. Emerson), Hsp68-LacZ [36] (kindly provided by A. Visel), and ptkEGFP [37] (kindly provided by M. E. Bronner). Stagia3 has a minimal TATA-box promoter coupled to the *EGFP reporter gene*, Hsp69-LacZ has a mouse Hsp68 minimal promoter [39] coupled to the *LacZ reporter gene*, and ptkEGFP has a herpes simplex virus thymidine kinase promoter coupled to the *EGFP reporter gene*. The piggyBac "helper" construct, pBase, and the "donor" construct, pB-CAG-LoxP-STOP-LoxP-GFP-pB with a cytoplasmatic GFP [14] (kindly provided by A. Klar), were used for the piggyBac integration system.

To construct Stagia3-cytomegalovirus (CMV), the CMV enhancer was amplified with PCR (95 °C 1 min, 35 cycles of 95 °C 30 s, Tm-5 °C 30 s, 72 °C 1 min/1000 bp, followed by 72 °C 10 min) from pCIG-DV and inserted into the SalI/EcoRI restriction sites of Stagia3. To construct Staria3-CMV (Stop TAta Rfp Ires Ap version 3), red fluorescence protein (RFP) was amplified with PCR from pRFPRNAiC and replaced the GFP at the AgeI and BsrGI restriction sites of Stagia3-CMV. To construct Stacia3-CMV (Stop TAta Cre Ires Ap version 3), the Cre recombinase was amplified with PCR from pKK 735 iCre [40] (kindly provided by K. Kullander) and replaced GFP at the AgeI and BsrGI restriction sites of Stagia3-CMV. To construct Stacia3-Pax6.300, a 300 bp Pax6 sequence was amplified with PCR from chicken gDNA and inserted into the SalI/EcoRI restriction sites of Stacia3. To construct Stacia3-Otx2, the retina-specific Otx2 enhancer element [25] was amplified from mouse gDNA with PCR and inserted into the SalI/EcoRI restriction sites of Stacia3. To construct Stacia3-RXRγ208, a 208 bp regulatory

sequence from the chicken *RXRγ gene* [41] was synthesized by Eurofins Genomics (Eurofins Genomics, Ebergsberg, Germany) and inserted into the Stacia3 construct at the SalI/EcoRI restriction sites.

The following sequences were used: chicken Pax6: 5′-TGC GGG ACT TTA CGG CTC TTT TCC CGA GCG TTT GTG TGC AAA TGA AGG GTC TCG TTA TTG CGC GAG CGG AGA GGG AGG CTT TAA TGA CGG GAG ATC TTT CCG CTC ATT GCC CTT TCA AAT ACA ATT GTA GAT CGA ACT CAG CCT TGT CAC GTT GAG GAG CAG TGC GTC CCT AAC ATC CAG GAC GTG CCT GTC GGC TCT CGG CGG ATT GCA TCC CAT CAC CCC CGG GGA ATG CAG CCC GCG TCG GGG CTG CGC GGG GCG CGG GAG GCG CAC GGC CCG AGC GGG GTC CCG AGT CGC GGT TAT-3′, mouse Otx2: 5′-CCG AGC CGC CAG TCA GCG AAG TTT TGT TTC CTT TCT ATC ATG CA GAA AAT TAA TCA GCC AGG ACG AGA AGC AGA GCT GAG CAC GAC GGC CTG TAA TTA AGG GAC GTG TGC CCC TCG GAT TAT CTC GTT AGT TTA TCA AGA AAA CAT TTA TTA TAA TTA ATT CTC GGA CGA GGT AAT TAT TAT TGA GCG AGG ACA CAG CAA CTG GTA GAT GGG CTT CTT GGA AGA AGG GGA AAA AAA ACC ACC AAG GAG GGG GGG CGA TCT GGA GGG GGA AGC GAC AGA TTG CAC GAA TTG ACC G-3′, chicken RXRγ: 5′-TAC AAG GAC TGG AGC CTC TCC CTA ACA CAA ACC CAC GTG TTC CCC AAA ACG AGT TGC CTG ATG CTT GCT TAT CTT GAG GGG GGG GGT TCT TTT GGA GGT GCT CGG TGT GCA TGT GTT GTG CAG CAC TAA GCA CTC ACT AAC CCA GAT CTG CTA AAA ATC AAT AAG GTA ATC CAC TTA CAG CCT GGG ACT GTG GCC TTT CGA AGG AGC T-3′. The following PCR primers were used: RFP Fwd (5′-ATG GCC TCC TCC-3′), RFP Rev (5′-TTA GGC GCC GGT-3′), Cre Fwd (5′-ATG GTG CCC AAG A-3′), Cre Rev (5′- CTATGC-GGCCCCA-3′), CMV Fwd (5′-ACA TAA CTT A-3′), CMV Rev (5′-GATGACTAATA-3′), Pax6 Fwd (5′-ATA ACC GCG ACT CGG G-3′), Pax6 Rev (5′-TGC GGG ACT TTA CG-3′), Otx2 Fwd (5′-CGG TCA ATT CGT G-3′), Otx2 Rev (5′-CCG AGC CGC CAG T-3′). All PCR products were verified with sequencing (Eurofins Genomics).

Electroporation:

Retinal explants—Retinal explants were prepared as previously described [6]. Briefly, the eyes were enucleated, and the sclera and pigment epithelium were removed using fine forceps. Eyes were put in a cuvette containing 100 µl DNA solution (1 µg of each construct/10 µl 1X Dulbecco´s Phosphate Buffered Saline (DPBS) +$MgCl_2$ +$CaCl_2$). Five 50 ms 15 V pulses were applied using an ECM 830 square wave evaporation system (BTX, Harvard Apparatus, Holliston, MA). After electroporation, the retinal explants were cultured in medium containing 1:1 DMEM:F12 Nutrient mix, 10% fetal bovine serum, 10 U/ml penicillin-streptomycin, 5 µg/ml insulin, and 2 mM L-glutamine for 24 h at 37 °C, 5% CO_2 on a rotator shaker with a constant speed of 50 rpm.

In ovo—For coelectroporation, DNA constructs with the same concentration (5 µg/µl) were mixed at the ratio of 1:1:1 of helper:donor:Cre construct [42]. Fast Green (F7252, Sigma-Aldrich, St. Louis, MO) was added to the mix to help visualize the injection. Approximately 0.2 µl solution was injected into the subretinal space (≥stage 22) of the eye or into the optic vesicle (stage 12), and five 50 ms 12 V (stage 12) or 15 V (≥stage 22) pulses were applied using an ECM 830 square wave evaporation system (BTX, Harvard Apparatus). After electroporation, the eggs were sealed with tape and put back into the incubator to allow for further development.

DF1-cells—Chicken fibroblast DF1-cells were electroporated using the Gene Pulser II (BioRad, Hercules, CA), set to 250 V and 250 µF. Ten micrograms of each construct at a 1:1:1:1 ratio of helper construct:donor construct:Cre recombinase construct:RFP control construct was used. The electroporated cells were cultured on coverslips in DMEM (D5671, Sigma-Adrich) containing 12% fetal bovine serum, 2% L-glutamine, and 100 U/ml penicillin-streptomycin at 37 °C and 5% CO_2. Coverslips were put into the dish before seeding of electroporated cells. On day 3, 7, and 14 post-electroporation, coverslips from the same dish of electroporated cells were removed and analyzed.

