

# Embryonic markers of cone differentiation

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**Purpose:** Photoreceptor cells are born in two distinct phases of vertebrate retinogenesis. In the mouse retina, cones are born primarily during embryogenesis, while rod formation occurs later in embryogenesis and early postnatal ages. Despite this dichotomy in photoreceptor birthdates, the visual pigments and phototransduction machinery are not reactive to visual stimulus in either type of photoreceptor cell until the second postnatal week. Several markers of early cone formation have been identified, including *Otx2*, *Crx*, *Blimp1*, *NeuroD*, *Trβ2*, *Rorβ*, and *Rxry*, and all are thought to be involved in cellular determination. However, little is known about the expression of proteins involved in cone visual transduction during early retinogenesis. Therefore, we sought to characterize visual transduction proteins that are expressed specifically in photoreceptors during mouse embryogenesis.

**Methods:** Eye tissue was collected from control and phosducin-null mice at embryonic and early postnatal ages. Immunohistochemistry and quantitative reverse transcriptase-PCR (qPCR) were used to measure the spatial and temporal expression patterns of phosducin (*Pdc*) and cone transducin  $\gamma$  (*Gngt2*) proteins and transcripts in the embryonic and early postnatal mouse retina.

**Results:** We identified the embryonic expression of phosducin (*Pdc*) and cone transducin  $\gamma$  (*Gngt2*) that coincides temporally and spatially with the earliest stages of cone histogenesis. Using immunohistochemistry, the phosducin protein was first detected in the retina at embryonic day (E)12.5, and cone transducin  $\gamma$  was observed at E13.5. The phosducin and cone transducin  $\gamma$  proteins were seen only in the outer neuroblastic layer, consistent with their expression in photoreceptors. At the embryonic ages, phosducin was coexpressed with *Rxry*, a known cone marker, and with *Otx2*, a marker of photoreceptors. *Pdc* and *Gngt2* mRNAs were detected as early as E10.5 with qPCR, although at low levels.

**Conclusions:** Visual transduction proteins are expressed at the earliest stages in developing cones, well before the onset of opsin gene expression. Given the delay in opsin expression in rods and cones, we speculate on the embryonic function of these G-protein signaling components beyond their roles in the visual transduction cascade.

Over the past two decades, components of the vertebrate visual transduction cascade have been characterized, and their functions in light-regulated signaling are well established. Visual signaling in the mouse retina does not begin until postnatal day (P) 13–14 [1]. Correspondingly, the onset of expression for rhodopsin and the cone opsins precedes eye opening and visual signaling by several days. However, rod and cone histogenesis begins even earlier, occurring in two distinct phases in vertebrate retinogenesis, with cones born embryonically and rods formed primarily during late embryogenesis and the postnatal period in rodents [2,3].

One of the mysteries of vertebrate retinogenesis is the lag between cone histogenesis and cone opsin expression. A similar lag exists between rod histogenesis and rhodopsin expression, although the delay is not as extended and occurs postnatally [4]. In mouse cone development, the earliest cones

become postmitotic around embryonic day (E)11.5 while cone opsin transcription starts days later, with S-cone opsin mRNA expressed by E15.5 [5,6] followed by faint mRNA expression for M-cone opsin around P7 [6,7]. Cone histogenesis and cone opsin protein expression shows an even greater lag, with S-cone opsin protein expression detected at P0 [5], and M-cone opsin protein expression detected around P14 [8,9].

Several genes involved in photoreceptor fate specification and differentiation are expressed during early cone histogenesis, including *Otx2*, *NeuroD*, *Blimp1*, *Rorβ*, and *Crx* [10–17], but these factors are all expressed in rods and cones. Early determinants of cone differentiation include *Rxry* and *Trβ2*, although again these factors are thought to regulate cone cell fate [5,18–20]. Alternatively, proteins involved in visual transduction (potential markers of differentiated cones) are expressed at or slightly preceding the onset of cone opsin expression, which is still days before the onset of visual transduction. However, one cone marker involved in visual transduction is expressed embryonically; cone transducin  $\gamma$  expression was detected at E15.5 in mice [21].

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Transducin is a heterotrimeric G protein found in rod and cone photoreceptors. Transducin is composed of three subunits, designated as  $G\alpha_{11}\beta_1\gamma_1$  and  $G\alpha_{12}\beta_3\gamma_8$  in rods and cones, respectively. The genes encoding the transducin subunits in cones are *Gnat2*, *Gnbt2*, and *Gngt2*. Transducin is an essential component of the phototransduction cascade. When photons are absorbed by either rhodopsin or cone opsin, transducin is activated. Once activated, transducin separates into  $\alpha$ - and  $\beta\gamma$ -subunits. The  $\alpha$ -subunit subsequently activates phosphodiesterase 6, which, in turn, reduces the intracellular cGMP levels and leads to hyperpolarization. The  $\beta\gamma$ -subunit forms a complex with phosducin, a cytosolic phosphoprotein expressed in rods and cones [22-27]. Phosducin is involved in the translocation of transducin  $\beta\gamma$  within photoreceptors during light adaptation [28].

We sought to characterize the expression of visual transduction proteins that are active embryonically during cone histogenesis. We report that phosducin and cone transducin  $\gamma$  are expressed early in cone histogenesis, with phosducin expression detected at E12.5 and cone transducin  $\gamma$  at E13.5. Here, we show the spatial and temporal profiles for the expression of these two genes and their corresponding proteins during the embryonic and postnatal periods.

## METHODS

**Animals and tissue collection:** Embryonic and postnatal eyes were collected from time-pregnant FVB/N female mice that had been mated with C57Bl/6J male mice to prevent the retinal degeneration (*rd<sup>1</sup>/rd<sup>1</sup>*) present in the FVB/N strain [29-31]. Eyes from mice at the selected ages (E13.5 and P0) were also collected from pure C57Bl/6J mice (to test for strain differences) and from a phosducin-null strain [28] (to test for antibody and primer specificity). Midnight of the mating date was considered embryonic day (E)0. The day of birth was counted as postnatal day (P)0. For mouse pups E10.5-E17.5, the mother was euthanized by cervical dislocation, fetuses were then surgically dissected from the uterus and decapitated. Newborn mouse pups (P0) were euthanized via decapitation following induced hypothermia and P21 pups were euthanized by cervical dislocation followed by decapitation. Following euthanasia, whole eyes were removed from the orbit using either a 26g beveled needle (E10.5-E13.5) or forceps (E15.5-P21). Eyes for immunohistology were fixed as whole heads (E10.5-17.5) or just eyes (P0-P21) in a 4% paraformaldehyde solution of phosphate-buffered saline (1X PBS; 150 mM NaCl, 1.06 mM  $KH_2PO_4$ , 2.97 mM  $Na_2HPO_4 \cdot 7H_2O$ , pH 7.4) at 4 °C overnight, and cryoprotected in 30% sucrose, 1X PBS. The eyes for the quantitative reverse transcriptase-PCR (qPCR) analysis were frozen on dry ice

in pairs. All animal procedures were approved by the West Virginia University (WVU) Institutional Animal Care and Use Committee and followed the guidelines set out by the Association for Research in Vision and Ophthalmology.

**Immunohistochemistry and immunofluorescence:** Cryoprotected eyes (n = 6 for each age) were mounted in TBS tissue freezing media (Triangle Biomedical Sciences, Durham, NC), frozen and sectioned on a Leica CM3050S cryostat (Buffalo Grove, IL) at 10  $\mu$ m thickness, and then transferred to glass slides. Before the antibody processing, as part of our standard protocol, the samples were subjected to an antigen retrieval procedure of 0.1 M Tris pH 9.5 incubation at 95 °C for 20 min. Test runs with and without antigen retrieval showed similar labeling for the phosducin and cone transducin  $\gamma$  antibodies. Following antigen retrieval, the sections were blocked with normal serum and treated with primary and secondary antibodies, following our published procedure [32]. The primary antibodies used in this study were anti-phosducin (as previously described by Sokolov et al. [28]; 1:1,000), anti-cone transducin  $\gamma$  (rabbit; CytoSignal, Irvine, CA; 1:500-1,000), anti-S cone opsin (rabbit; Chemicon, Temecula, CA; 1:100), anti-mouse cone arrestin (mouse; gift from Dr. Cheryl Craft; 1:500), anti-retinoid X receptor  $\gamma$  (rabbit; Santa Cruz Biotechnology, Dallas, TX; 1:1000), anti-cone phosphodiesterase (rabbit; Thermo Fisher Scientific, Waltham, MA; 1:500), and anti-Otx2 (rabbit; Millipore, Billerica, MA; 1:1000). Secondary antibodies were anti-sheep, anti-mouse, or anti-rabbit antibodies that were either biotin-labeled for Elite-ABC reactions (Vector Laboratories Inc.; Burlingame, CA) or fluorophore-tagged for immunofluorescence (Molecular Probes, Eugene, OR). Images were captured on an Olympus AX70 microscope (Olympus; Center Valley, PA) equipped with a MicroFire digital camera (Optronics; Goleta, CA) or a Zeiss 710 confocal microscope (Carl Zeiss, Inc.; Thornwood, NY).

