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The role of the *Rx* homeobox gene in retinal progenitor proliferation and cell fate specification

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Abstract

The Retinal homeobox gene (*Rx;* also *Rax*) plays a crucial role in the early development of the vertebrate eye. Germline deletion of *Rx* in mice results in the failure of optic vesicle formation, leading to anophthalmia. Recent research using conditional mouse knockout models provides some clues to the role of Rx in eye development following optic vesicle formation. However, the functions of Rx in embryonic retinogenesis are still not fully understood. We investigated the function of Rx in the mouse neural retina using a conditional knockout where the Pax6α-Cre driver deletes *Rx* activity in early retinal progenitors. The deletion of *Rx* activity causes a loss of retinal lamination, a depletion of retinal progenitors, and a change in retinal cell fate in our

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conditional knockout model. The deletion of *Rx* leads to an absence of late-born retinal neurons (rods and bipolar cells) and Müller glia at postnatal ages, as well as a loss of the early-born cone photoreceptors. Decreased BrdU labeling in the *Rx*-deleted portion of the retina suggests a loss of retinal progenitors via early cell cycle exit, which likely prevents the formation of late-born cells. As early-born cells, cone photoreceptors should not be as affected by early cell cycle exit of retinal progenitors. However, embryonic cone photoreceptor labeling is also markedly reduced in *Rx*-deleted retinas. Together these data demonstrate the importance of Rx for retinal progenitor proliferation and a specific requirement of Rx for cone formation in mice.

Introduction

Vertebrate eye development begins with the formation of the optic vesicles at embryonic day (E) 8.5 in the mouse. The optic vesicle folds inward forming the optic cup, with the innermost layer becoming the neural retina. The neural retina is a highly organized structure arranged in layers of specific cell types. Generation of the retinal cells types follows a conserved developmental pattern in vertebrates. Retinal progenitor cells generate retinal neurons in a biphasic temporal order, resulting in early- and late-born retinal cell types. Birth-dating studies show the first cell type to be generated is retinal ganglion cells around E10.5 followed by horizontal cells, cones, and amacrine cells (Young, 1985; Rapaport et al., 2004). These cells comprise the early-born cell types that are primarily formed during the embryonic period. Late-born cells, including rods, bipolar cells, and Müller glia, start forming during embryogenesis but are primarily born postnatally (Carter-Dawson and LaVail, 1979; Young, 1985; Rapaport et al., 2004). Many factors regulate cell proliferation, specification and differentiation for retinal formation, including the transcription factors, Pax6, Six3, Otx2, Sox2, and Rx (also known as Rax) (Zagozewski et al., 2014).

The *Rx* homeobox gene is highly conserved among vertebrates and plays multiple important roles in eye development (Bailey et al., 2004). Mice have a single *Rx* gene (Furukawa et al., 1997; Mathers et al., 1997), whereas the number of *Rx* genes is variable among other species. Germline deletion of *Rx* in mice leads to anophthalmia (the absence of eyes) as a result of a failure of the optic vesicles to form (Mathers et al., 1997). In humans, *RAX* has been associated with anophthalmia, microphthalmia (small eyes) and coloboma (Voronina et al., 2004; Lequeux et al., 2008; London et al., 2009; Gonzalez-Rodriguez et al., 2010; Abouzeid et al., 2012; Chassaing et al., 2014). Due to the very early requirement for Rx activity at the optic vesicle/cup stages, there is a relative paucity of information on the role *Rx* plays following mammalian eye initiation. Conditional alleles for the embryonic inactivation of the *Rx* gene in mice allows the investigation of *Rx* gene function during this time period (Muranishi et al., 2011; Voronina et al., 2005). Embryonic conditional knockout (CKO) of *Rx* was briefly explored using a Chx10-Cre driver to inactivate *Rx* in retinal progenitors at E11.5 and using an inducible Crx-CreERT2 to inactivate *Rx* at E14.5 (Muranishi et al., 2011). Decreases in Otx2 and Crx were observed in both CKO models at E15.5, suggesting a possible role for Rx in the formation of photoreceptors (Muranishi et al., 2011). An inducible conditional knockout model that deleted *Rx* postnatally showed that *Rx*, along with *Crx*, is important for photoreceptor maturation and survival during the postnatal period of retinal development (Irie et al., 2015).

The role of *Rx* in embryonic retinal development from optic vesicle formation through birth is still not well understood. Using an embryonic conditional deletion of *Rx* in mice, we sought to assess its role following initial eye development, specifically examining retinal progenitor proliferation and cell fate decisions. Research shows Rx expression correlates with proliferation (Furukawa et al., 1997; Mathers et al., 1997; Rohde et al., 2011), and *Xenopus* Rx is important for both proliferation and multipotency of retinal progenitors (Andreazzoli et al., 2003; Casarosa et al., 2003). However, whether Rx is necessary for retinal progenitor proliferation in mammals has not yet been established.

Studies also suggest a role for Rx in photoreceptor determination. Embryonic conditional deletion models of *Rx* in mice showed decreases in Crx and Otx2 and showed that Rx can transactivate Otx2 (Muranishi et al., 2011). Postnatal conditional deletion of *Rx* showed that it is necessary for photoreceptor survival (Irie et al., 2015). Further, evidence for a role of Rx in photoreceptor development is provided in studies of other species such as *Xenopus* (Pan et al., 2010). Currently, several questions remain unanswered regarding the function of Rx during retinogenesis, including its effects on cell fate beyond photoreceptors, possible differences in the functions of Rx between cone and rod photoreceptor development, and whether Rx is necessary for photoreceptor generation or just maturation and survival.

In the present study, we performed an embryonic conditional deletion of *Rx* using Pax6α-Cre to delete *Rx* in very early retinal progenitors, and assessed the effects on retinal development. We show that Rx is important in maintaining the proliferative state of retinal progenitors in mice, and therefore indirectly important for the development of late-born retinal neurons, which are lost in the *Rx* conditional knockout. Finally, we show that Rx has a direct role in the development of cone photoreceptors.

Methods

Animals and Tissue Collection

All animal procedures were approved by the WVU Institutional Animal Care and Use Committee and followed the guidelines set out by the Association for Research in Vision and Ophthalmology. Conditional deletion of *Rx* was achieved using Rx^{flox} transgenic mice (as described in Voronina et al., 2005). In these mice exon 2 of the *Rx* gene is flanked by loxP sites. In the presence of Cre recombinase, exon 2 is then excised, thus creating a nonfunctional allele. Two strains of reporter mice were used, from Jackson Laboratory (Bar Harbor, ME), Rosa26R and Rosa26-EGFP (Soriano, 1999; Mao et al., 2001). Rx^{flox} mice were mated with reporter mice to generate a strain in which either β-galactosidase or EGFP activity is activated upon exposure to Cre-recombinase, thus allowing us to visualize cells where Cre-mediated recombination has occurred and *Rx* has been inactivated. Our conditional knockout was generated using mice expressing Cre recombinase under the control of Pax6α enhancer (supplied by P. Gruss, Max-Planck) that were crossed with *Rx* null ($Rx^{\Delta 1,2}$) mice (Mathers et al., 1997). The Pax6α enhancer is active in the distal neural retina only starting at embryonic day (E)10.5 (Marquardt et al., 2001). Therefore, the final cross for our *Rx* conditional deletion was homozygous Rx^{flox}; Rosa (EGFP or LacZ) mice crossed with mice heterozygous for Pax6α-cre; $Rx^{\Delta 1,2}$. From this cross we used Pax6α *Cre*/+; Rx^{flox}/*Rx*-null; Rosa/+ mice, the conditional knockout mice hereafter called *Rx*

CKO, and Pax6α *Cre*/+; *Rx*$^{\text{flox}}$/+; Rosa/+ mice were used as controls. Tail DNA was collected for genotyping by PCR using the primers described in Voronina et al. (2005).