Tissue collection and immunohistochemistry: Retinal explants and enucleated eyes were fixed in 4% paraformaldehyde (PFA) in 1X PBS (10X; 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4xH_2O$, 2 g KH_2PO_4, pH 7.4) at 4 °C for 15 min and cryoprotected in 30% sucrose in 1X PBS. The tissue was embedded in optimum cutting temperature (OCT) compound (NEG50, Richard-Allan Scientific, San Diego, CA), and 10 µm sections were collected on Superfrost Plus slides (J1800AMNZ, Menzel-Gläser, Braunschweig, Germany). DF1-cells were fixed in 4% PFA at 4 °C for 15 min.

For immunohistochemistry, the retinal sections were rehydrated in 1X PBS for 15 min and incubated in blocking solution (1% fetal calf serum, 0.02% Thimerosal, and 0.2% Triton X-100 in 1X PBS) for 30 min. Primary and secondary antibodies were diluted in blocking solution and incubated on slides in a humidified chamber overnight at 4 °C and for 2 h at room temperature, respectively.

Table 1. List of primary antibodies.

Antibody	Host	Dilution	Company	Catalog number
GFP	Goat	1:4,000	Abcam	ab5450
GFP	Rabbit	1:4,000	Abcam	ab28283
Isl1	Mouse	1:200	Developmental studies hybridoma bank	40.2D6
Lim1/2	Mouse	1:20	Developmental studies hybridoma bank	4F2-s
TrkA	Rabbit	1:2,000	Gift from Louis Reichardt[1]	-
Visinin	Mouse	1:1,000	Developmental studies hybridoma bank	7G4
β-galactosidase	Rabbit	1:2,000	Bio-Rad Laboratories	4600–1505
GABA	Rabbit	1:1,000	Sigma-Aldrich	A2052
Red/green opsin	Rabbit	1:2,000	Millipore	AB5405
Rhodopsin	Rabbit	1:500	Cosmo Bio Co Ltd	LSL-LB-5555

[1]Lefcort F, Clary DO, Rusoff AC, Reichardt LF. Inhibition of the NT-3 receptor TrkC, early in chick embryogenesis, results in severe reductions in multiple neuronal subpopulations in the dorsal root ganglia. J Neurosci. 1996;16(11):3704–13.

Primary antibodies are listed in Table 1. A GFP antibody was used to stain retinal sections to allow for better image capturing; the GFP expressed from the vector was sufficient for all other analyses. Secondary antibodies were obtained from Invitrogen (Carlsbad, CA). ProLong Gold (P36935, Life Technologies, Eugene, OR) with 4',6-diamidino-2-phenylindole (DAPI) was used to visualize the nuclei.

Cell counting: Retinas from electroporated animals were cryosectioned, and the GFP positive (+) cells were counted. GFP+ cells were allocated to the ONL, INL, or ganglion cell layer (GCL), based on their nuclear position. Four animals per time point were analyzed and used for cell counting. Distribution is presented as mean ± standard deviation (SD).

Image analysis: Images of whole retinas were captured using a Leica M165FC (Leica Microsystems, Wetzlar, Germany) stereomicroscope equipped with a Leica DFC495 camera. Images of sectioned retinas were captured using a Zeiss Axioplan 2 microscope equipped with an AxioCam C camera or a Zeiss LSM 510 Meta confocal microscope. Confocal images were acquired with laser line 633 nm using BP filter 505–530 and a Plan-Apochromat 20x/0.8 or 63x/1.4 oil DIC objective lens. Figures were assembled in Adobe Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA).

RESULTS

Analysis of minimal promoter activity: We tested the HSP68-LacZ [36], ptkEGFP [37], and Stagia3 [12] trap vectors with electroporation of Hamburger and Hamilton stages 21–25 (E3.5–4.5; Figure 1A) [38] whole retinal explants, a technique that allows for rapid screening of multiple vectors [6]. LacZ expression from the HSP68-LacZ vector was detected with immunohistochemistry for β-galactosidase, whereas GFP expression from ptkEGFP and Stagia3 was visualized with fluorescence microscopy (Figure 1B–E, n≥4).

The HSP68-LacZ trap vector has been used to visualize the expression pattern of human cis-regulatory elements in mice embryos [36]. The Hsp68 basal promoter does not have background expression in the mouse [43]; however, we detected LacZ expression in the chicken retina without any inserted enhancer (Figure 1B). The ptkEGFP trap vector also gave background expression (Figure 1C). Stagia3 [12] has been used for studies in the mouse and chicken retina [25]. Consistent with this previous work, we did not detect reporter gene expression in the absence of an enhancer element (Figure 1D). To confirm that Stagia3 could drive expression, the CMV enhancer was inserted. Stagia3-CMV produced robust GFP expression, thus verifying the function of Stagia3-CMV (Figure 1E).

Evaluation of the Cre-LoxP piggyBac transposon system in the developing chicken retina: To confirm that the reporter gene was integrated into the genome, we electroporated chicken fibroblast cells (DF1-cells) with the pBase "donor" vector, pB-CAG-LoxP-STOP-LoxP-GFP-pB, and Stacia3-CMV, the modified Stagia3 vector with the CMV enhancer and EGFP replaced with the Cre recombinase sequence (Figure 2A,B). Stacia3-CMV gave strong ubiquitous Cre expression and was used as a positive control. Staria3-CMV, a modified Stagia3 vector with a CMV enhancer and EGFP replaced with an RFP sequence, was coelectroporated with the other constructs to serve as an electroporation control

(Figure 2B′). Three days post-electroporation, strong GFP and RFP signals were seen, but after 14 days, RFP was no longer detectable, while GFP remained strong in clone-like clusters of cells (Figure 2A). The result indicated that the RFP vector that was not integrated had been lost while the GFP vector was integrated and produced stable expression.

We confirmed the functionality of the Cre-LoxP piggyBac transposon system in the embryonic chicken retina. Stage 12 (E2) optic vesicles were electroporated in ovo with the constructs (Figure 2B′′) followed by analysis 6 days later at stage 34 (E8; Figure 2C, n = 4). The intact electroporated retinas showed a large area of GFP+ cells (Figure 2C), and immunohistochemical analysis showed clone-like clusters of GFP+ cells along the apicobasal axis of the retina (Figure 2C). We also electroporated retinas at stage 22, 25, or 28 (E3.5, 4.5, and 5.5) and analyzed them at stage 36, to confirm functionality (Figure 2D, n>4 for each stage).

Analysis of cis-regulatory elements of the Pax6, Otx2, and RXRγ genes: We used the ECR Browser (evolutionary conserved genomic regions), a tool especially designed for comparison of multiple vertebrate genomes [44] and the VISTA database [36] and searched the literature for relevant regulatory elements. We searched genes known to be expressed in HCs, such as *Lim1*, *Prox1*, and *Pax6* [19]. Negative results, that is, conserved regulatory elements that did not produce detectable reporter gene expression in the chicken retina, are not reported.