**Quantification of colabeled cells:** Eyes from E13.5, E15.5, E17.5, and P0 mouse pups were sectioned at 12  $\mu$ m thickness. Immunofluorescence following the above protocol was performed on the retinal sections using anti-phosducin and anti-cone transducin  $\gamma$  antibodies and propidium iodide as a nuclear counterstain. Three animals per age, with multiple quadrants of at least three sections per animal, were imaged on a Zeiss 710 confocal microscope. Labeled cells were counted within the imaged Z-stacks using the cell counter plugin of Fiji imaging software (Madison, WI) [33]. All labeled cells were counted and designated as colabeled, phosducin only, or cone transducin  $\gamma$  only. The percentage of labeled cells was then calculated by dividing the number of cells in each category by the total number of labeled cells

for each age. Data are presented as percentage  $\pm$  standard deviation (SD) for each category.

**Quantitative reverse transcriptase–PCR:** Total RNA was isolated from pairs of eyes from three mice at each age according to the manufacturer's instructions using either the Absolutely RNA Miniprep kit (Agilent Technologies, Inc.; Santa Clara, CA) with modifications for small samples for E13.5–P8 or the Absolutely RNA Nanoprep kit (Agilent Technologies) for E10.5–12.5. Each RNA sample was double DNase-treated, and the RNA concentration was quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific; West Palm Beach, FL). Select RNA samples were also analyzed on Bioanalyzer chips (Agilent Technologies). Fifty nanograms of total RNA were reverse transcribed using oligo(dT) primers and the AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies), and cDNA samples originating from the same animals were pooled. Primers were designed using GenBank mouse mRNA sequences so that the amplicon would cover an exon–exon boundary. The following primer sequences were synthesized and high-performance liquid chromatography (HPLC)–purified by Integrated DNA Technologies (IDT; Coralville, IA): phosducin (*Pdc*; 5'-GCA CAC AGG ACC CAA AGG AGT AAT-3' and 5'-ACA CAA ACC CAT ACC TAG GCC CAA-3'), cone transducin  $\gamma$  (*Gngt2*; 5'-GGA AGT GAA GAA CCC ACG TGA TCT GA-3' and 5'-AGC ACA CAA GTG CCT TTC TCC TTG-3'), and hypoxanthine-guanine phosphoribosyltransferase (*Hprt*; 5'-CAG GCC AGA CTT TGT TGG AT-3' and 5'-GGA CGC AGC AAC TGA CAT T-3'). Before beginning, the primer concentrations were optimized for each forward and reverse primer. The qPCR reaction efficiencies for each pair were confirmed to be within a range of 90–110%. Reactions were prepared in technical triplicate using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies), including reference dye, and 5 ng of each cDNA with 100 nM of each forward and reverse primer. Reactions were incubated at 95 °C for 10 min and then cycled at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s using a Stratagene (San Diego, CA) Mx3000P real-time PCR system. A melting curve analysis was added at the end to verify a single product from each reaction, and the fluorescence was recorded during every PCR cycle at the annealing step (55 °C) and the extension step (72 °C). Finally, select PCR products were also verified by size on agarose gels to ensure single band amplifications. The final relative quantities of *Pdc* and *Gngt2* expression were determined after normalization to *Hprt* by the MxPro™ QPCR software version 3.00 (Stratagene). In addition, three reference P0 samples were run in triplicate on every plate so that interplate variations could be controlled. As a result,

the data presented are also normalized to the P0 expression levels.

## RESULTS

**Expression of phosducin in the developing mouse retina:** Phosducin (*Pdc*) expression is known to be specific to photoreceptors in the retina, with expression found in rods and cones [24,26]. Previous studies have shown phosducin expression primarily in the early postnatal and adult retina [27,34,35], but the expression of phosducin during embryogenesis has not been explored. Using a well-characterized sheep antibody against phosducin [28,36,37], we identified phosducin expression in sections of embryonic mouse retina (Figure 1). This prompted us to perform a developmental series of phosducin expression, covering the earliest stages of mouse retinogenesis (E10.5) until after the maturation of rod photoreceptors (P21, Figure 1I). No phosducin protein expression was detectable at E10.5 or E11.5 (Figure 1A,B). Starting at E12.5, a small number of cells expressing phosducin protein were present in the central retina (arrows in Figure 1C). Most reactive cells were present in the outer neuroblastic layer (Figure 1D). This location is consistent with the position of the future outer nuclear layer, where differentiated photoreceptors will reside. Given the ventricular location of these phosducin-positive cells and the reported expression specificity of phosducin [24,26], the labeled cells likely represent photoreceptors that have either fully or nearly completed migration into their mature location within the neural retina.

As development proceeded, a rapid increase in the number of phosducin-expressing cells occurred between E12.3 and E13.5 (compare Figure 1C,D). As might be expected from the central to peripheral gradient of retinal differentiation [38], no phosducin-positive cells were found in the distal retina at this age. From E13.5 to E15.5, a gradual increase in the number of phosducin-reactive cells was observed, with the further progression of stained cells toward the distal retina. By E17.5, the number of phosducin-positive cells increased, and this trend continued through the last stage tested, P21 (Figure 1F–I). To demonstrate that the staining observed in these images accurately represents the expression of phosducin protein, retinal sections from a phosducin knockout mouse line [28] were processed and reacted with anti-phosducin antibody. No reactivity was observed anywhere within the phosducin-knockout retinas at either E13.5 (Figure 2A) or P0 (Figure 2B), demonstrating that the antibody showed no cross-reactivity in the retina at embryonic or neonatal ages. For positive controls, phosducin-knockout retinas and similarly aged controls were colabeled with anti-phosducin and either anti-Otx2 at E13.5 or anti-cone