Eyes were collected at a variety of time points: E12.5, E13.5, E14.5, E16.5, E17.5, E18.5, postnatal day (P) 0 and P21. Following euthanasia, whole eyes were removed from the orbit and fixed in a 4% paraformaldehyde solution of phosphate-buffered saline (1× PBS; 150 mM NaCl, 1.06 mM KH2PO4, 2.97 mM Na2HPO4-7H2O, pH 7.4) overnight at 4°C. After fixation, eyes were cryoprotected in 30% sucrose in PBS. Cryoprotected eyes were frozen in TBS tissue freezing media (Triangle Biomedical Sciences, Durham, NC), and sectioned on a Leica CM3050S cryostat at a thickness of 12μm, then transferred to Super Frost plus-coated glass slides (Thermo Fisher Scientific, Waltham, MA).

BrdU Labeling

Timed pregnancies were determined by the presence of a vaginal plug the morning after mating; noon the day after mating was considered E0.5. Time-mated female mice received an intraperitoneal injection of BrdU at a dose of 50mg/kg bodyweight and then were sacrificed 2 hours later. Heads (E12.5) or whole eyes (E14.5 and E18.5) were collected and processed as described below for immunofluorescence. To calculate the percentage of BrdU-positive cells, the total number of cells (labeled by propidium iodide) and the number of BrdU-labeled cells were counted in three separate slices from at least four different animals of both control and *Rx* CKO mice.

Histology

Frozen sections were stained with either hematoxylin and eosin or X-gal. For hematoxylin and eosin staining (H & E staining), slides were processed by the Pathology/Histology Core Facilities at West Virginia University and National Cancer Institute-Frederick.

For X-gal staining, frozen sections were rinsed with 1× PBS and incubated with X-gal staining solution as described in Marrs et al., 2013. After histological staining, images were visualized using an Olympus AX70 microscope (Olympus, Center Valley, PA) equipped with a MicroFire digital camera (Optronics; Goleta, CA)

Immunofluorescence

Sections were subjected to an antigen retrieval procedure of 0.1M Tris pH 9.5 incubation at 95°C for 20 min prior to antibody processing. Following antigen retrieval, sections were blocked with normal serum at room temperature and treated with primary and secondary antibodies, following our published procedure (Howell et al., 2007). Additionally, select retinas were counterstained with propidium iodide (Molecular Probes, Eugene, OR; 1:200).

Primary antibodies used were: anti-activated caspase-3 (Sigma-Aldrich, St Louis, MO; 1:600), anti-BrdU (Abcam, Cambridge, MA: 1:10), anti-Brn3b (Santa Cruz Biotechnology, Dallas, TX; 1:50), anti-blue cone opsin (Chemicon, Temecula, CA; 1:100), anti-calbindin (Swant, Marly, Switzerland; 1:2000), anti-cone transducin γ (CytoSignal, Irvine, CA; 1:500), anti-Chx10 (Exalpha Biologicals, Shirley, MA; 1:1000), anti-CRALBP (Abcam; 1:1000), anti-GFP (chicken; Abcam; 1:1500), anti-GFP (rabbit; Abcam; 1:1000), antineurofilament 165kD (Hybridoma bank, Iowa City, IA; 1:1000), anti-Otx2 (Millipore, Billerica, MA; 1:1000), anti-phosducin (as previously described by Sokolov et al., 2004; 1:1000), anti-red/green cone opsin (Chemicon; 1:100), and anti-syntaxin 1A (Sigma-Aldrich; 1:1000). Secondary antibodies were fluorophore-tagged for immunofluorescence (Molecular Probes). Images were captured on an Olympus AX70 microscope (Olympus, Center Valley, PA) equipped with a MicroFire digital camera (Optronics; Goleta, CA), an LSM 510 Meta confocal microscope (Carl Zeiss, Inc.; Thornwood, NY) or an LSM 710 confocal microscope (Carl Zeiss, Inc.; Thornwood, NY).

Results

Rx deletion results in histological changes in the retina

To study the functions of Rx during retinogenesis, we generated a retinal specific conditional knockout using the Pax6α-Cre driver (Marquardt et al., 2001) to induce recombination in retinal progenitors of the distal retina. The resulting Cre recombination excises exon 2 of *Rx* at approximately E10.5, the beginning of retinal neurogenesis.

X-gal staining to detect β-galactosidase activity from the Rosa26R reporter shows *Rx* deletion results in a decreased area of X-gal staining in the distal retina in *Rx* CKO mice compared to control mice (Figure 1A). Further histological analysis shows that deletion of *Rx* leads to a disruption in retinal lamination and creates an expanded mass in the distal portion of the P21 retina as shown by H & E staining in comparison to control (Figure 1B). However, the central retina (the non-deleted portion) in *Rx* CKO mice appears normal and shows well defined lamination and normal retinal thickness.

Deletion of *Rx* in the embryonic retina does not increase cell death

Next, we assessed if the reduction in X-gal labeling upon *Rx* deletion was caused by an increase in cell death in the retina. To determine the role of apoptosis in the *Rx* CKO phenotype, we performed immunolabeling using the cell death marker, activated caspase-3 and Rosa-EGFP, which labels cells that have undergone Cre recombination and thus *Rx* deletion. Examination of retinal sections across various ages (E13.5-P21; Figure 2 shows P0 labeling as a representative sample) shows no differences between *Rx* CKO and control retinas at any of the ages tested. Quantification of the activated caspase-3- and GFP-positive cells showed an average of 1.5 ±0.5 (SD) activated caspase 3-positive cells in the control and 2.1 ± 1.4 (SD) activated caspase-3-positive cells in the *Rx* CKO; t-test analysis was nonsignificant ($p = 0.14$). The limited activated caspase-3 labeling in the *Rx*-deleted portion of the retina indicates that the embryonic loss of *Rx* in the retina does not significantly increase cell death.

Rx is important for retinal progenitor proliferation

The reduction in the X-gal-labeled domain of *Rx* CKO mice combined with previous research that proposes a role for Rx in proliferation (Furukawa et al., 1997; Mathers et al., 1997; Casarosa et al., 2003) suggests a possible decline in the number of retinal progenitors. Therefore we sought to determine whether deletion of *Rx* affects retinal progenitors and proliferation. To assess changes in proliferation in the *Rx*-deleted retina, we used BrdU incorporation and compared *Rx* CKO mice with littermate controls. For BrdU immunostaining, which labels cells in the S phase of mitosis, we counterstained with the nuclear marker, propidium iodide (PI), at three ages (E12.5, E14.5 and E18.5, see Figure 3). We observed an age-related decrease in the percentage of BrdU-positive cells in the *Rx* CKO distal retina compared to controls (Figure 3). At E12.5, there is no significant difference in BrdU incorporation in the distal retina of *Rx* CKO and control mice (Figure 3A–C). By E14.5, a significant difference emerges such that the percentage of BrdU-positive cells in the control retinas is 37.0 2.1% and in the *Rx* CKO is 34.3 2.1% ($P < 0.05$) (Figure 3D–F). Finally, at E18.5 the percentage of BrdU-positive cells in the controls is 33.9 1.4%, and the central retina of *Rx* CKO mice is comparable at 34.4 2.3%, whereas the distal, deleted portion of the *Rx* CKO shows a drastic reduction ($P <0.001$), with only 3.6 1.1% BrdU-positive cells (Figure 3G–J). An overview of a whole retinal section at E18.5 shows the dramatic decrease in BrdU labeling in the distal regions of the *Rx* CKO retina compared to control (Figure 4A). Examining the expression of Cre-mediated GFP and BrdU incorporation in the *Rx* CKO (Figure 4B) allows the determination of proliferation within the areas of the distal retinal that have undergone *Rx* deletion. Areas where GFP expression is not activated show robust BrdU incorporation, whereas GFP-positive regions show a paucity of BrdU labeling (Figure 4B). The BrdU results suggest that *Rx* is important in maintaining mouse retinal progenitor proliferation and that deletion of *Rx* results in a decrease in the progenitor pool likely due to early cell cycle exit.