A 300 bp conserved region located in close proximity to the transcription initiation site for the *PAX6 gene* was PCR amplified and cloned into the Stacia3 vector (Stacia3-Pax6.300; Figure 3A,B). The Pax6.300 element was analyzed after in ovo electroporation of the stage 25 (E4.5) embryos (GFP expression driven by a specific gene sequence, enhancer, or promoter is referred to as gene::GFP). Analysis at stage 34 (E8), a time point when all the different cell types and layers in the retina have been established [28], showed GFP expression (Figure 3B, n = 4) in several cells located along the apicobasal axis of the stage 34 retina.

A conserved regulatory sequence of the *Otx2 gene* has been shown to direct expression to immature PR, HC, and some retinal ganglion cell progenitors [25]. We PCR amplified the 294 bp *Otx2* sequence and cloned it into the Stacia3

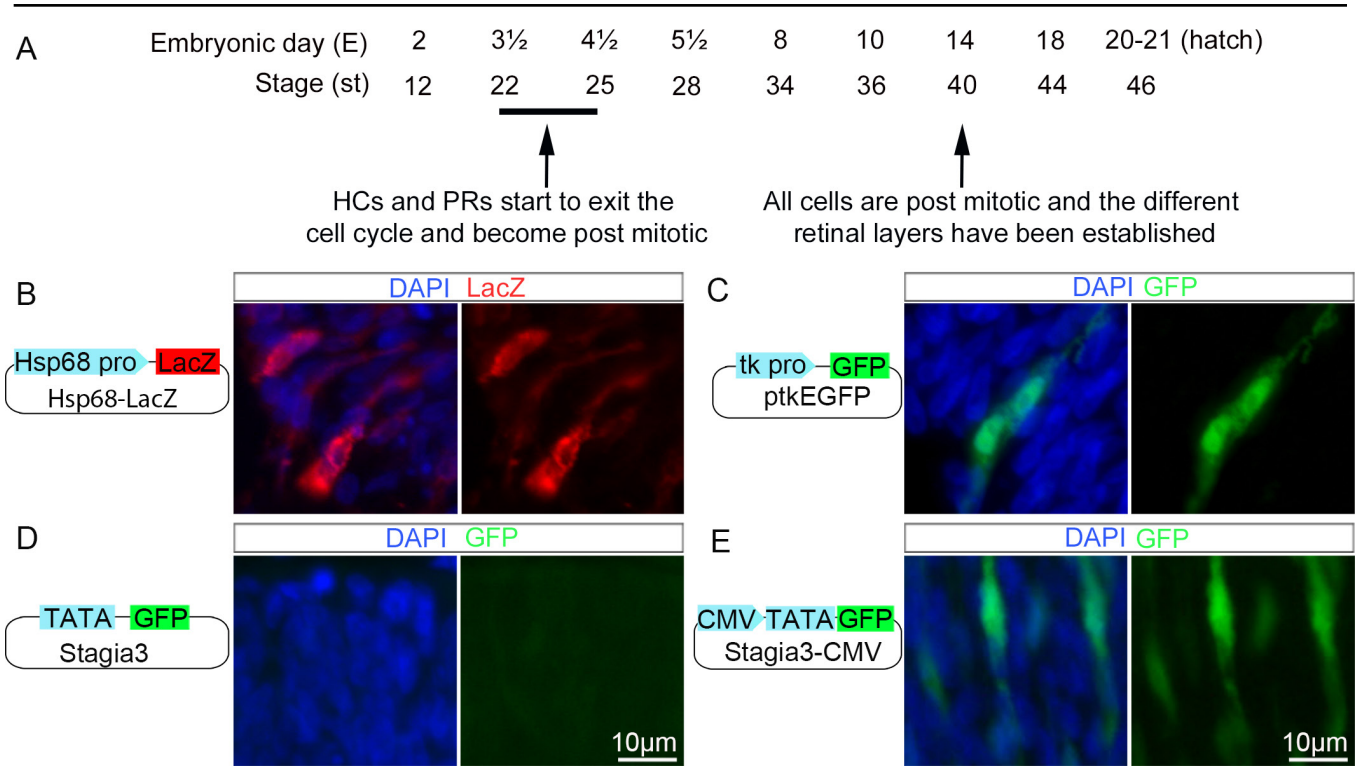

Figure 1. Evaluation of enhancer trap vectors. To test background expression from the enhancer trap vectors Hsp68-LacZ, ptkEGFP, or Stagia3, embryonic chicken retinas were electroporated, and reporter gene expression analyzed. **A**: Chicken embryonic development indicated by days and stages, according to Hamburger and Hamilton [38]. Important time points in neurogenesis [28] are indicated by arrows. **B**–**E**: Diagrams of the trap vectors and fluorescence micrographs of the retinal explants electroporated at stages 22–25 and analyzed after 24 h.