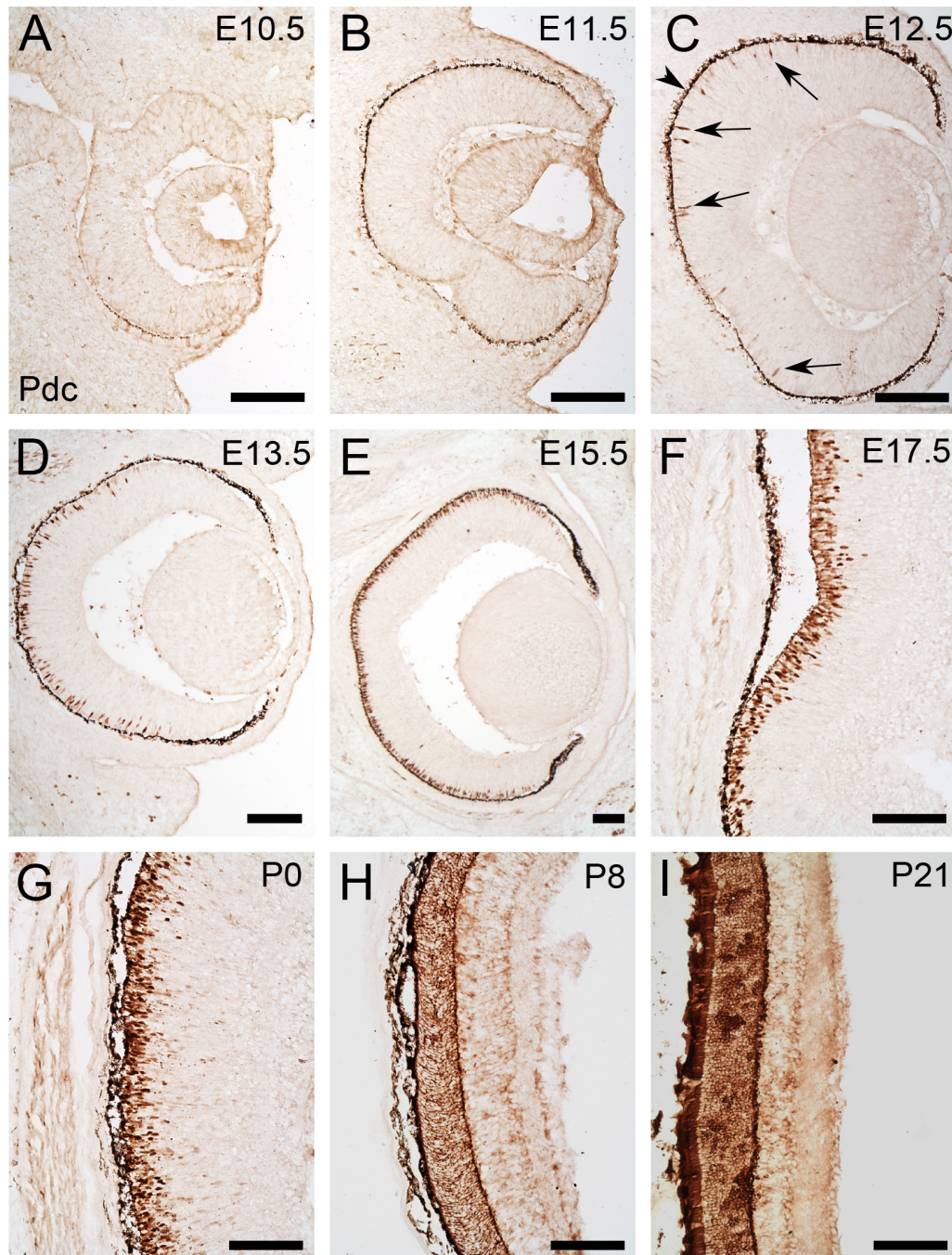


Figure 1. Developmental expression of phosducin protein in the embryonic and postnatal retina. Immunohistochemical staining was performed on coronal sections starting at (A) E10.5 (before cone birth) through (I) P21. Anti-phosducin antibody labeled photoreceptors starting at E12.5 (arrows in C) and continued through all time points tested. Arrowhead indicates the RPE. Scale bars = 100  $\mu$ m.

transducin  $\gamma$  at P0. Expression of Otx2 (Figure 2A) and cone transducin  $\gamma$  (Figure 2B) was observed in the control and phosducin-knockout retinas, whereas phosducin expression was lacking in the phosducin-knockout retinal sections.

*Colocalization of phosducin and photoreceptor markers:* Examination of the coexpression of phosducin with a

known photoreceptor marker, orthodenticle homeobox 2 protein (Otx2), and a known cone-specific marker, retinoid X receptor gamma (Rrx $\gamma$ ), was performed to determine the cellular specificity of phosducin labeling in the embryonic retina. Otx2 is important for cell fate determination of photoreceptors and is expressed in photoreceptors and the

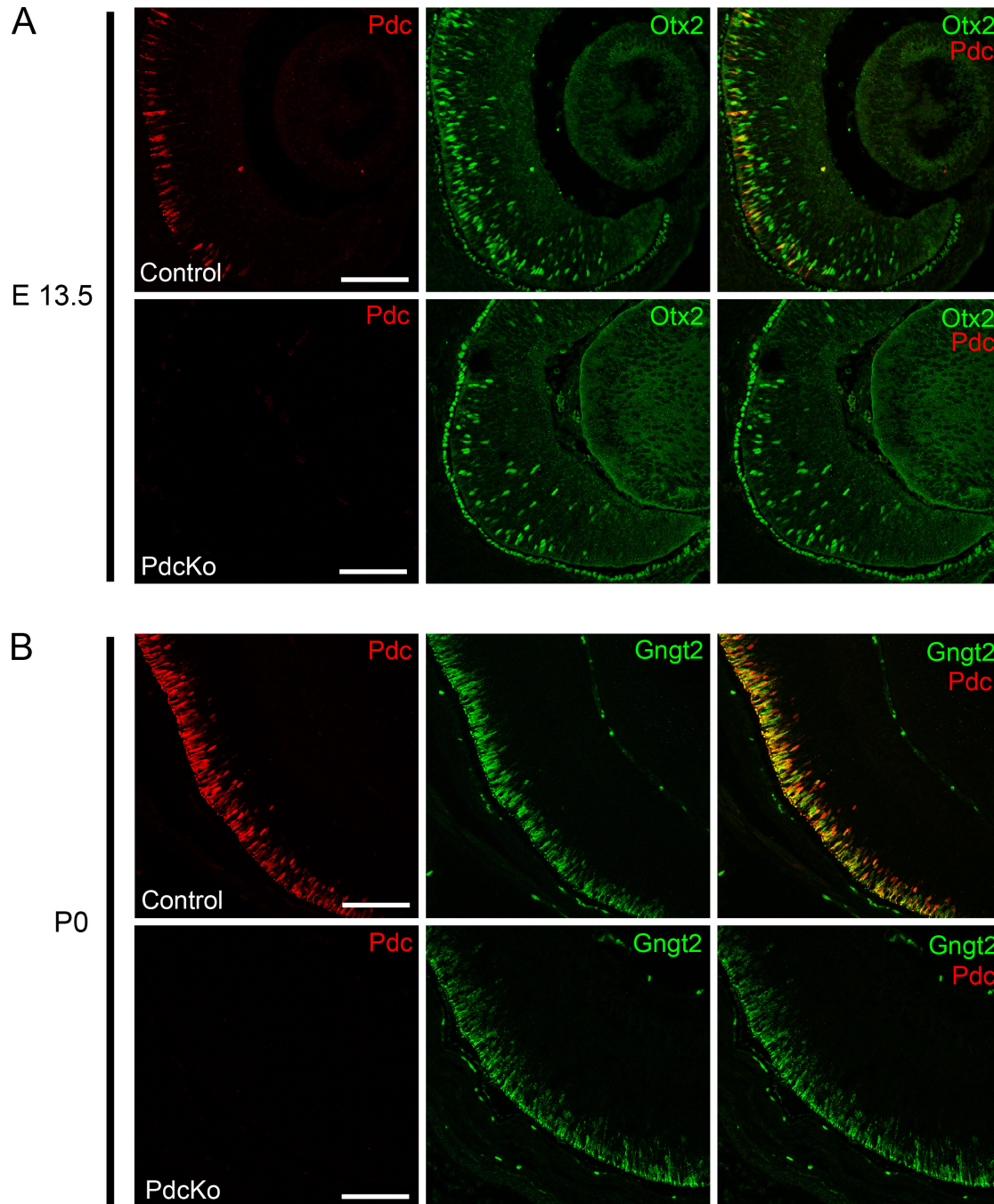


Figure 2. Comparison of PdcKO and control retinas. Immunofluorescence was performed on coronal sections of control and phosducin knockout (PdcKO) mice at ages E13.5 (**A**) using anti-Pdc and anti-Otx2 antibodies and P0 (**B**) using anti-Pdc and anti-cone transducin  $\gamma$  (Gngt2) antibodies. Pdc expression is seen in the control retinas and is absent in the PdcKO retinas, whereas Otx2 and Gngt2 are expressed in the control and PdcKO retinas. Scale bars = 100  $\mu$ m.

RPE until P6 [15]. Expression of Otx2 is seen in migrating and post-migratory developing photoreceptors. We used immunofluorescence to determine whether phosducin and Otx2 are expressed in the same cells. Both proteins localized to the ventricular surface with a large amount of

colocalization (Figure 3A). Phosducin-positive cells almost always expressed Otx2; however, not all Otx2-positive cells expressed phosducin. Otx2 was expressed prominently in migrating (arrows in Figure 3A) photoreceptor precursors and post-migratory photoreceptor cells. Thus, these expression