Rx deletion alters cell fate specification

Since the loss of *Rx* significantly reduces the number of retinal progenitors and affects retinal lamination, we sought to determine if there are any changes in cell fate specification in the *Rx*-deleted portion of the *Rx* CKO retina. To determine the identity of cells in the deleted portion of the *Rx* CKO retina, we conducted immunolabeling studies using retinal cell-specific markers in mature retinas along with the Rosa-EGFP reporter. Comparing control and *Rx* CKO retinas at P21, we observed GFP- and Brn3b-colabeled cells, indicating the presence of ganglion cells in the *Rx*-deleted distal retina (Figure 5A,B). Immunolabeling with syntaxin 1A shows extensive labeling in the distal (GFP-positive) portion of the *Rx* CKO retina, suggesting abundant amacrine cell expression in the *Rx*-deleted retina (Figure 5D) compared to control retinas (Figure 5C). The presence of horizontal cells was detected using immunolabeling of calbindin 28kD and neurofilament 165 kD. Co-labeling of these markers with GFP indicates the presence of horizontal cells in both control retinas (Figure 5E,G) and portions of the retina where *Rx* is deleted (Figure 5F,H). Examination of P21 retinal sections using cone-specific markers (S-opsin and M-opsin) shows opsin expression within the control retinas (Figure 6A,C) and a marked decrease in labeling in the distal retina of *Rx* CKO mice (Figure 6B,D). Labeling with GFP shows the Cre-recombined region overlapping with the region lacking cone photoreceptors. The results of immunolabeling for early-born retinal cell types in the *Rx* CKO retina revealed a reduction in cone photoreceptor labeling and an abundance in amacrine cell labeling.

Due to the reduction in the retinal progenitor pool, we hypothesized that the late-born retinal cell types would be affected by the loss of *Rx*. To test this prediction, we performed immunolabeling on P21 *Rx* CKO and control retinas with antibodies against rhodopsin, Chx10 and CRALBP, which label rod photoreceptors, bipolar cells and Müller glia, respectively. Labeling for all three cell types was observed in the control retinas (Figure 7A,C,E) and in the central region of the *Rx* CKO retina (Figure 7B,D,F). Examining the area of Cre recombination in the *Rx* CKO, as demarcated by GFP expression, shows an absence of rhodopsin, Chx10 and CRALBP labeling (Figure 7B,D,F), demonstrating a total loss of the late-born retinal cell types in the *Rx*-deleted region.

Deletion of *Rx* directly affects cone photoreceptor generation

The loss of the late-born cell types is consistent with Rx expression in the retinal progenitor pool (Furukawa et al., 1997; Mathers et al., 1997; Andreazzoli et al., 2003; Casarosa et al., 2003). The loss of the early-born cones was suggested in previous *Rx* CKO models showing a lack of Crx and Otx2 activity. However, the loss of cones is unlikely to be solely the result of a premature cell cycle exit phenotype given the presence of retinal ganglion, amacrine, and horizontal cells. There are two possible explanations for the loss of cones-1) Rx is necessary for cone histogenesis; or 2) the cones are initially formed but subsequently degenerate, suggesting a role for Rx in embryonic cone survival similar to the role of Rx in postnatal cones (Irie et al., 2015). To test these possibilities, we sought to explore the expression of cone markers in embryonic retinogenesis to determine if cones are being born normally in the *Rx*-deleted retina. Cone histogenesis begins around E11.5 and is complete just prior to birth (Carter-Dawson and LaVail, 1979; Young, 1985; Rapaport et al., 2004). During retinogenesis, cells are first born in the central retina, and a wave of differentiation progresses to the periphery. The Pax6α-Cre expression domain is in the distal retina; therefore, the earliest we could examine cone generation in this model and expect to find cones in the periphery is during late cone histogenesis (Young, 1985; Rodgers et al., 2016). Since cone opsins are not expressed in the embryonic retina, we used the markers phosducin and cone transducin γ. A previous study showed phosducin labeling at E17.5 shows 87% co-expression with the cone-specific marker, cone transducin γ, thus making them useful markers of embryonic cones (Sakagami et al., 2009; Rodgers et al., 2016). Immunolabeling with phosducin and GFP at E17.5 and P0 shows robust phosducin labeling along the entire length of the retina at the ventricular surface in control samples (Figure 8A,C). In the *Rx* CKO retina, the central portion of the retina displays phosducin expression, but this expression is greatly reduced in the distal portion of the retina (Figure 8B,C). To determine the location of the *Rx* deletion in the CKO retina in relation to phosducin labeling, we examined GFP expression from the Cre-mediated, Rosa26-EGFP reporter. We observe mosaic GFP expression in the distal retina, and in the GFP-labeled areas, there is a sharp decrease in phosducin-positive cells compared to non GFP-labeled areas of the *Rx* CKO distal retina (Figure 8). Phosducin and cone transducin γ expression shows strong co-labeling at embryonic ages (Rodgers et al., 2016). These cone markers are abundant and co-expressed in the control and central retina of the *Rx* CKO and show reduced expression in the distal *Rx* CKO retina (Supplemental figure 1). To further test our findings, we next examined the expression of Otx2. Otx2 is known to label photoreceptors in the embryonic retina; however, it is not photoreceptor-specific. Immunolabeling for Otx2 and GFP expression in E17.5 control and *Rx* CKO retinal sections of shows strong Otx2 labeling in the control (Figure 9A) and central portion of the *Rx* CKO (Figure 9B). In the distal portion of the *Rx* CKO retina, there is sharp decline in Otx2-labeled cells (Figure 9B), with GFP-labeling indicating those cells have undergone recombination and thus *Rx* deletion. Taken together, the cone marker immunolabeling data suggest that there is a large decrease in cone expression during cone histogenesis. Based on of the lack of cell death and the presence of other early-born cell types, it is probable that Rx is necessary for cone photoreceptor generation.

Discussion

The role of *Rx* in retinogenesis following optic vesicle/cup initiation is not well understood. Here, we investigated the functions of *Rx* during embryonic neural retinal formation. Our data indicate that *Rx* plays multiple roles in eye development during retinal formation.