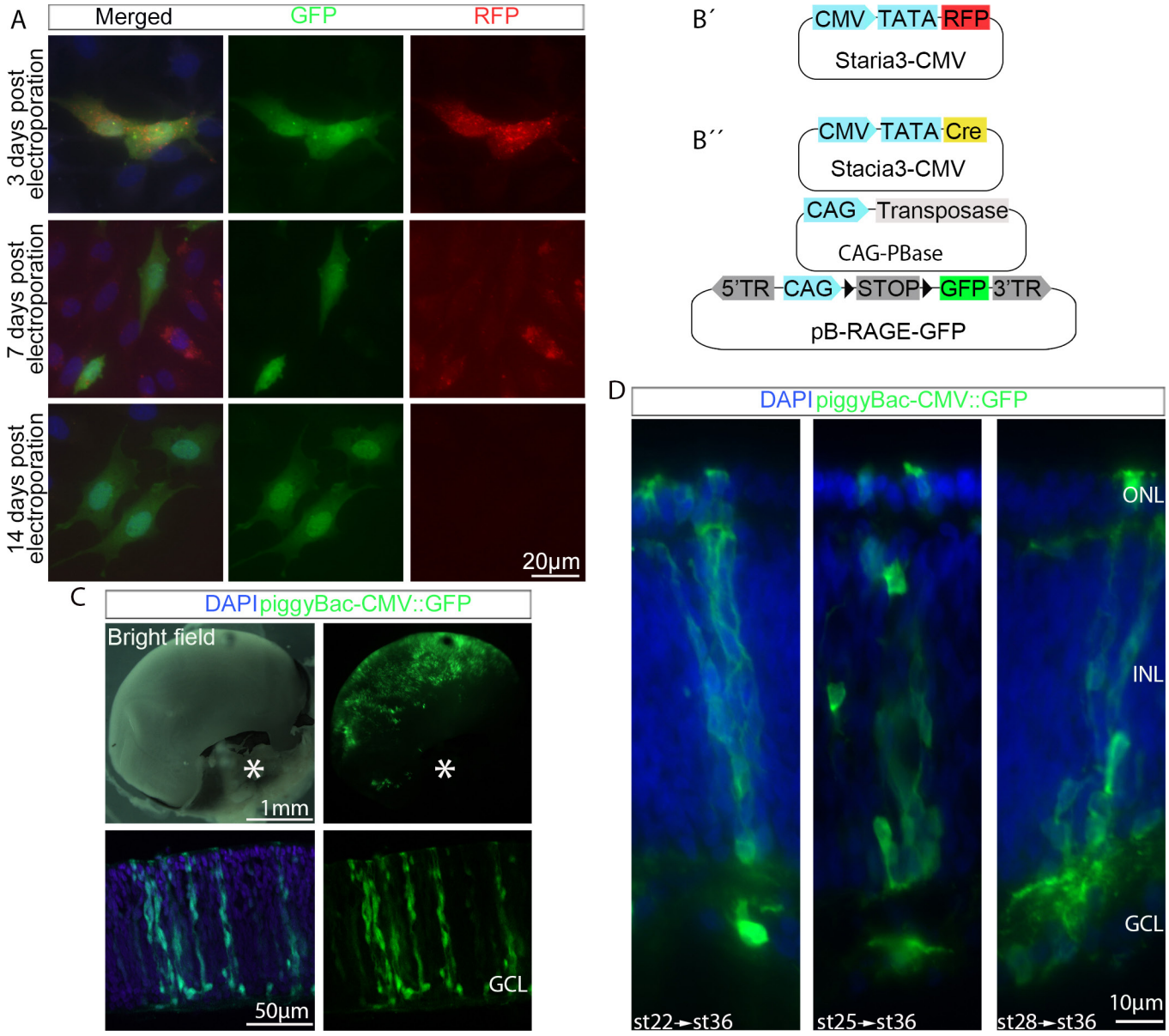

Figure 2. Evaluation of the Cre-LoxP piggyBac transposon system. **A**: Fluorescence micrographs of chicken fibroblast DF1-cells electroporated with the piggyBac constructs and a red fluorescence protein (RFP) control, and analyzed 3, 7, or 14 days post-treatment. **B´**: Diagram of the RFP-expressing control construct. **B´´**: Diagrams of the green fluorescent protein (GFP) reporter gene constructs used for integrations into the host cell genome. **C**: Micrographs of the whole retina and of the section from a stage 34 retina electroporated with the Stacia3-cytomegalovirus (CMV), CAG-PBase, and pB-RAGE-green fluorescent protein (GFP) constructs in ovo at stage 12. Asterisk (*) indicates the optic nerve exit. **D**: Fluorescence micrographs of stage 36 retinas that were electroporated with the Cre-LoxP piggyBac constructs in ovo at stages 22, 25, and 28. St = stage, ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.

vector (Stacia3-Otx2.294). In ovo electroporation of the stage 25 (E4.5) embryos showed GFP expression in cells along the apicobasal axis upon analysis of the stage 34 retina (Figure 3C, n = 4). Immunohistochemistry showed colocalization between the Otx2.294::GFP cells and markers for all cell types in the retina (data not shown); no evident cell specificity was seen. A 208 bp element from the *RXRγ gene* [41], previously shown to drive expression in the neural retina, was PCR amplified and inserted into the Stacia3 vector (Stacia3-RXRγ208). In ovo electroporation of the stage 25 (E4.5) embryos showed RXRγ208::GFP cells that were mainly, but not exclusively, localized to the ONL and the outer part of the INL in the stage 34 retina (Figure 3D, n>4).

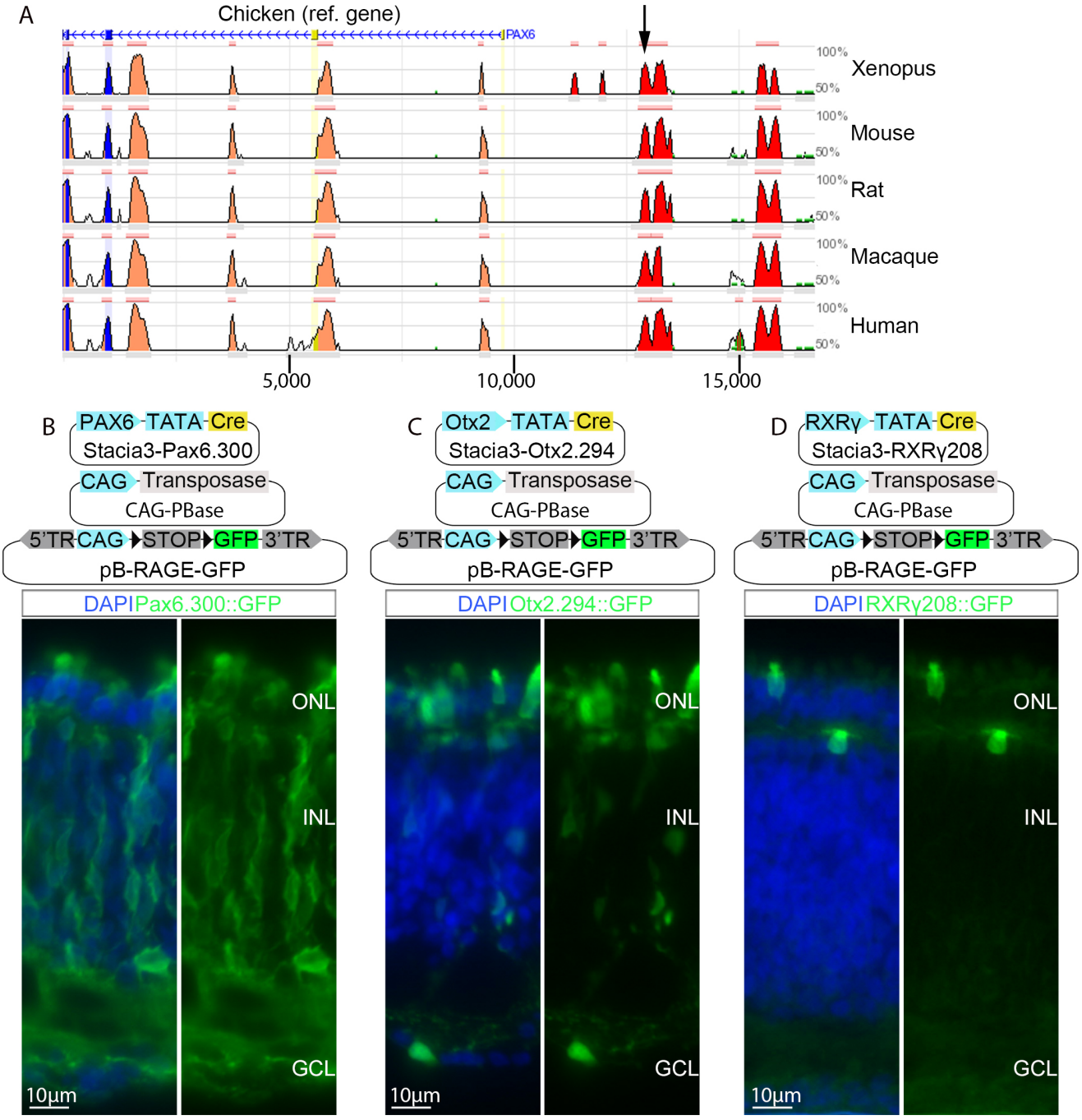

Figure 3. Assessment of the Pax6, Otx2, and RXRγ regulatory sequences in combination with the piggyBac integration system. **A**: An evolutionarily conserved sequence (indicated by the arrow) from the chicken *PAX6 gene*, positioned upstream of the transcription start [44]. Color-coded peaks correspond to sequences that are highly conserved between the species. The protein coding exons are blue, the conserved intronic regions are salmon, the 5′ untranslated regions (UTRs) are yellow, and the conserved upstream (intergenic) regions are red. The numbers underneath denote distance in base pairs. **B**–**D**: Diagrams of constructs and fluorescence micrographs of sectioned retinas electroporated at stage 25 with the Pax6.300, Otx2.294, or RXRγ208 driven Cre-LoxP piggyBac constructs and analyzed at stage 34. St = stage, ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.