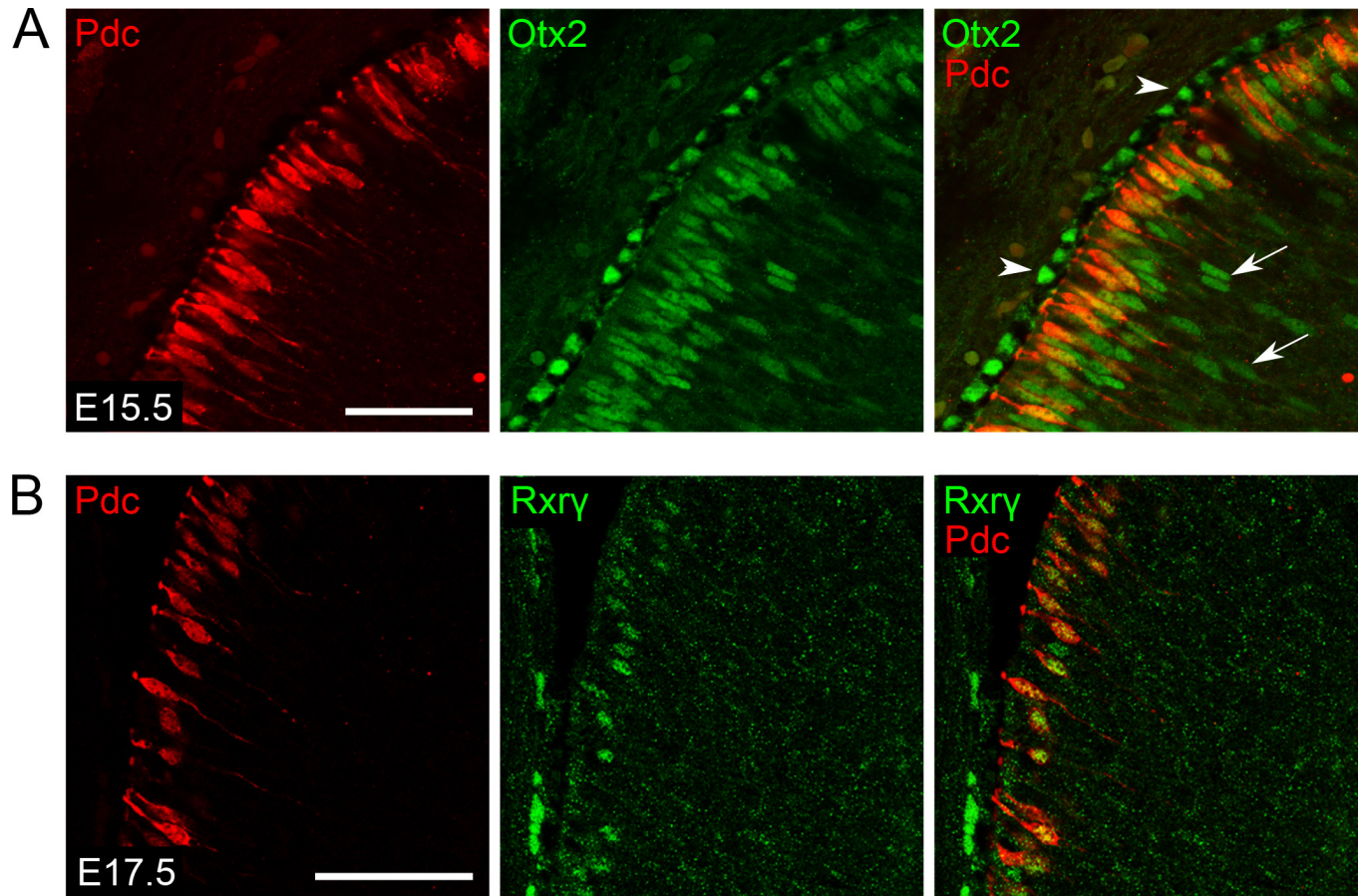


Figure 3. Colocalization of phosducin with known photoreceptor markers in the embryonic retina. **A:** Fluorescent imaging of coronal sections of mouse retinas at E15.5 shows the colocalization of phosducin and Otx2, a photoreceptor marker, along the ventricular surface. Otx2 shows RPE labeling (arrowheads) in addition to photoreceptor labeling. Otx2-positive, Pdc-negative migrating cells are also seen interior to the ventricular surface (arrows). **B:** Immunofluorescent imaging of the retinal sections at E17.5 for phosducin and the cone marker, Rxry, shows consistent colocalization along the ventricular surface of the retina. Scale bars = 50  $\mu$ m.

patterns supported the supposition that phosducin labels post-migratory photoreceptors during embryonic ages.

Rxry is involved in the formation of the S-opsin gradient of cone photoreceptors and is expressed in the embryonic neural retina in postmitotic cones and retinal ganglion cells with peak expression at E17.5 [20,39]. To examine the cellular specificity of phosducin expression in developing cones, we performed immunofluorescence with anti-phosducin and anti-Rxry antibodies in E17.5 retinal sections. Both proteins were expressed along the ventricular border of the retina and colocalized (Figure 3B), suggesting that the majority of phosducin-labeled cells in the embryonic retina, up to and including E17.5, were cones.

*Expression of cone transducin  $\gamma$  in the developing mouse retina:* Because phosducin is known to interact with transducin  $\beta\gamma$  and cone transducin  $\gamma$  is expressed embryonically [21], we sought to determine the earliest age at which cone

transducin  $\gamma$  could be detected. Similar to the results seen for phosducin expression, cone transducin  $\gamma$  was expressed in the outer neuroblastic layer of the embryonic mouse retina (Figure 4). Cone transducin  $\gamma$  protein could not be detected through E12.5 (Figure 4A–C) but was detected at E13.5 (Figure 4D). As with phosducin expression, cone transducin  $\gamma$ -immunoreactive cells were observed only along the ventricular edge of the retina. The expression timing and limited expression domain suggested that cone transducin  $\gamma$  may be expressed in differentiated cones only once they have fully migrated into position, similar to phosducin. Expression continued to expand from the central retina into the periphery over the course of embryonic retinal development (Figure 4D–F), similar to the pattern seen with phosducin expression (Figure 1). Unlike the expression pattern of phosducin, the cone transducin  $\gamma$ -reactive cell numbers appeared to remain

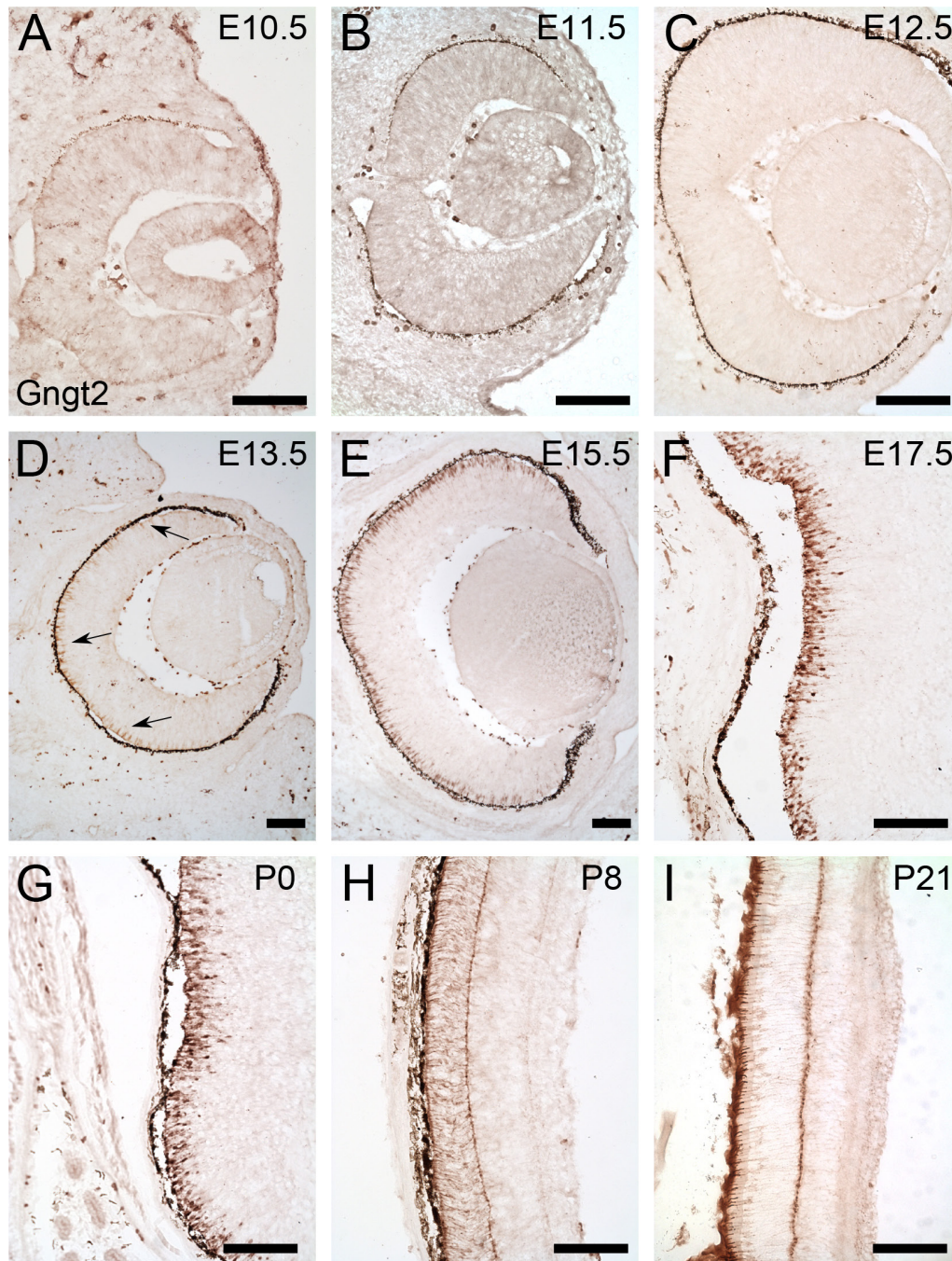


Figure 4. Developmental expression of cone transducin  $\gamma$  protein in the embryonic and postnatal retina. Immunohistochemical staining was performed on coronal sections starting at (A) E10.5 (before cone birth) through (I) P21. Beginning at E13.5, cone transducin  $\gamma$  expression is observed in cells along the ventricular surface (arrows in D), and this expression continues through all ages tested. Scale bars = 100  $\mu$ m.

constant from E17.5 to P21 (Figure 4F–I), consistent with the findings that 95% of cones are born by the day of birth [2,3].