Rx is required for appropriate retinal morphology

Conditional deletion of *Rx* at E10.5 in the Pax6α-Cre-mediated *Rx* CKO leads to several abnormalities in the postnatal retina. The first noted difference was a decrease in clonal size of the Cre-marked lineage domain in the *Rx* CKO, which complements the finding in other embryonic *Rx* CKO mice that showed a decrease in eye size (Muranishi et al., 2011). The limited Cre deletion mediated by the Pax6α-Cre driver suggests that the defects seen in our *Rx* CKO model are likely cell autonomous. Additional histological examination reveals that *Rx* deletion leads to a disorganized retina that had lost its typical lamination at postnatal ages. Disruptions in retinal lamination are seen in conditional knockout models of *Sufu,* a regulator of hedgehog signaling (Cwinn et al., 2011), and *Pax6, Lhx2,* and *Otx2,* retinal transcription factors (Marquardt et al., 2001; Nishida et al., 2003; Gordon et al., 2013). Similar to our *Rx* CKO mice, these models all show alterations in cell type specification, including a loss of photoreceptors. Koike et al. (2005) showed that aPKC lambda in differentiating photoreceptors is necessary for proper retinal lamination, suggesting that the loss of photoreceptors may have resulted in the abnormal retinal lamination seen in our *Rx* CKO mice and the other models. However, mice with a *Chx10* (*Vsx2*) homeobox null allele (Burmeister et al., 1996) show a disruption in normal retinal lamination and a lack of bipolar cells but retain their photoreceptors, demonstrating the possibility that the disruption in lamination is not due solely to disruptions in photoreceptor formation.

Rx maintains mouse retinal progenitors

Our study extends previous studies showing that Rx is expressed in retinal progenitors (Furukawa et al., 1997; Mathers et al., 1997; Casarosa et al., 2003) by providing evidence that *Rx* is necessary in maintaining the murine retinal progenitor pool. Using BrdU to label proliferating cells, we show a decrease in retinal progenitors in the *Rx*-deleted retina. We propose that deletion of *Rx* from the retinal progenitors at E10.5 causes premature cell cycle exit. Assessment of the retinal cell types found in the *Rx*-deleted portion of the retina shows a loss of all late-born cell types, suggesting that the loss of the progenitor pool prevents their formation, while allowing the generation of the early-born cell types. In addition, the decrease in retinal progenitors explains the decrease in clone size of the Cre-marked lineage domain in the *Rx* CKO as observed by X-gal staining (Figure 1). Further, the difference in activated caspase-3 labeling between control and *Rx* CKO retinal sections is too small to account for the changes seen in the retina, suggesting that apoptosis does not cause the reduction in the Cre recombination domain or loss of retinal progenitors in the *Rx* CKO.

Several genes have been associated with decreases in the retinal progenitor pool, including *Pax6, Ldb1*, and *Lhx2* (Marquardt et al., 2001; Gordon et al., 2013; Gueta et al., 2016). Rx is necessary for functional Pax6 expression (Zhang et al., 2000), and conditional knockout of *Pax6* does not affect expression of retinal progenitor markers, including *Rx* (Marquardt et al., 2001), suggesting that Rx functions upstream of Pax6 in its role in progenitor proliferation. Conditional knockout of *Ldb1/Ldb2 (Lim domain binding 1/2)* decreases *Rx* expression, suggesting that *Rx* may be downstream of or in a feedback loop with the *Ldb-Lhx2 complex* in the maintenance of the retinal progenitor pool (de Melo et al., 2016; Gueta et al., 2016).

Early deletion of *Rx* in retinal progenitors leads to alterations in cell type in the postnatal retina

Deletion of *Rx* in retinal progenitors at E10.5, the very beginning of neural retinal cell birth, leads to many changes in cell type in the postnatal retina. Late-born cell types were absent, along with early-born cone photoreceptors; in addition, amacrine marker expression is expanded. Previous studies of conditional *Rx* deletion in retinal progenitors did not explore cell types in the postnatal retina, however, they did show ectopic Pax6 expression in the embryonic *Rx* CKO retina which labels amacrine cells and retinal progenitors (Muranishi et al., 2011), which fits with our finding of abundant amacrine marker labeling at postnatal ages.

These alterations in cell type follow a pattern seen in other homeobox gene knockouts that show changes in cell fate specification, where some cell types are decreased and others are increased. Conditional knockout of *Otx2* results in an increase in amacrine cells, a decrease in rods and an absence of cones (Nishida et al, 2003) similar to what is seen in our *Rx* CKO. However, unlike our *Rx* CKO, Müller glia are unaffected in the *Otx2* CKO and there is a large amount of cell death (Nishida et al., 2003). *Lhx2* conditional knockout at E10.5 shows an overproduction of retinal ganglion cells and a decrease in all other cell types (Gordon et al, 2013). Conditional deletion of Pax6 allows only amacrine cells, with all other cell types absent (Marquardt et al, 2001).

Similarly, changes in cell fate are observed in the conditional knockout of the transmembrane receptor, Notch1 (Jadhav et al., 2006; Yaron et al., 2006). *Notch1* CKO mice generated with Pax6α-Cre display altered retinal morphology, decreased retinal progenitors and an increase in cone photoreceptor at the expense of other early born cell types (Yaron et al., 2006). A similar phenotype was observed in *Notch1* CKO mice generated using Chx10 Cre; *Notch1* CKO mice showed alterations in retinal morphology, a decrease in retinal progenitors and an increase in rod and cone photoreceptors (Jadhav et al., 2006).

The absence of all late-born cell types within the early *Rx*-deleted retina at postnatal ages, coupled with the large decrease in proliferative cells that have incorporated BrdU at E18.5, suggests an indirect effect of *Rx* deletion on these late-born cell types. Changes in proliferation are known to affect the type of cell generated; late-born cells require a sufficient pool of progenitors for their formation (Dyer and Cepko, 2001). Therefore, the absence of the late-born cells is likely due to the loss of the progenitor pool in our *Rx* CKO model.

Rx is important for the formation of cone photoreceptors

Previous studies on Rx suggest it has a function in photoreceptor development. In our model, the loss of rods is likely due to the lack of progenitors so we chose to specifically focus on further studying cone photoreceptors. The loss of cone photoreceptors in the *Rx* CKO at P21 could be the result of a lack of cone formation, suggesting a role in cone cell fate, or caused by degeneration, suggesting a role in cone survival. Thus, we proceeded to investigate cone histogenesis in the *Rx* CKO. Our results show that deletion of *Rx* leads to a large decrease in the birth of cone photoreceptors without increases in cell death. However, our study did not show a complete absence of cones in the distal portion of the *Rx* CKO. The few cells that were labeled with cone markers in the distal retina could be the result of the mosaic nature of Pax6α-Cre expression.

We propose that *Rx* is necessary for cone histogenesis. In support of this proposal, we observe a lack of cones in embryonic *Rx*-deleted retina with no evidence of increased cell death, suggesting that the absence of cones is due to a failure to form and not due to degeneration. A possible role of mouse Rx in early photoreceptor development was previously suggested in a study using two conditional knockout models of *Rx* (Muranishi et al., 2011). Conditional deletion of *Rx* at E11.5 and E14.5 results in reduced levels of Otx2 and Crx expression at E15.5 (Muranishi et al, 2011), which is similar to our results showing decreased levels of Otx2 (Fig. 9). However, our results show a less dramatic Otx2 decrease possibly due to the mosaic nature of the Pax6α-Cre expression. Our results are consistent with those previous findings showing decreases of photoreceptor markers in the embryonic retina. We extended those findings by studying cell death in the *Rx* CKO models and more fully exploring proliferation and specific cell types produced. Postnatal conditional deletion of *Rx* showed decreases in photoreceptor markers and increases in cell death, suggesting Rx is necessary for maturation and survival of photoreceptors during the postnatal time period (Irie et al., 2015). Taken together, the results of the *Rx* conditional knockout models suggest that Rx has multiple roles in photoreceptor development, including the formation of cones and survival of photoreceptors.