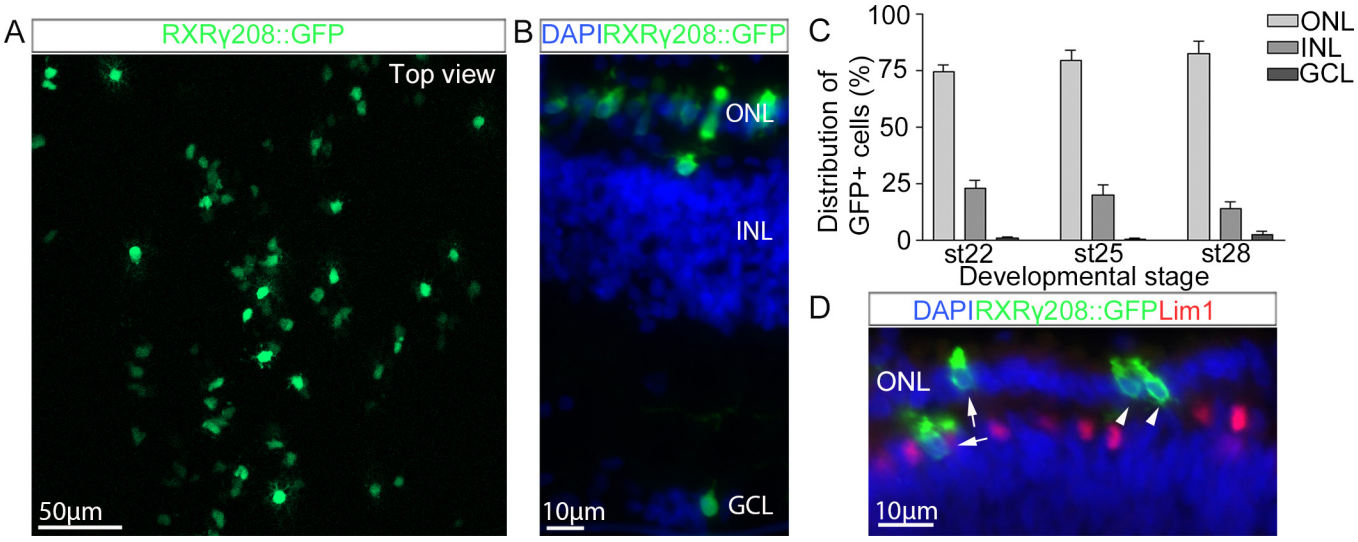

Figure 4. Activity of the RXRγ208 sequence produced specific reporter gene expression. **A**: Z-stacked confocal fluorescence micrograph of a flatmounted retina from a stage 40 embryo electroporated with the RXRγ208 Cre-LoxP piggyBac constructs in ovo at stage 25. **B**: Fluorescence micrograph of a retina from a stage 40 embryo electroporated with the RXRγ208 Cre-LoxP piggyBac constructs in ovo at stage 28. **C**: Bar graph showing the distribution of green fluorescent protein–positive (GFP+) cells in sectioned retinas from embryos electroporated with the RXRγ208 Cre-LoxP piggyBac constructs in ovo at stages 22, 25, and 28 (mean ± standard deviation, SDSD, n = 4 per stage). **D**: Fluorescence micrograph of a retina showing a pair of photoreceptors (PRs; arrow heads) and a pair with a horizontal cell (HC) and a PR (arrows). St = stage, ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.

The RXRγ208 element directs gene expression to the PRs and the HCs: We analyzed RXRγ208::GFP cells in seven flatmounted intact stage 40/44 (E14/18) retinas that had been electroporated at stage 22/25/28, and the results showed a "stars in the sky"-like pattern of GFP+ cells. A representative image of an E14 retina electroporated at stage 25 is shown in Figure 4A. Sections confirmed the scattered pattern with separate GFP+ cells mainly in the ONL and the horizontal cell layer (HCL; Figure 4B, n>4).

Next, we quantified the distribution of the RXRγ208::GFP cells in the different layers. GFP+ cells were allocated to the ONL, INL, or GCL, based on their nuclear position [45]. Retinas were electroporated at stages 22, 25, and 28. For stage 22, 74±3.0% of the cells were localized to the ONL, 23±4.0% were localized to the INL, and a small proportion, 1.0±1.0%, were found in the GCL (Figure 4C, 603 cells counted, mean ± SD, n = 4). For stage 25, 79±4.0% was localized to the ONL, 20±4.0% to the INL, and 0.5±0.5% to the GCL (Figure 4C, 368 cells counted, mean ± SD, n = 4). For stage 28, 83±5.0% was localized to the ONL, 14±3.0% to the INL, and 2.0±2.0% to the GCL (Figure 4C, 560 cells counted, mean ± SD, n = 4). The fraction of RXRγ208::GFP cells located in the INL was further subdivided into the HCL and non-HCL cells, based on their nuclear position. For the retinas electroporated at stage 22, 91±9.0% were located at HCL (131 cells counted, mean ± SD, n = 4). For stage 25, 89±7.0% were located at the HCL (71 cells counted, mean ± SD, n = 4). For stage 28, 94±4.0% were located at the HCL (79 cells counted, mean ± SD, n = 4).

We noted that cells appeared in pairs with one PR and one HC or two PRs (Figure 3D and Figure 4D). This was further quantified, and PRs were considered to be a pair if their nuclei were located <10 µm apart, and a PR and an HC that were arranged in a line on the apicobasal axis were considered to be a pair. In the retinas electroporated at stages 22–28 and analyzed ≥8 days later, 14±2.0% of all GFP+ cells were part of a PR pair, and 8.0±2.0% of all GFP+ cells were part of a pair consisting of a PR and an HC (513 cells counted, mean ± SD, n = 4). There was a significant difference in the number of cells involved in a PR pair compared to those involved in a PR and HC pair ($p<0.01$). The cells in a PR pair were not part of a PR and HC pair.