*Colocalization of phosducin and cone transducin  $\gamma$  protein expression:* Given the similar embryonic localization patterns seen between phosducin and cone transducin  $\gamma$ , we used immunofluorescence to determine whether these two proteins

colocalize in retinal cells. Examination of the expression of both proteins from E13.5 to P8 (Figure 5A–E) showed they were distributed widely across the retina along the central to peripheral axis but were restricted to the ventricular layer. Prominent colocalization of phosducin and cone transducin  $\gamma$  was seen in coronal eye sections at all ages examined.

Using E13.5–P0 retinal sections, we quantified the amount of coexpression by determining the percentage of cells that were labeled with phosducin only, cone transducin  $\gamma$  only, and those colabeled with phosducin and cone transducin  $\gamma$ . At E13.5, 97% ( $\pm 0.4\%$ ; SD) of the labeled cells in the retina were colabeled. The percentage of colabeled cells dropped at E15.5 and E17.5 to 88% ( $\pm 1.1\%$ ; SD) and 87% ( $\pm 2.4\%$ ; SD), respectively, and by P0, the percentage of colabeled cells was 79% ( $\pm 6.2\%$ ; SD; Figure 5F). This quantification of colocalization suggests that most phosducin-labeled cells are cones at most embryonic ages, although rod histogenesis has begun by this time [2,3].

*Phosducin and cone transducin  $\gamma$  mRNA expression levels show different dynamics:* After early detection of phosducin

and cone transducin  $\gamma$  expression in the developing mouse retina with immunohistochemistry (E12.5; Figure 1C and E13.5; Figure 4D), we sought to explore phosducin and cone transducin  $\gamma$  gene expression. We analyzed mRNA expression levels for phosducin (*Pdc*) and cone transducin  $\gamma$  (*Gngt2*) by qPCR, using gene-specific primer sets. Samples were tested from littermates used for the immunolocalization presented above for consistency. A minimum of three biologic replicates were analyzed and averaged for each age. *Pdc* expression above the threshold was detected at E10.5 and E11.5, but these levels were only 0.4% and 0.2% that of P0 levels, respectively (Figure 6A). The extremely low expression levels and relative decrease from E10.5 to E11.5 suggest that these values may reflect the sporadic phosducin-positive protein staining seen

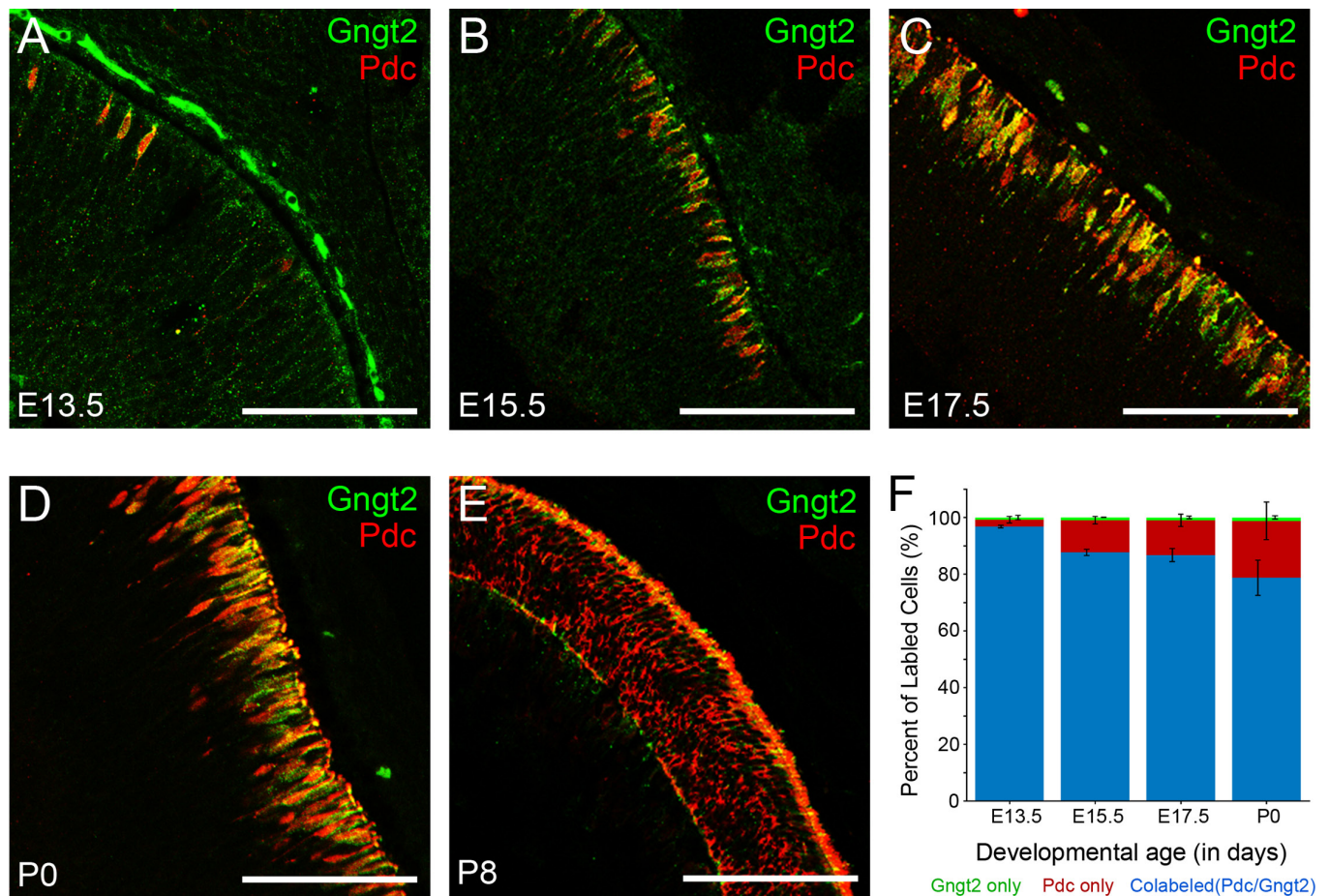


Figure 5. Colocalization of phosducin and cone transducin  $\gamma$  expression in the retina. Fluorescent imaging of coronal sections of mouse retina from E13.5–P8 (A–E) was performed using anti-phosducin and anti-cone transducin  $\gamma$  antibodies. Both markers localized almost exclusively to the ventricular surface at all ages tested. Scale bars = 50  $\mu$ m. Quantification of the colabeled cell for ages E13.5–P0 (F) shows a high percentage of colabeled cells (97%  $\pm$  0.4%; SD) at E13.5 that progressively decreased with age (E15.5 was 88%  $\pm$  1.1%; E17.5 was 87%  $\pm$  2.3%; P0 was 79%  $\pm$  6.2%). Phosducin-only cells comprised 2.4%  $\pm$  1.1% at E13.5, 11.3%  $\pm$  1.3%, at E15.5, 12.3%  $\pm$  2.2% at E17.5, and 20%  $\pm$  6.6% at P0. Cone transducin  $\gamma$ -only cells constituted 0.8%  $\pm$  0.7% at E13.5, 1%  $\pm$  0.4% at E15.5, 1%  $\pm$  0.4% at E17.5, and 1.2%  $\pm$  0.7% at P0. The blue bars indicate cells that are colabeled, the phosducin-only cells are shown in red, the cone transducin  $\gamma$ -only cells appear in green, and the error bars represent standard deviations.