The mechanism of action for Rx in photoreceptor development has been partially explored. In *Xenopus* and humans, *Rx* can bind with PCE-1, a conserved element found in the promoter regions of photoreceptor-specific genes (Kimura et al., 2000; Pan et al., 2010). Further, the expression of Rx in *Xenopus* co-localizes with red opsin and rhodopsin promoters in photoreceptors (Pan et al., 2010). A group of genes phylogenetically related to *Rx* has been identified, termed *Rx-like. Rx-like* genes are found in humans, frogs, and chickens and are expressed in photoreceptors and activate photoreceptor genes (*QRX*: Wang et al., 2004; *Rx-L*: Pan et al., 2006 and *RaxL;* Chen and Cepko, 2002). Mice have a single *Rx* gene and lack *Rx-like* genes (Wang et al., 2004), suggesting that the single *Rx* gene may play a similar role to the multiple *Rx* and *Rx-like* genes found in other species in photoreceptor development. Studies in mice show that Rx can transactivate Otx2 and, along with Crx, can transactivate photoreceptor genes (Irie et al., 2015; Muranishi et al., 2011).

Further studies are needed to fully determine if Rx plays separate roles in rod versus cone photoreceptor development. In chickens, *RaxL*, plays a role in cone differentiation (Chen and Cepko, 2002), and the results of our study suggest that murine *Rx* has a direct role in cone photoreceptor development. In summary, the data presented here suggest multiple roles for *Rx* during neural retinogenesis, including proper retinal lamination, maintenance of retinal progenitors, and cone photoreceptor generation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The authors declare no conflicts of interest.

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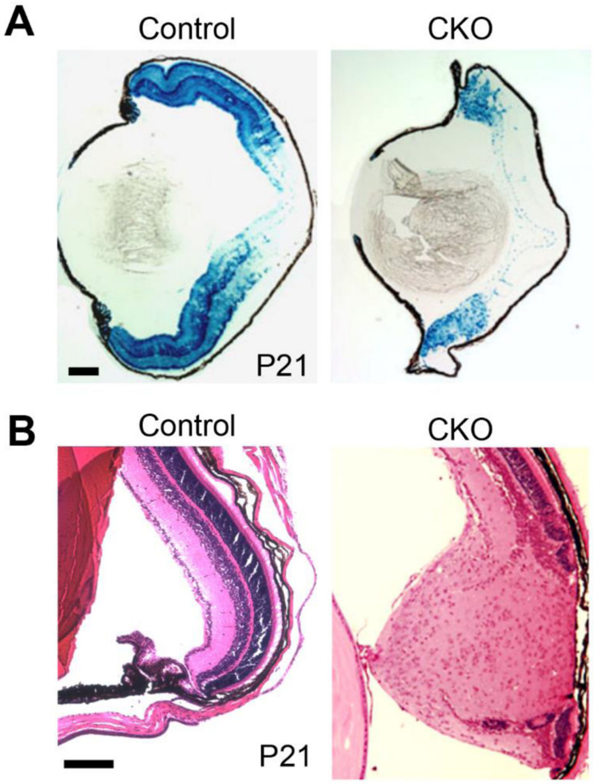

Figure 1. Deletion of *Rx* leads to a reduced Cre-lineage domain and a loss of lamination

(A) X-gal staining of P21 retinal sections from control (*Pax6α-Cre/+; Rx-flox/+; R26R/+*) and *Rx* CKO mice (*Pax6α-Cre/+; Rx-flox/Rx-null; R26R/+*) shows a reduction in the lineage of Cre-recombined cells in the *Rx*-deleted retina. Scale bar is 400 μm (B) H & E staining on P21 retinal sections of control and *Rx* CKO mice demonstrates a loss of lamination and an expansion in the distal portion of the *Rx* CKO retina. Light eosin staining is likely indicative of cellular processes rather than cell bodies. Scale bar is 200 μm.

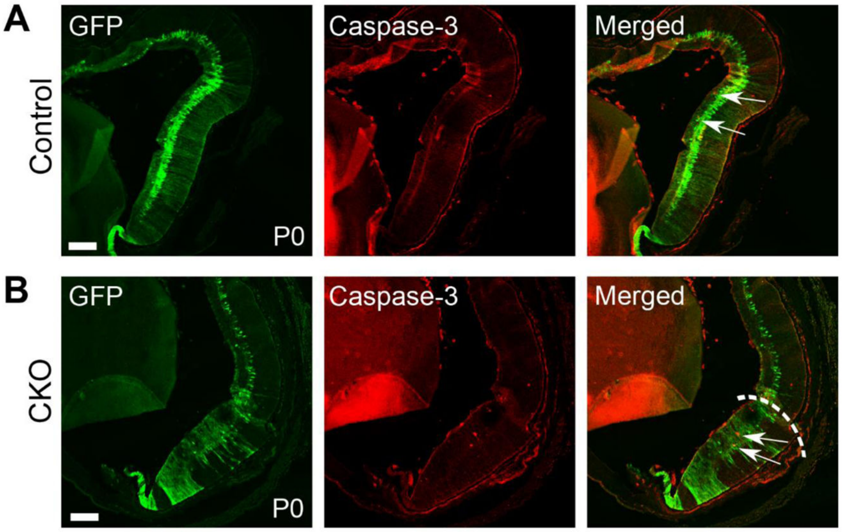

Figure 2. Levels of apoptosis are unaffected by conditional deletion of *Rx*

(A) Retinal sections at P0 from control mice (*Pax6α-Cre/+; Rx-flox/+; R26-EGFP/+*) immunolabeled for the programmed cell death marker, activated caspase-3, and Cre-activated GFP show similar numbers of activated caspase-3- and GFP-co-labeled cells compared to *Rx* CKO mice (*Pax6α-Cre/+; Rx-flox/Rx-null; R26-EGFP/+*) (B). Dashed line (panel B, merged image) indicates the approximate region of *Rx* deletion based on GFP-positive cell labeling. Arrows indicate cells that are labeled for both GFP and caspase-3. Scale bars are 100 μm.

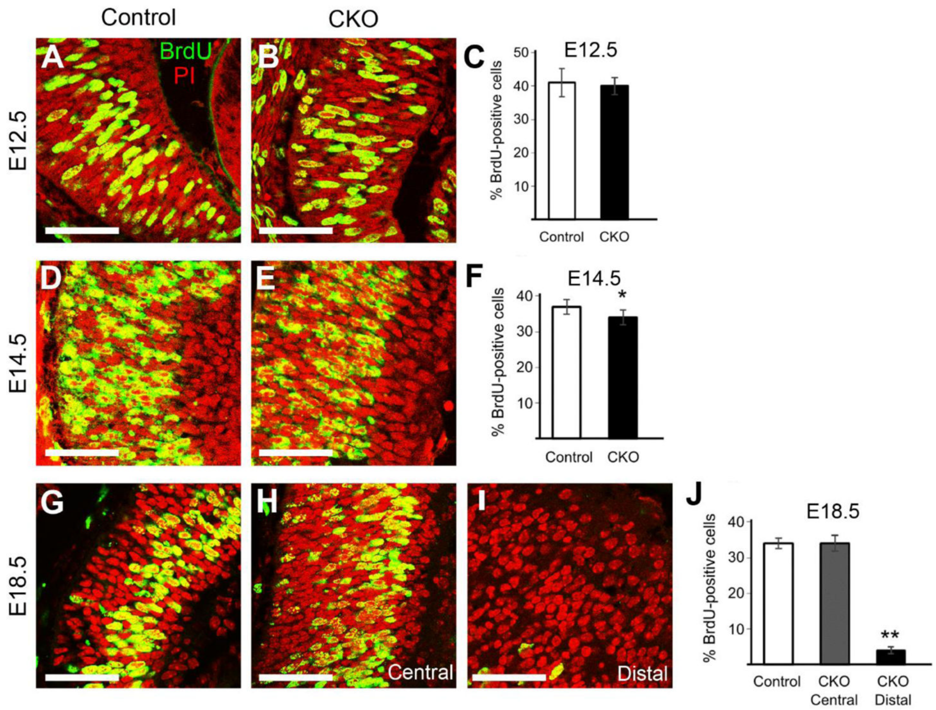

Figure 3. *Rx* deletion reduces the retinal progenitor pool in an age-dependent manner