Most of the RXRγ208::GFP cells (about 99%) were present either in the ONL or in the outer part of the INL, suggesting that they were PRs and HCs. The PRs (cones and rods) were specifically identified by the Ca^{2+} binding protein visinin [29,46-48] (Figure 5A). In the retinas electroporated at stages 22–28 and analyzed ≥8 days later, 86±6.0% of the GFP+ cells in the ONL were visinin+ (167 cells counted, mean ± SD, n = 5). The chicken retina has three types of HCs (H1, H2, H3) that are distinguished by morphology and molecular markers [49]: axon-bearing subtype (H1)

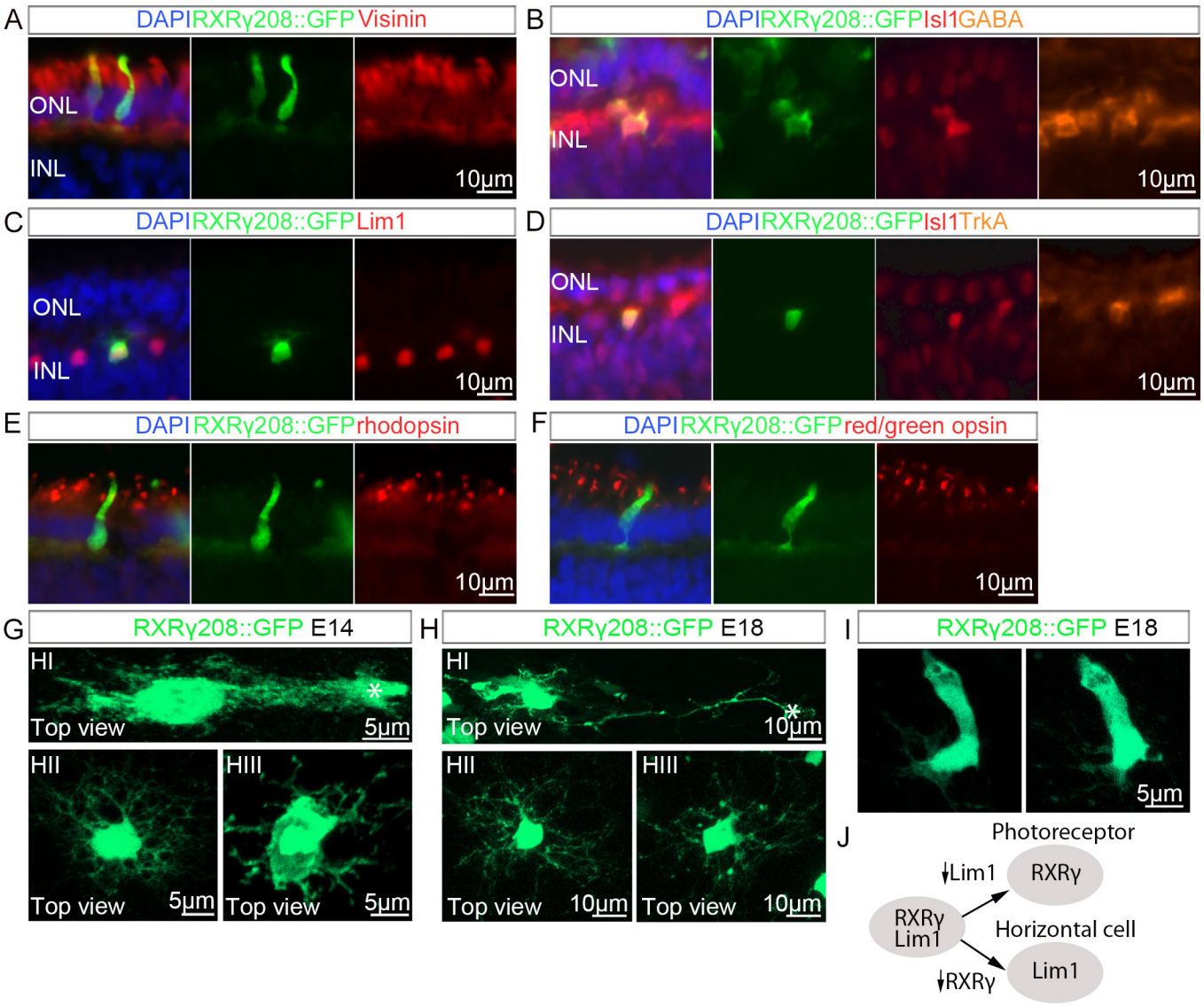

Figure 5. RXRγ208-activated expression in PRs and all three HC subtypes. **A**–**D**: Fluorescence micrographs of embryonic day 14 (E14) retinas showing green fluorescent protein–positive (GFP+) cells that colocalize with markers for photoreceptors (PRs), axon-bearing horizontal cells (HCs; H1), and axon-less HCs (H2 and H3). **E**–**F**: Fluorescence micrographs of E18 retinas showing GFP+ cells colocalizing with rhodopsin but no colocalizing with the red or green opsin marker. **G**–**H**: Z-stacked confocal fluorescence micrographs of flatmounted retinas showing the presence of all three HC subtypes (H1, H2, H3) in the E14 and E18 embryos. Asterisk (*) denotes the axon terminal. **I**: Z-stacked confocal fluorescence micrographs of a flatmounted E18 retina showing the presence of PRs. **J**: Schematic model of the roles of RXRγ and Lim1 in photoreceptor and horizontal cell genesis. E = embryonic day; ONL = outer nuclear layer; INL = inner nuclear layer.

expressing Lim1, and two axon-less subtypes (H2 and H3) expressing Isl1 and the neurotransmitter γ-aminobutyric acid (GABA) or Isl1 and the neurotrophin receptor TrkA, respectively [20]. Immunohistochemistry showed colocalization of the GFP and HC subtype markers (Figure 5B–D). There are 50% axon-bearing and 50% axon-less cells in the chicken retina [20]. In the retinas electroporated at stages 22–28 and analyzed ≥8 days later, 70±21% of the GFP+ cells were Lim1+ axon-bearing H1 HCs (166 cells counted, mean ± SD, n=8). The GFP+ cells located in the ONL were further analyzed regarding the different PR subtypes. The chicken retina consists of five different opsin-expressing cells: those expressing rhodopsin, red opsin, green opsin, blue opsin, or violet opsin [29]. Immunohistochemistry showed colocalization of GFP and rhodopsin (Figure 5E), but no colocalization with the marker for red or green opsin (Figure 5F) in the retinas electroporated at stage 22 and analyzed at E18.