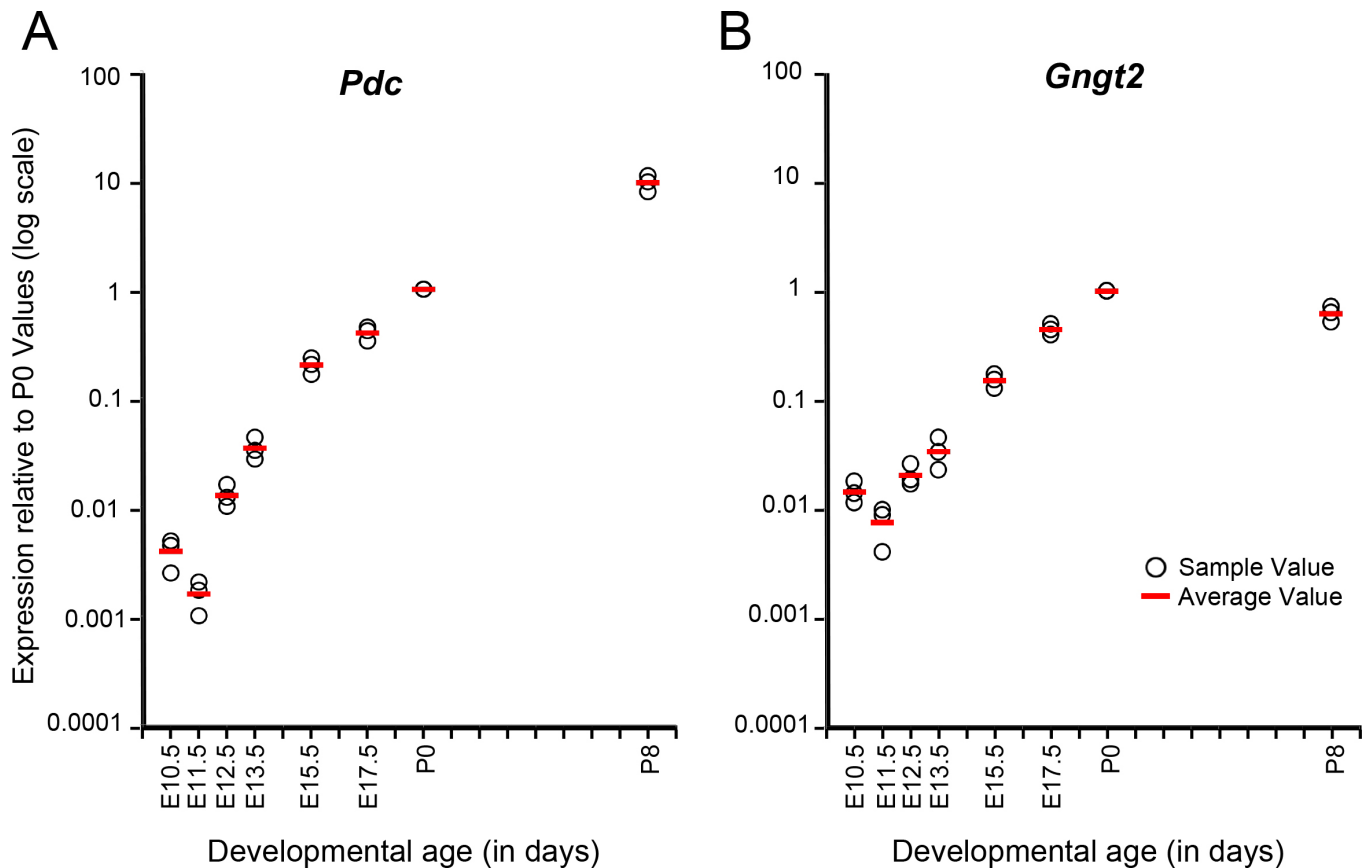


Figure 6. mRNA expression across developmental ages measured with qPCR. **A:** *Pdc* expression above the threshold was detected with qPCR at E10.5 and E11.5 but at extremely low levels. The relative *Pdc* expression levels increase at E12.5, which is consistent with protein expression (see Figure 1). Expression increases gradually through P0, and then at P8, there is a dramatic increase in the *Pdc* mRNA levels. **B:** Expression of *Gngt2* mRNA is detected with qPCR at E10.5 and E11.5, but similar to phosducin, at low levels. A steady increase in *Gngt2* expression occurs from E12.5 to P0. The *Gngt2* levels at P8 decrease slightly relative to the P0 values. All numbers are expressed relative to P0 and normalized to the *Hprt* levels in each sample and graphed on a log scale. At each age, three independent samples were collected, and each of these samples was run in triplicate as technical replicates. Red lines indicate the average for that age group; black circles are individual samples at each age group.

at E12.5, and that decreases between E10.5 and E11.5 could represent stochastic variations in small numbers. No values above the threshold were detected in samples from either the E13.5 or P0 phosducin-knockout animals, suggesting that other cellular RNAs do not contribute a background signal and that these extremely low levels of phosducin expression at E10.5 and E11.5 may represent real expression.

The relative *Pdc* expression levels increased dramatically at E12.5, with levels six-fold higher than at E11.5 and approximately 1.3% those of P0 samples (Figure 6A). Given that the phosducin protein is first detected at this stage, this would seem to be a minimum age at which *Pdc* expression can reliably be demonstrated, but earlier expression is certainly a distinct possibility. Dramatic increases in *Pdc* mRNA levels were observed between E13.5 (3.7% of P0 levels) and E15.5 (19.7% of P0 levels). Although the E17.5 levels for *Pdc* were

roughly one-third those of P0, the levels at P8 were more than nine-fold greater than the P0 levels. This large increase in postnatal phosducin levels is consistent with the increase seen in phosducin protein expression and could reflect the fact that rod photoreceptors are primarily formed during postnatal stages of mouse development [2,38] and rods represent about 97% of the photoreceptors in the mouse retina [2,40].

As with *Pdc* gene activation, expression of *Gngt2* mRNA was detected at E10.5 with qPCR, with levels at 1.4% of those values at P0 (Figure 6B). A similar decrease was observed for *Pdc* and *Gngt2* from E10.5 to E11.5, with levels jumping up 2.6-fold by E12.5. A steady increase in *Gngt2* expression occurred from E12.5 to P0. However, unlike the situation with *Pdc* in which a large increase in mRNA expression occurred between P0 and P8, the relative *Gngt2* levels at P8 decreased to 61.5% of those seen at P0. This decrease potentially reflects

the overall dilution of cone photoreceptor cell contribution to the total RNA pool with the rapid growth of the retina during the postnatal time period and the paucity of new cone histogenesis that takes place postnatally.

## DISCUSSION

*Embryonic photoreceptor-specific expression for visual transduction proteins:* We present evidence that proteins important for phototransduction are expressed early in embryonic photoreceptors. We show the onset of phosducin protein expression at E12.5, which precedes earlier reports by nearly 2 weeks (E12.5 versus P5 [27,35]), and cone transducin  $\gamma$  protein expression at E13.5, preceding the earliest reported detection by 2 days [21]. Expression of both proteins was nearly 3 weeks before the onset of M-opsin protein expression at P14 and nearly 1 week before S-opsin protein expression at P0 [5,9].

We infer that the embryonic expression of phosducin and cone transducin  $\gamma$  is labeling cone photoreceptors. Several lines of evidence support this inference. First, in the postnatal retina, cone transducin  $\gamma$  is specific for cones, and phosducin is specific for photoreceptors [24,41]. Second, cone transducin  $\gamma$ - and phosducin-positive cells reside along the ventricular border, the location of photoreceptors. Additionally, immunofluorescence showed phosducin and cone transducin  $\gamma$  to be coexpressed in cells. Given the regionalized expression and colocalization of these proteins, these findings suggest that cone transducin  $\gamma$  and phosducin are expressed in photoreceptors that have migrated into their adult location, similar to the expression pattern seen for early photoreceptor markers, Crx [12,42] and Tr $\beta$ 2 [11,18]. Third, colocalization of phosducin with Otx2 and Rxry, known markers for photoreceptors and cones, respectively, suggests cone-specific labeling of phosducin at embryonic ages. Quantification of colocalization data suggests that, at E13.5, virtually all of phosducin-labeled cells are cones, and by E17.5, most labeled cells (87%) are still cones, as they are phosducin- and cone transducin  $\gamma$ -positive.

*Rapid increases in phosducin, but not cone transducin  $\gamma$ , in the postnatal retina:* From P0 to P8, we observed a nine-fold increase in *Pdc* mRNA expression with qPCR. This increase likely reflects the massive production of rod photoreceptors that occurs during early postnatal development, such that 50% of all retinal cells and 97% of all mouse photoreceptors are rods [2,40]. The possibility also exists that, in addition to a dramatic increase in rod cell numbers during this period, the expression levels of phosducin within each photoreceptor could be increasing. With this caveat in mind, however, the magnitude of change for phosducin expression was consistent

with the increase in rod cell number during early postnatal retinal development [3].