The distal region of retinal sections from control (*Pax6α-Cre/+; Rx-flox/+; R26-EGFP/+*) and *Rx* CKO (*Pax6α-Cre/+; Rx-flox/Rx-null; R26-EGFP/+*) mice were labeled with the nuclear marker, propidium iodide, and the progenitor marker, BrdU, at E12.5 (A,B), E14.5 (D,E) and E18.5 (G,I). The central (non *Rx*-deleted) region of E18.5 retinas were examined (H) as an internal control compared to the distal *Rx*-deleted retina. Scale bars are 50 μm. Quantitative analysis of progenitor cells at E12.5 (C), E14.5 (F), and E18.5 (J) in retinal sections show a decrease in the percentage of BrdU-positive cells in the *Rx* CKO distal region. The decrease in progenitors was age dependent. There was no detectable difference between control and *Rx*-deleted retinas at E12.5 (p=0.37), but at E14.5 a small difference emerged (p=0.04) and at E18.5 there was a drastic reduction in progenitors in the *Rx*-deleted region (p<0.0001). Open bars represent control retinas, and closed bars represent *Rx* CKO distal retinas. The grey bars in J represents *Rx* CKO central retinas. All bars are the mean ± SD of 3 retinal slices from at least 4 separate animals. Statistical significance is based on t-test analysis between percentages from control and *Rx* CKO distal region and is indicated by asterisks (* indicates p<0.05 and ** indicates p<0.001).

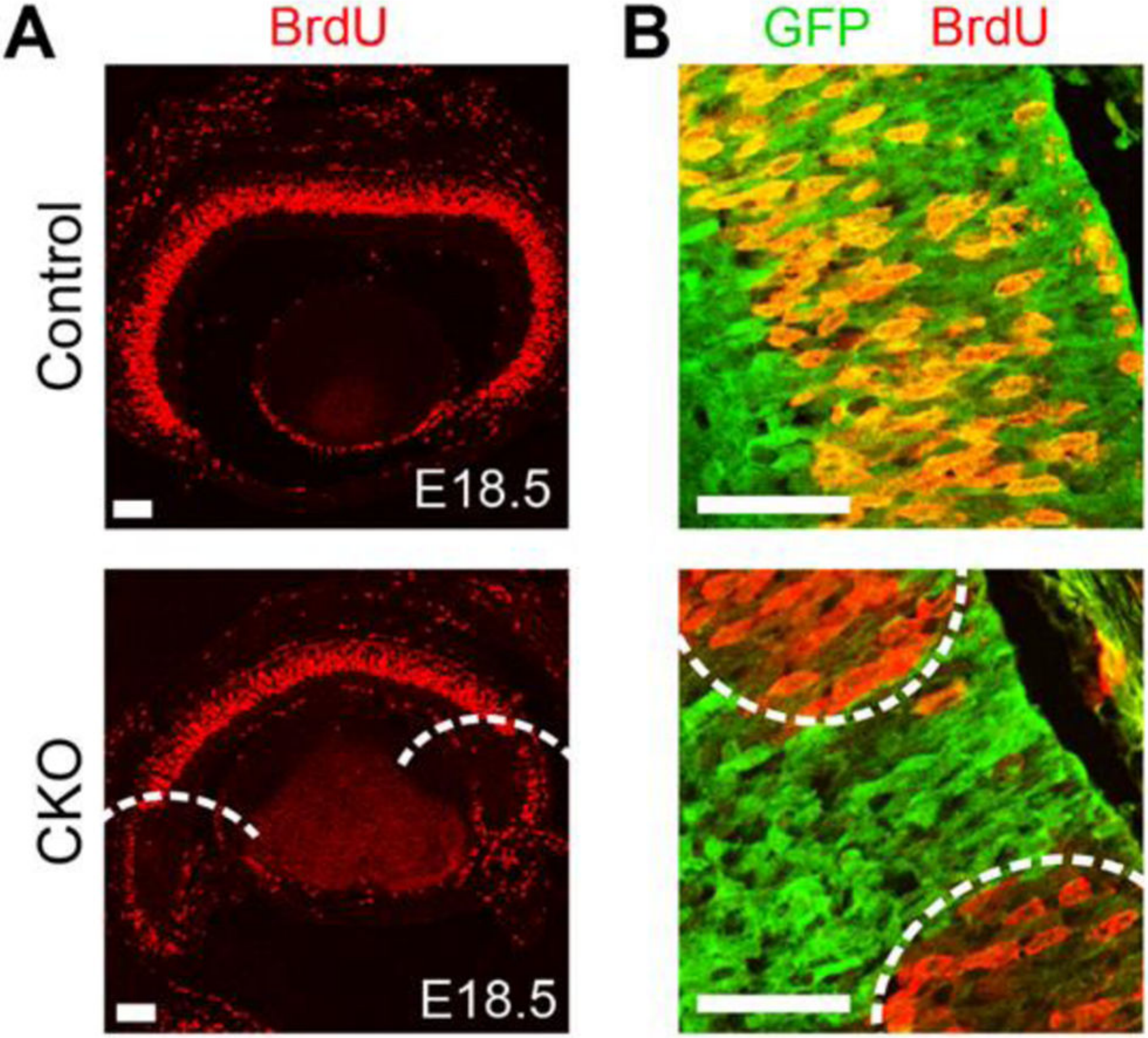

Figure 4. BrdU-labeled progenitors are reduced in *Rx*-deleted regions of the *Rx* CKO retina

(A) Sections from control (*Pax6α-Cre/+; Rx-flox/+; R26-EGFP/+*) and *Rx* CKO (*Pax6α-Cre/+; Rx-flox/Rx-null+; R26-EGFP/+*) mice showing the span of the retina were labeled with the progenitor marker, BrdU, at E18.5, showing the decrease in retinal progenitors in the distal region of the *Rx* CKO. (B) Higher magnification images demonstrate Cre-activated GFP expression co-labeling with BrdU incorporation throughout control retinal sections. In contrast, GFP expression is mostly excluded from BrdU incorporation areas within the *Rx* CKO, indicating the loss of progenitors within the *Rx*-deleted regions. Dashed lines represent the approximate boundary of the *Rx*-deleted region in the *Rx* CKO retina. Scale bars are 100 μm in A and 50 μm in B.