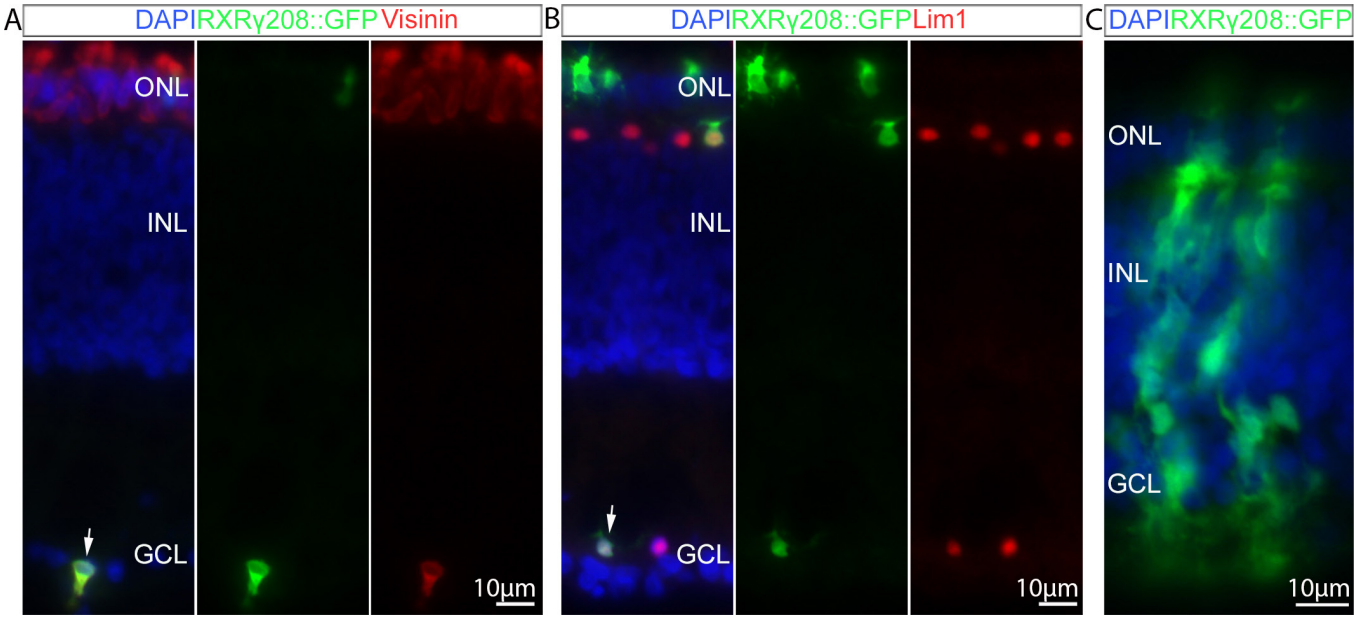

Figure 6. Displaced cells and electroporation of a stage 12 optic vesicle. **A**–**B**: Fluorescence micrograph of a sectioned retina from an embryonic day 14 (E14) embryo electroporated with the RXRγ208 Cre-LoxP piggyBac constructs in ovo at stage 28, showing a displaced photoreceptor (PR; arrow) and a displaced horizontal cell (HC; arrow). **C**: Fluorescence micrograph of a sectioned retina from an E14 embryo electroporated with the RXRγ208 Cre-LoxP piggyBac constructs in ovo at stage 12.

Unfortunately, we were unable to find an antibody that could detect the blue or violet opsin.

The E14 (n = 4) and E18 (n = 3) retinas were flatmounted and analyzed with confocal microscopy. Based on previous morphological descriptions [50], the axon-bearing (H1) and the two axon-less subtypes, H2 with long, thin processes and H3 with thicker dendritic terminals, were identified (Figure 5G,H). The PRs, with their distinct morphology [51], were easily distinguished (Figure 5I). Based on these results, and previous studies, a model of the expression and potential roles of *RXRγ* and *Lim1* in PR and HC genesis is presented in Figure 5J.

Displaced RXRγ208::GFP cells in the GCL: A fraction of the RXRγ208::GFP cells (about 1%) were found in the GCL. The displacement of HCs and PRs has been previously described [52-55]. Retinal sections were stained with either Lim1 (which marks the H1 subtype) or visinin (which marks the PRs). Lim1 and visinin colocalized with the RXRγ208::GFP cells in the GCL (Figure 6A,B). Non-GFP-expressing Lim1+ cells (Figure 6B) and visinin+ cells were also noted in the GCL, consistent with normal development.

RXRγ208::GFP cells in the E14 retina after electroporation of the optic vesicle: Studies performed in quail [56] show RXRγ expression in the optic vesicle and neural tube during early embryogenesis. Stage 12 (E2) chicken optic vesicles were therefore electroporated in ovo, and the retinas were analyzed at E14. RXRγ208::GFP cells were seen in all retinal layers along the apicobasal axis (Figure 6C).

DISCUSSION

Combining the techniques of episomal vector gene-specific Cre expression and genomic integration using the piggyBac transposon system opens up possibilities for studies of gene expression–specific cell lineage tracing in the chicken [14,57]. In this work, we used an expression vector with a 208 bp gene regulatory sequence from the chicken *RXRγ gene* (RXRγ208) [41] to drive Cre expression. The vector was combined with a piggyBac "donor" vector containing a floxed STOP sequence followed by EGFP, as well as a piggyBac helper vector for efficient integration into the host cell genome. The vectors were introduced into the embryonic chicken retina with in ovo electroporation. Cells that drove Cre expression from the regulatory RXRγ208 sequence excised the floxed STOP sequence and expressed GFP. The approach generated a stable lineage with robust expression of GFP in retinal cells that had activated transcription from the RXRγ208 element. The results showed that GFP was expressed in cells that express HC or PR markers when electroporation was performed between stage 22 and stage 28. Electroporation of the stage 12 optic cup gave multiple cell types in accordance with *RXRγ gene* expression.

All retinal cells share a common progenitor [58], and through the expression or absence of specific genes, the final identity of the retinal cells is established. Recent studies have shown that PRs and HCs share an immediate progenitor [22-24]. The cells also share other features, such as their final cell number is not regulated by apoptosis [59,60] and they have been implicated to be involved in retinoblastoma [18,61,62]. In addition, Lim1-expressing HCs are able to undergo an S-phase and remain with a duplicated genome [16], as well as enter mitosis in the presence of DNA damage [63]. Studies that further address the development and behavior of these highly interesting cells need to be performed.

In this study, we focused on genes important for PR and HC differentiation in the chicken embryonic retina. To identify relevant regulatory elements, we tested sequences located close to the transcription initiation site. Although many were highly conserved during evolution, most of the sequences tested did not produce any detectable reporter gene expression in the early developing retina. This is expected as gene regulation is complex, and to drive expression, the selected sequences have to attract transcriptional activators. Transcriptional activation is often dependent on cooperative interactions between several cis-regulatory elements where a defined distance between the elements and the promoter is important. We used an episomal vector with a minimal promoter, and to activate expression, the introduced regulatory sequences must be able to recruit and activate the transcription initiation complex. Therefore, transcriptional activators that remodel the chromatin structure or factors that are chromatin context-dependent are less likely to work. Taken together, these factors highlight the difficulty of identifying elements that drive cell-specific expression in episomal expression vectors, which was reflected in our results.

We tested three enhancer trap expression vectors: HSP68-LacZ, ptkEGFP, and Stagia3. The HSP68-LacZ vector has previously been successfully used in transgenic mice [36]. Our results showed that the HSP68-LacZ vector produced reporter background expression without any inserted regulatory sequence (Figure 1B), and therefore was not useful for our studies. A similar result has been seen in the chicken neural tube [64]. The leakage from the HSP68-LacZ minimal promoter may be explained by the fact that the construct was episomally located in the chicken but was integrated into the genome in the mouse studies. The ptkEGFP vector has been used for functional analysis of enhancers in the central nervous system (CNS) of chicken embryos [37]; however, this vector also produces background reporter gene expression in the neural retina (Figure 1C). The Stagia3 trap vector was constructed and tested to work episomally [12,25], and we did not detect any background expression (Figure 1D).

Electroporation of episomal expression vectors gives transient expression during a few days (Figure 2A) [57,65]. However, GFP has been detected after longer periods in post-mitotic neurons [8]. To achieve stable expression after electroporation, a donor unit with a strong CAG promoter was incorporated into the genome of the host cell. There are several such systems, including the piggyBac, tol2, and Sleeping Beauty among which the piggyBac system generates the highest efficiency in terms of stable gene integration [66]. Our results showed that the piggyBac system worked well in embryos of developmental stages ranging from stages 12 to 28 (E2–5.5; Figure 2C,D). Dividing retinal progenitor cells and post-mitotic cells, such as stage 28 ganglion cells, could be targeted. The moth-derived piggyBac system is not species dependent, and it has been used in plants, insects, fish, birds, and mammals [42,67-70], making it a highly versatile tool.