*Potential functions for visual transduction proteins in the embryonic retina:* Surprisingly, the temporal pattern of phosducin and cone transducin  $\gamma$  was coincident with the expression of early cone-determining transcription factors, suggesting that photoreceptor precursor cells are fully committed to a cone differentiation pattern at these early ages. Since the phototransduction process is weeks removed from this expression, these visual transduction factors likely play other functional roles in the embryonic photoreceptor cell. We observed an absence of other visual transduction proteins (mouse cone arrestin, cone phosphodiesterase and S-cone opsin) in the mouse retina at E15.5. Although visual transduction proteins have been found embryonically in the primate retina [43,44], this expression is at a stage of development that matches the postnatal period in rodents, suggesting that cone transducin  $\gamma$  and phosducin may have specific functions in the embryonic retina unrelated to their roles in visual transduction.

As a result, we sought clues in the literature to identify potential activity of cone transducin  $\gamma$  and phosducin in the embryonic mouse retina, if any. Previous studies on phosducin show that it can interact with Crx in cultured cells, where phosducin acts to inhibit Crx-mediated transcription at the interphotoreceptor retinoid-binding protein (IRBP) promoter [45]. In addition, phosducin possesses a transcriptional activation domain at the C-terminal end [46], although this would appear to conflict with the reported repression of Crx transcriptional activity [45]. Previous studies have yielded conflicting information regarding the localization of phosducin. Whereas Zhu et al. [45] demonstrated that phosducin colocalized to the nucleus when coexpressed with Crx in Cos7 cells and in the adult bovine retina (where phosducin shows nuclear and cytoplasmic localization), the same group [46] and others [47,48] found phosducin to be cytoplasmic. If phosducin localized to the nucleus, this finding would suggest a possible role in transcription; however, the cytoplasmic localization seen in our study would suggest that perhaps there is an additional function for embryonic phosducin beyond any transcriptional activities.

Another possible function for embryonic phosducin expression is that phosducin may interact with cone transducin  $\beta\gamma$  as phosducin does postnatally [49]. This possibility would lead to the obvious question regarding the potential function for G protein  $\beta\gamma$  at embryonic ages. Although the transducin  $\beta\gamma$  complex is known to function only in phototransduction, the function of the transducin  $\beta\gamma$  complex during embryonic stages has not been explored to our

knowledge. Given the large number of G protein-coupled receptors (GPCRs) and their expression in multiple tissues and cell types, it is conceivable that the transducin  $\beta\gamma$  complex has additional functions during embryonic retinal development that have yet to be fully understood or explored. Some potential GPCR signaling pathways involved in retinal function include dopamine signaling [50,51], hedgehog (Hh) signaling [52,53], and Wnt signaling [54], along with others [55,56]. Wnt signaling in the retina appears to function primarily in the RPE at embryonic ages [54], and therefore is unlikely to involve phosducin and cone transducin  $\gamma$  activity in photoreceptor cell differentiation. Dopamine signaling appears to occur at later embryonic and postnatal stages than seen for the earliest phosducin and cone transducin  $\gamma$  expression [51]. Hh signaling plays a significant role in retinal progenitor cell proliferation [57], and factors induced by Hh signaling (e.g., Gli1) overlap spatially and temporally with the expression for phosducin shown above [58]. However, functional manipulation of Hh signaling [58-60] suggests that it either antagonizes cone photoreceptor specification or has a transient role in cone differentiation. Therefore, further work is necessary to elucidate the early functions for phosducin and cone transducin  $\gamma$  in the developing retina and their potential roles in photoreceptor cell differentiation.

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#### REFERENCES

- Hoffpauir BK, Marrs GS, Mathers PH, Spirou GA. Does the brain connect before the periphery can direct? A comparison of three sensory systems in mice. *Brain Res* 2009; 1277:115-29. [PMID: 19272365].
- Carter-Dawson LD, LaVail MM. Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. *J Comp Neurol* 1979; 188:263-72. [PMID: 500859].
- Rapaport DH, Wong LL, Wood ED, Yasumura D, LaVail MM. Timing and topography of cell genesis in the rat retina. *J Comp Neurol* 2004; 474:304-24. [PMID: 15164429].
- al-Ubaidi MR, Pittler SJ, Champagne MS, Triantafyllos JT, McGinnis JF, Baehr W. Mouse opsin. Gene structure and molecular basis of multiple transcripts. *J Biol Chem* 1990; 265:20563-9. [PMID: 1978723].
- Applebury ML, Farhangfar F, Glösmann M, Hashimoto K, Kage K, Robbins JT, Shibusawa N, Wondisford FE, Zhang H. Transient expression of thyroid hormone nuclear receptor TRbeta2 sets S opsin patterning during cone photoreceptor genesis. *Dev Dyn* 2007; 236:1203-12. [PMID: 17436273].
- Fei Y. Development of the cone photoreceptor mosaic in the mouse retina revealed by fluorescent cones in transgenic mice. *Mol Vis* 2003; 9:31-42. [PMID: 12592228].
- Fujieda H, Bremner R, Mears AJ, Sasaki H. Retinoic acid receptor-related orphan receptor alpha regulates a subset of cone genes during mouse retinal development. *J Neurochem* 2009; 108:91-101. [PMID: 19014374].
- Glaschke A, Glösmann M, Peichl L. Developmental changes of cone opsin expression but not retinal morphology in the hypothyroid Pax8 knockout mouse. *Invest Ophthalmol Vis Sci* 2010; 51:1719-27. [PMID: 19834026].
- Katoh K, Omori Y, Onishi A, Sato S, Kondo M, Furukawa T. Blimp1 suppresses Chx10 expression in differentiating retinal photoreceptor precursors to ensure proper photoreceptor development. *J Neurosci* 2010; 30:6515-26. [PMID: 20463215].
- Baas D, Bumsted KM, Martinez JA, Vaccarino FM, Wikler KC, Barnstable CJ. The subcellular localization of Otx2 is cell-type specific and developmentally regulated in the mouse retina. *Brain Res Mol Brain Res* 2000; 78:26-37. [PMID: 10891582].
- Brzezinski JA, Lamba DA, Reh TA. Blimp1 controls photoreceptor versus bipolar cell fate choice during retinal development. *Development* 2010; 137:619-29. [PMID: 20110327].
- Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ. Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* 1997; 19:1017-30. [PMID: 9390516].
- Martinez-Morales JR, Signore M, Acampora D, Simeone A, Bovolenta P. Otx genes are required for tissue specification in the developing eye. *Development* 2001; 128:2019-30. [PMID: 11493524].
- Morrow EM, Furukawa T, Lee JE, Cepko CL. NeuroD regulates multiple functions in the developing neural retina in rodent. *Development* 1999; 126:23-36. [PMID: 9834183].
- Nishida A, Furukawa A, Koike C, Tano Y, Aizawa S, Matsuo I, Furukawa T. Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. *Nat Neurosci* 2003; 6:1255-63. [PMID: 14625556].
- Samson M, Emerson MM, Cepko CL. Robust marking of photoreceptor cells and pinealocytes with several reporters under control of the Crx gene. *Dev Dyn* 2009; 238:3218-25. [PMID: 19882727].