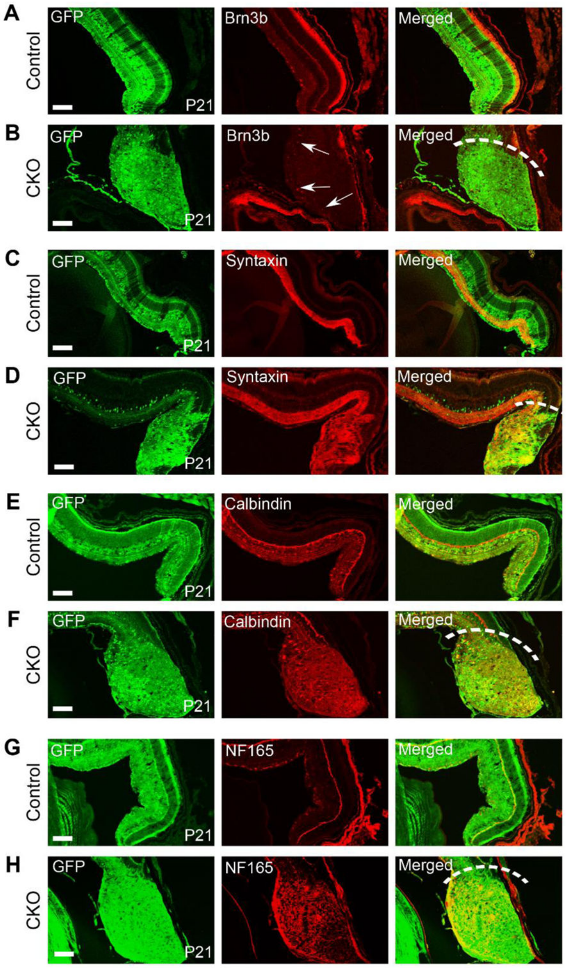

Figure 5. Amacrine cell markers are altered in the P21 *Rx* CKO retina

Immunolabeling for Brn3b and GFP in retinal sections from control (A) and *Rx* CKO (*Pax6α-Cre/+; Rx-flox/Rx-null; R26-EGFP/+*) (B) shows the presence of retinal ganglion cells in the control and *Rx*-deleted retina. Arrows indicate examples of Brn3B-expressing cells. An abundance of amacrine cell-specific expression in the *Rx*-deleted portion of the *Rx* CKO retina (D) is shown with the labeling of synataxin 1A and GFP compared to control retinal sections (C). Calbindin and GFP immunolabeling in retinal sections of control (E) and *Rx* CKO (F) show the presence of amacrine and horizontal cells in both control retina and in *Rx* CKO. (D) Co-labeling of neurofilament 165 kD and GFP shows the presence of horizontal cells in the *Rx* CKO retina (G) as well as in control retinal sections (H). Dashed lines indicate the approximate boundary of the *Rx*-deleted portion in the *Rx* CKO retina. Scale bars are 100 µm.

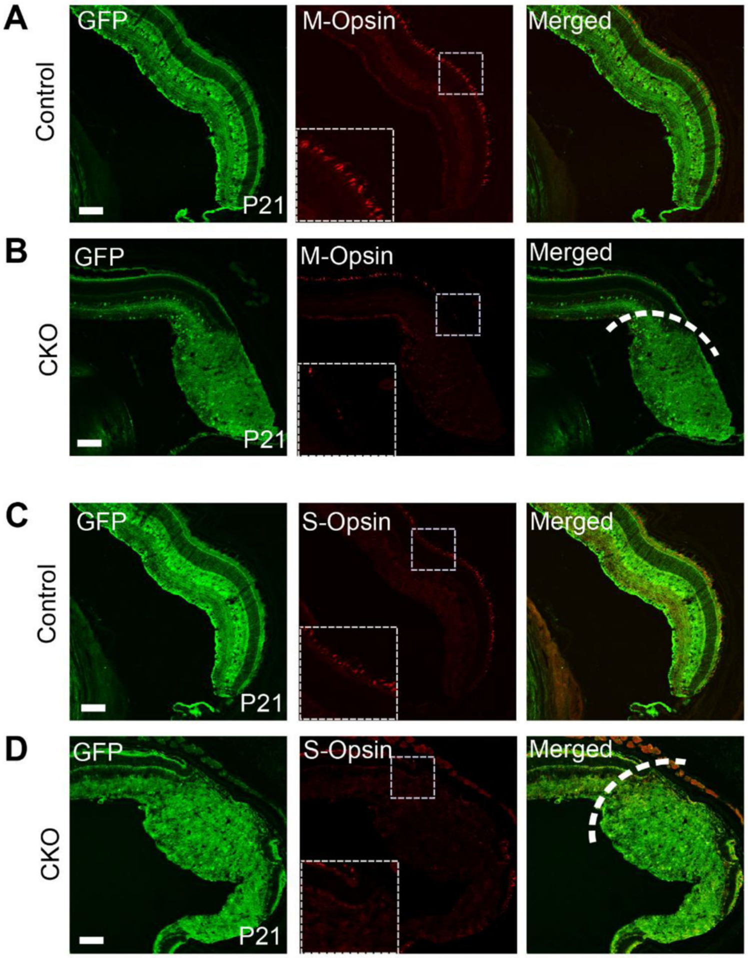

Figure 6. Cone opsin expression is absent in the *Rx* CKO P21 retina

Co-labeling with GFP and M-opsin shows expression along the ventricular surface of the control retina (A) and the central portion of the *Rx* CKO (*Pax6α-Cre/+; Rx-flox/Rx-null; R26-EGFP/+*) retina (B); however, the *Rx*-deleted portion of the *Rx* CKO retina shows a lack of M-opsin labeling (B). The presence of S-cone opsin co-labeled with GFP shows normal expression in the control (C) and central region of the *Rx* CKO retina (D), whereas the distal portion of the *Rx* CKO (D) labeled with GFP shows a lack of S-opsin expression. Dashed boxes indicate a region that was magnified by 2× in the lower left hand corner of the opsin images. Dashed curved lines indicate the approximate boundary of the *Rx*-deleted region in the *Rx* CKO retina. Scale bars are 100 µm.