We observed expression in the retina with regulatory sequences from the *Pax6*, *Otx2*, and *RXRγ genes* (Figure 3). The transcription factor Pax6 is often considered the master regulator of eye formation and is expressed during early retinal development [71]. The expression remains and increases in differentiated HCs, amacrine cells, and retinal ganglion cells [19]. The Pax6 promoter and proximal elements have been extensively studied in the mouse [72-74]. A sequence upstream of the mouse P0 promoter drives expression to the HCs [74]. In contrast to the mouse *Pax6 gene* that has multiple transcripts, only one transcript has been found in the chicken. A 300 bp conserved sequence located proximal to the transcription initiation site of the chicken *PAX6 gene* drove expression in the neural retina, but without cell-type specificity (Figure 3A,B). The GFP+ cells were located all along the apicobasal axis of the retina, stretching from the ONL to the GCL.

The *Otx2 gene* is already expressed in the optic vesicle and is important for proper eye and retinal development [75-77]. We tested a 294 bp conserved sequence from the retinal transcription factor Otx2 [25] in combination with the piggyBac system, and it drove expression in retinal neurons, but not with a clear cell-type specificity (Figure 3C). The *Otx2 gene* has multiple promoters and enhancers that act at different times and cell types in a context-dependent fashion [78,79]. In contrast, Cre expression driven by a 208 bp gene regulatory sequence from the chicken *RXRγ gene* [41] produced stable GFP expression (RXRγ208::GFP) in PRs and HCs (Figure 4 and Figure 5). RXRγ is expressed in retinal progenitor cells during development, and its expression is

later restricted to the ONL in the chicken and cow retina [26,27] and to the ONL, INL, and GCL in the mouse [30].

The RXRγ208::GFP cells were sometimes seen in pairs with one PR and one HC or two PRs (Figure 4D). The results showed that the RXRγ208 sequence directed Cre expression to cells that generate PRs and HCs in a pattern that indicates the onset of the *RXR*γ208 element in a progenitor cell that gives rise to HCs and PRs. This result is consistent with previous data from chicken, zebrafish, and mouse showing that HCs and PRs are generated from a common progenitor cell [22-24] and that cone photoreceptors can be generated by symmetric terminal division of dedicated precursors in zebrafish [22]. We defined a pair of cells as two cells located close together at a maximum distance of 10 µm. Quantification showed that about 14% of all GFP+ cells were part of a PR pair and about 8% of all GFP+ cells were part of a pair consisting of a PR and an HC. However, these numbers may not fully account for all the pairs generated because tangential movement of PRs and HCs that separate the cells has been reported [80,81]. To study the formation of pairs, live cell imaging may be the best option. However, this method is difficult to carry out in chicken embryos due to the dense pigmentation of the RPE.

The distribution of RXRγ208::GFP cells was approximately 80% in the ONL, 20% in the INL, and 1% in the GCL (Figure 4C). The numbers are in agreement with a study in zebrafish in which dedicated retinal progenitor cells gave rise to PRs and HCs or only PRs [22]. In addition, post-mitotic PRs that activate RXRγ208 will become GFP+ and increase the number of GFP+ cells in the ONL. Our result strengthens the hypothesis that some progenitors produce PRs and HCs, and some produce only PRs.

Immunohistochemistry with subtype markers and analysis of cell morphology using confocal microscopy showed that all three subtypes of HCs are represented among the RXRγ208::GFP cells (Figure 5), indicating that RXRγ208 is activated in the progenitor for all three subtypes. This is consistent with a previous study that showed all three subtypes can be generated following labeling of retinal progenitor cells at E4 [81]. The ratio of GFP+ axon-bearing HCs was slightly higher than the normal ratio of 50% [20,81]. This could be due to the time point at which the reporter was integrated. A similar conclusion was reached based on results from a virus-mediated reporter transfer study in which the ratio of HC subtypes varied depending on developmental age [81].

Immunohistochemistry with PR subtype markers showed colocalization between GFP+ cells and rhodopsin (Figure 5E), indicating that the RXRγ208 sequence drives expression in cells destined to become rods. This conclusion was further supported by the fact that the rods tend to have their nuclei localized in the part of the ONL that is toward the INL [82,83], and that treatment with the signaling molecule RA, which acts via nuclear receptors such as RXRγ, promotes rod differentiation [32,35]. No colocalization was detected with the red or green opsin marker (Figure 5F). Unfortunately, we were unable to find an antibody that stains the blue or violet opsin-expressing cells. However, based on the nuclear position of the GFP+ cells that were red or green negative, closer to the outer segments compared to the nuclei of rods [82,83], they may be blue or violet opsin-expressing cells. Which subtype of PR cell that is produced following activation of the RXRγ208 sequence used in this study may depend on the specific time point for the electroporation. In addition, the sequence investigated here may not fully reflect the endogenous expression pattern as other important sequences or factors may be needed to replicate the exact endogenous RXRγ expression. Our results showing that this RXRγ208 element directs expression to the HCs and PRs opens up for further studies of the *RXRγ gene* and its regulation, as well as PR subtype specification.

We have summarized our results with previous studies in a model that displays the involvement of *RXRγ* and *Lim1* in PR and HC genesis (Figure 5J). Previous studies showed that RXRγ is expressed in the neural retina [26,27,30-33]. However, neither of the publications reported any colocalization between RXRγ and HC markers. Therefore, our results add new insight into the role of RXRγ during retinal development.

When we electroporated the optic cup of the stage 12 (E2) embryos and analyzed the retinas after cell differentiation, we found RXRγ208::GFP cells spread out across the whole apicobasal axis of the retina representing several retinal cell types (Figure 6C). This is consistent with results showing expression of RXRγ in the optic cup neuroepithelium in quail [56] in multipotent retinal progenitors at a time well before cell type formation and neuronal differentiation [28].

By using the system described here, it is possible to detect cells that at some point during development expressed the gene of interest even if that gene is no longer actively transcribed, an advantage that will aid in the necessary work to characterize and better understand the expression and function of genes involved in development. In addition, the chicken embryo provides an easily accessible, and cost-effective, model where regulatory sequences can be tested in a time-efficient manner. Electroporation of piggyBac transposons enables integration and stable expression of reporter genes without the need to generate transgenic animals. It also

allows for site- and time-specific integration or expression, which may be important if the gene that needs to be investigated causes gross malformations or is embryonically lethal. Electroporation of DNA constructs is a method that can be, and has been, applied to other model organisms, as well as other cell types. However, to target the retinal cells, the availability of the embryo poses a limiting factor, and the protocol presented here may have to be adjusted.

To conclude, it is possible to selectively target and trace PRs and HCs in the embryonic chicken retina by using the *RXR*γ208 element in combination with the piggyBac integration system and in ovo electroporation. The results confirm the hypothesis that HCs and PRs share a common progenitor. In addition, the technique opens up the possibility of specific expression of effector genes, such as regulators of cell fate or cell cycle progression, which will benefit further studies that address the behavior of the HCs, PRs, and their common progenitor. The genetic network governing the generation of PRs and HCs is far from fully understood, and in this study, we have added one more component.

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