17. Roberts MR, Srinivas M, Forrest D, Morreale de Escobar G, Reh TA. Making the gradient: thyroid hormone regulates cone opsin expression in the developing mouse retina. *Proc Natl Acad Sci USA* 2006; 103:6218-23. [PMID: 16606843].
18. Ng L, Hurley JB, Dierks B, Srinivas M, Saltó C, Vennström B, Reh TA, Forrest D. A thyroid hormone receptor that is required for the development of green cone photoreceptors. *Nat Genet* 2001; 27:94-8. [PMID: 11138006].
19. Ng L, Ma M, Curran T, Forrest D. Developmental expression of thyroid hormone receptor beta2 protein in cone photoreceptors in the mouse. *Neuroreport* 2009; 20:627-31. [PMID: 19282790].
20. Roberts MR, Hendrickson A, McGuire CR, Reh TA. Retinoid X receptor (gamma) is necessary to establish the S-opsin gradient in cone photoreceptors of the developing mouse retina. *Invest Ophthalmol Vis Sci* 2005; 46:2897-904. [PMID: 16043864].
21. Sakagami K, Gan L, Yang X-J. Distinct effects of Hedgehog signaling on neuronal fate specification and cell cycle progression in the embryonic mouse retina. *J Neurosci* 2009; 29:6932-44. [PMID: 19474320].
22. Gaudet R, Bohm A, Sigler PB. Crystal structure at 2.4 angstroms resolution of the complex of transducin beta-gamma and its regulator, phosducin. *Cell* 1996; 87:577-88. [PMID: 8898209].
23. Lee RH, Lieberman BS, Lolley RN. A novel complex from bovine visual cells of a 33,000-dalton phosphoprotein with beta- and gamma-transducin: purification and subunit structure. *Biochemistry* 1987; 26:3983-90. [PMID: 3477288].
24. Lee RH, Whelan JP, Lolley RN, McGinnis JF. The photoreceptor-specific 33 kDa phosphoprotein of mammalian retina: generation of monospecific antibodies and localization by immunocytochemistry. *Exp Eye Res* 1988; 46:829-40. [PMID: 2461862].
25. Loew A, Ho YK, Blundell T, Bax B. Phosducin induces a structural change in transducin beta gamma. *Structure* 1998; 6:1007-19. [PMID: 9739091].
26. von Schantz M, Szél A, van Veen T, Farber DB. Expression of soluble phototransduction-associated proteins in ground squirrel retina. *Invest Ophthalmol Vis Sci* 1994; 35:3922-30. [PMID: 7928190].
27. Lee RH, Lieberman BS, Lolley RN. Retinal accumulation of the phosducin/T beta gamma and transducin complexes in developing normal mice and in mice and dogs with inherited retinal degeneration. *Exp Eye Res* 1990; 51:325-33. [PMID: 2401349].
28. Sokolov M, Strissel KJ, Leskov IB, Michaud NA, Govardovskii VI, Arshavsky VY. Phosducin facilitates light-driven transducin translocation in rod photoreceptors. Evidence from the phosducin knockout mouse. *J Biol Chem* 2004; 279:19149-56. [PMID: 14973130].
29. Taketo M, Schroeder AC, Mobraaten LE, Gunning KB, Hanten G, Fox RR, Roderick TH, Stewart CL, Lilly F, Hansen CT. FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc Natl Acad Sci USA* 1991; 88:2065-9. [PMID: 1848692].
30. Sidman RL, Green MC. Retinal degeneration in the mouse: location of the rd locus in linkage group XVII. *J Hered* 1965; 56:23-9. .
31. Yang J, Nan C, Ripps H, Shen W. Destructive Changes in the Neuronal Structure of the FVB/N Mouse Retina. *PLoS One* 2015; 10:e0129719-[PMID: 26091175].
32. Howell DM, Morgan WJ, Jarjour AA, Spirou GA, Berrebi AS, Kennedy TE, Mathers PH. Molecular guidance cues necessary for axon pathfinding from the ventral cochlear nucleus. *J Comp Neurol* 2007; 504:533-49. [PMID: 17701984].
33. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012; 9:676-82. [PMID: 22743772].
34. Babila T, Schaad NC, Simonds WF, Shinohara T, Klein DC. Development of MEKA (phosducin), G beta, G gamma and S-antigen in the rat pineal gland and retina. *Brain Res* 1992; 585:141-8. [PMID: 1511297].
35. Brown BM, Carlson BL, Zhu X, Lolley RN, Craft CM. Light-driven translocation of the protein phosphatase 2A complex regulates light/dark dephosphorylation of phosducin and rhodopsin. *Biochemistry* 2002; 41:13526-38. [PMID: 12427013].
36. Krispel CM, Sokolov M, Chen Y-M, Song H, Herrmann R, Arshavsky VY, Burns ME. Phosducin regulates the expression of transducin betagamma subunits in rod photoreceptors and does not contribute to phototransduction adaptation. *J Gen Physiol* 2007; 130:303-12. [PMID: 17724163].
37. Song H, Belcastro M, Young EJ, Sokolov M. Compartment-specific phosphorylation of phosducin in rods underlies adaptation to various levels of illumination. *J Biol Chem* 2007; 282:23613-21. [PMID: 17569665].
38. Young RW. Cell differentiation in the retina of the mouse. *Anat Rec* 1985; 212:199-205. [PMID: 3842042].
39. Mori M, Ghyselinck NB, Chambon P, Mark M. Systematic Immunolocalization of Retinoid Receptors in Developing and Adult Mouse Eyes. *Invest Ophthalmol Vis Sci. The Association for Research in Vision and Ophthalmology* 2001; 42:67-1318. .
40. Jeon CJ, Strettoi E, Masland RH. The major cell populations of the mouse retina. *J Neurosci* 1998; 18:8936-46. [PMID: 9786999].
41. Ong OC, Yamane HK, Phan KB, Fong HK, Bok D, Lee RH, Fung BK. Molecular cloning and characterization of the G protein gamma subunit of cone photoreceptors. *J Biol Chem* 1995; 270:8495-500. [PMID: 7721746].
42. Furukawa T, Morrow EM, Cepko CL. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* 1997; 91:531-41. [PMID: 9390562].

43. Sears S, Erickson A, Hendrickson A. The spatial and temporal expression of outer segment proteins during development of Macaca monkey cones. *Invest Ophthalmol Vis Sci* 2000; 41:971-9. [PMID: 10752930].
44. Ross EJ, Graham DL, Money KM, Stanwood GD. Developmental consequences of fetal exposure to drugs: what we know and what we still must learn. *Neuropsychopharmacology* 2015; 40:61-87. [PMID: 24938210].
45. Zhu X, Craft CM. Modulation of CRX transactivation activity by phosducin isoforms. *Mol Cell Biol* 2000; 20:5216-26. [PMID: 10866677].
46. Zhu X, Craft CM. The carboxyl terminal domain of phosducin functions as a transcriptional activator. *Biochem Biophys Res Commun* 2000; 270:504-9. [PMID: 10753654].
47. Schulz R, Schulz K, Wehmeyer A, Murphy J. Translocation of phosducin in living neuroblastoma x glioma hybrid cells (NG 108-15) monitored by red-shifted green fluorescent protein. *Brain Res* 1998; 790:347-56. [PMID: 9593987].
48. Wehmeyer A, Schulz R. Phosducin expression in NG 108-15 hybrid cells enhances prostaglandin E1 stimulated adenylate cyclase activity. *Life Sci* 1998; 62:PL127-34. [PMID: 9496706].
49. Willardson BM, Howlett AC. Function of phosducin-like proteins in G protein signaling and chaperone-assisted protein folding. *Cell Signal* 2007; 19:2417-27. [PMID: 17658730].
50. Deming JD, Shin J-A, Lim K, Lee E-J, Van Craenenbroeck K, Craft CM. Dopamine receptor D4 internalization requires a beta-arrestin and a visual arrestin. *Cell Signal* 2015; 27:2002-13. [PMID: 26169958].
51. Reis RAM, Ventura ALM, Kubrusly RCC, de Mello MCF, de Mello FG. Dopaminergic signaling in the developing retina. *Brain Res Brain Res Rev* 2007; 54:181-8. [PMID: 17292477].
52. Levine EM, Roelink H, Turner J, Reh TA. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. *J Neurosci* 1997; 17:6277-88. [PMID: 9236238].
53. Stenkamp DL, Frey RA, Prabhudesai SN, Raymond PA. Function for Hedgehog genes in zebrafish retinal development. *Dev Biol* 2000; 220:238-52. [PMID: 10753513].
54. Westenskow P, Piccolo S, Fuhrmann S. Beta-catenin controls differentiation of the retinal pigment epithelium in the mouse optic cup by regulating Mitf and Otx2 expression. *Development* 2009; 136:2505-10. [PMID: 19553286].
55. Odani N, Pfaff SL, Nakamura H, Funahashi J-I. Cloning and developmental expression of a chick G-protein-coupled receptor SCGPR1. *Gene Expr Patterns* 2007; 7:375-80. [PMID: 17251065].
56. Perry KJ, Johnson VR, Malloch EL, Fukui L, Wever J, Thomas AG, Hamilton PW, Henry JJ. The G-protein-coupled receptor, GPR84, is important for eye development in *Xenopus laevis*. *Dev Dyn* 2010; 239:3024-37. [PMID: 20925114].
57. Wallace VA. Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina. *Brain Res* 2008; 1192:61-75. [PMID: 17655833].
58. Wang Y, Dakubo GD, Thurig S, Mazerolle CJ, Wallace VA. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. *Development* 2005; 132:5103-13. [PMID: 16236765].
59. Yu C, Mazerolle CJ, Thurig S, Wang Y, Pacal M, Bremner R, Wallace VA. Direct and indirect effects of hedgehog pathway activation in the mammalian retina. *Mol Cell Neurosci* 2006; 32:274-82. [PMID: 16815712].
60. Cwinn MA, Mazerolle C, McNeill B, Ringuette R, Thurig S, Hui CC, Wallace VA. Suppressor of fused is required to maintain the multipotency of neural progenitor cells in the retina. *J Neurosci* 2011; 31:5169-80. [PMID: 21451052].

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