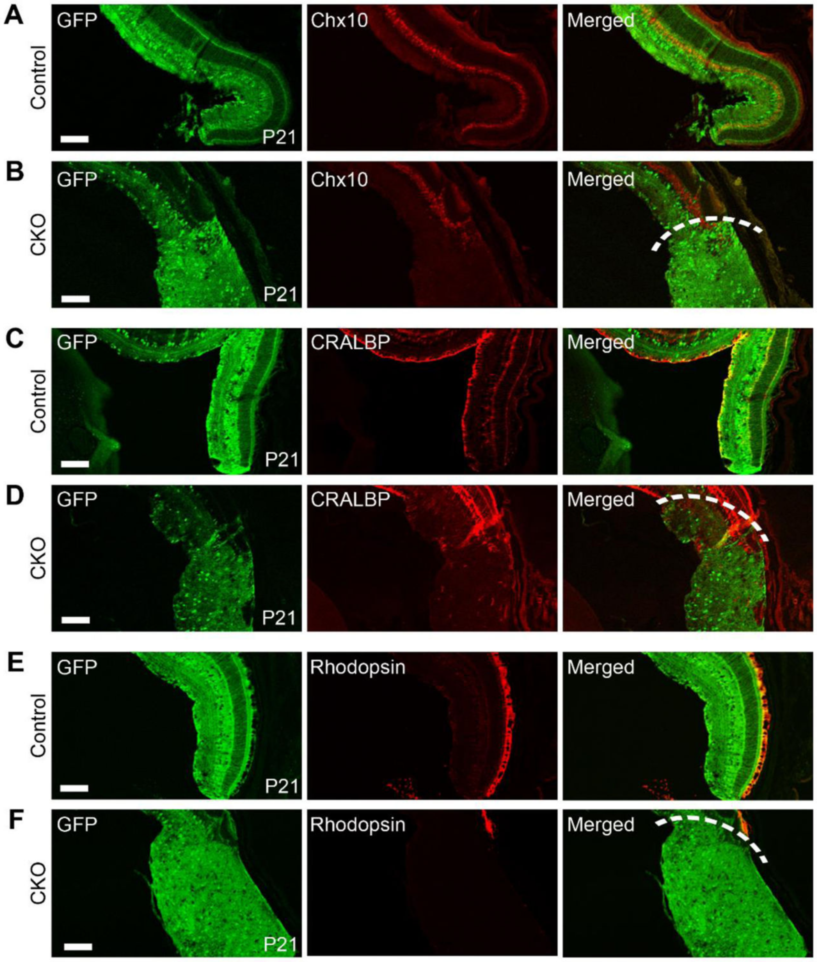

Figure 7. Absence of late-born cell types in P21 *Rx* CKO retinal sections

Immunolabeling against Chx10 shows the presence of bipolar cells in the control (A) and central region of the *Rx* CKO (*Pax6α-Cre/+; Rx-flox/Rx-null; R26-EGFP/+*) (B), whereas co-labeling with GFP shows a loss of bipolar cells in the *Rx*-deleted portion of the *Rx* CKO retina (B). Retinal sections labeled with CRALBP and GFP show Müller glia in the control (C) and central part of the *Rx* CKO (D). The region of the *Rx* CKO (D) where *Rx* deletion occurred (labeled with GFP) shows a loss of Müller glia. Labeling with rhodopsin displays rod photoreceptors throughout control retinal sections (E) but only in the central portion in the *Rx* CKO retina (F). Co-labeling with GFP shows a lack of rhodopsin expression in the GFP-labeled, distal region of the *Rx* CKO, which demarcates where *Rx* has been deleted. Dashed lines represent the approximate boundary of the *Rx*-deleted portion of the retina. Scale bars are 100 μm.

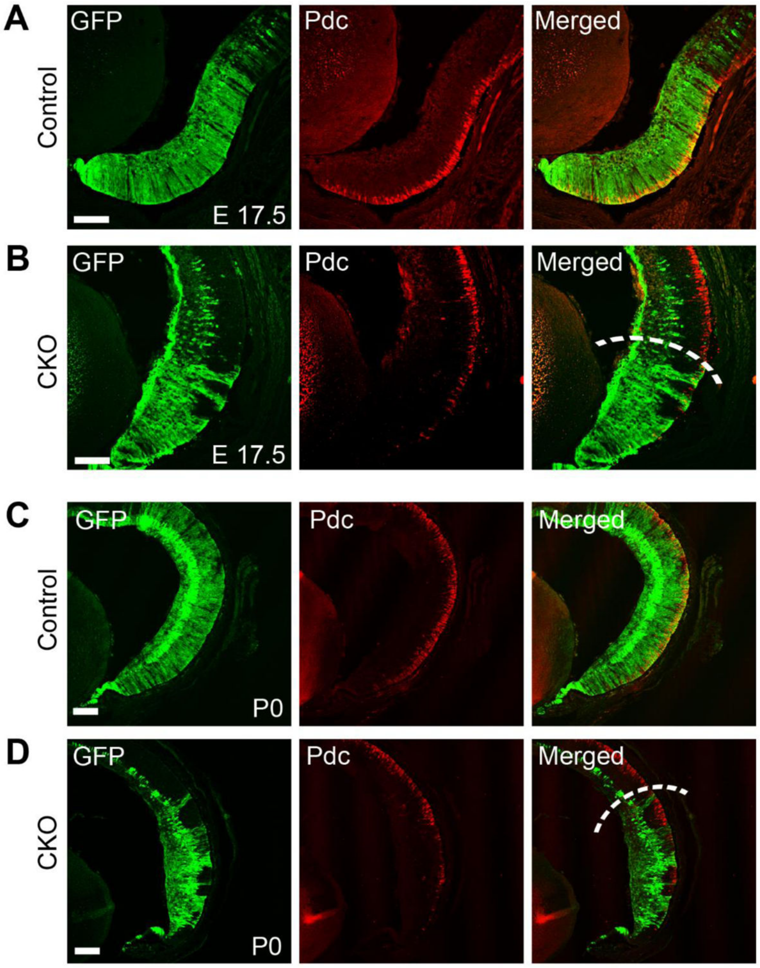

Figure 8. Phosducin expression is reduced in embryonic and early postnatal *Rx* CKO retina

Co-labeling with phosducin (Pdc) and GFP, to visualize the regions of the *Rx* CKO (*Pax6α-Cre/+; Rx-flox/Rx-null; R26-EGFP/+*) retina (B) where *Rx* deletion occurred shows a decrease in phosducin-labeling at E17.5 compared to control (A). Similarly at P0, co-labeling with GFP and phosducin shows a decrease in phosducin expression in the *Rx*-deleted portion of the *Rx* CKO retina (D) compared to control (C). Phosducin-labeled cells in the distal retina are primarily in regions that have not undergone Cre-mediated recombination (i.e. GFP-negative). Dashed lines represent the approximate region of *Rx* deletion. Scale bars are 100 μM.

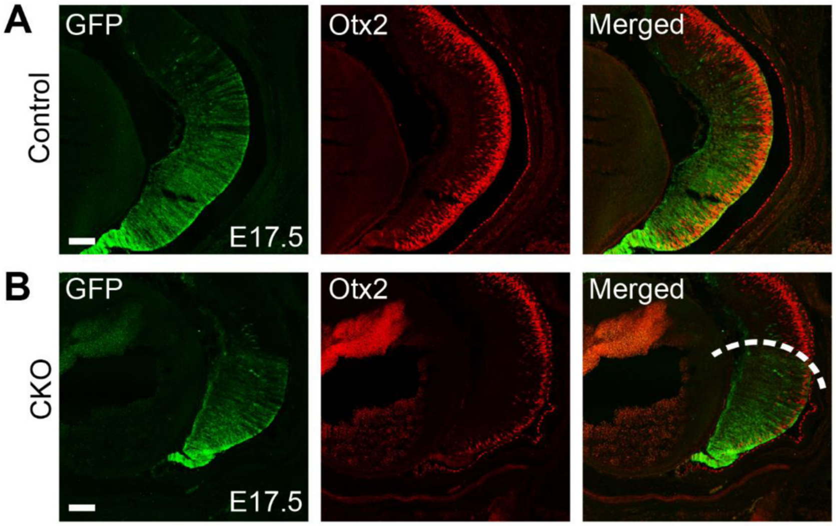

Figure 9. Decreased Otx2 expression in embryonic *Rx* CKO retina

(A) Immunolabeling in control retinal sections with GFP and Otx2 (labeling photoreceptors) shows strong Otx2 labeling along the length of the retina at E17.5. (B) Retinal sections of E17.5 *Rx* CKO (*Pax6α-Cre/+; Rx-flox/Rx-null; R26-EGFP/+*) mice show strong Otx2 labeling in the central portion of the retina, but decreased labeling in the distal region that corresponds with the area labeled by the GFP reporter. Dashed line represents the approximate region of *Rx* deletion. Scale bars are 100 μm.