THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 45, pp. 27239–27247, November 6, 2015

Conditional Ablation of Retinol Dehydrogenase 10 in the Retinal Pigmented Epithelium Causes Delayed Dark Adaption in Mice*

Received for publication, July 28, 2015, and in revised form, September 14, 2015 Published, JBC Papers in Press, September 21, 2015, DOI 10.1074/jbc.M115.682096

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Background: RDH10 is a candidate 11-*cis*-retinol dehydrogenase in the retinal pigmented epithelium (RPE) capable of regenerating the visual chromophore, 11-*cis*-retinal.
Results: Loss of *Rdh10* in RPE cells caused delayed regeneration of 11-*cis*-retinal.
Conclusion: Rdh10 is an NAD^+-dependent 11-*cis*-retinol dehydrogenase localized to RPE cells.
Significance: This study provides evidence that RDH10 plays a complimentary role in producing 11-*cis*-retinal in the RPE.

Regeneration of the visual chromophore, 11-*cis*-retinal, is a crucial step in the visual cycle required to sustain vision. This cycle consists of sequential biochemical reactions that occur in photoreceptor cells and the retinal pigmented epithelium (RPE). Oxidation of 11-*cis*-retinol to 11-*cis*-retinal is accomplished by a family of enzymes termed 11-*cis*-retinol dehydrogenases, including RDH5 and RDH11. Double deletion of *Rdh5* and *Rdh11* does not limit the production of 11-*cis*-retinal in mice. Here we describe a third retinol dehydrogenase in the RPE, RDH10, which can produce 11-*cis*-retinal. Mice with a conditional knock-out of *Rdh10* in RPE cells (*Rdh10* cKO) displayed delayed 11-*cis*-retinal regeneration and dark adaption after bright light illumination. Retinal function measured by electroretinogram after light exposure was also delayed in *Rdh10* cKO mice as compared with controls. Double deletion of *Rdh5* and *Rdh10* (*cDKO*) in mice caused elevated 11/13-*cis*-retinyl ester content also seen in $Rdh5^{-/-}Rdh11^{-/-}$ mice as compared with $Rdh5^{-/-}$ mice. Normal retinal morphology was observed in 6-month-old *Rdh10* cKO and *cDKO* mice, suggesting that loss of Rdh10 in the RPE does not negatively affect the health of the retina. Compensatory expression of other retinol dehydrogenases was observed in both $Rdh5^{-/-}$ and *Rdh10* cKO mice. These results indicate that RDH10 acts in cooperation with other RDH isoforms to produce the 11-*cis*-retinal chromophore needed for vision.

Upon absorption of a photon of light, the 11-*cis*-retinal chromophore is photoisomerized to all-*trans*-retinal, which then initiates signal transduction that culminates in visual sensation (1, 2). To maintain vision, the photo-activated chromophore, all-*trans*-retinal, is isomerized back to the photo-sensitive form, 11-*cis*-retinal, via the visual (retinoid) cycle (3). All-*trans*-retinal is first reduced to all-*trans*-retinol by enzymes of the short-chain dehydrogenase (SDR)[2] family, retinol dehydrogenase 12 (RDH12) and RDH8, present in the inner and outer segments of the retina, respectively (4, 5). All-*trans*-retinol is transported to the retinal pigmented epithelium (RPE), where it is sequentially esterified by lecithin retinol acyltransferase (LRAT) to all-*trans*-retinyl ester (6). All-*trans*-retinyl ester is then isomerized and hydrolyzed by retinal pigment epithelium-specific protein 65 kDa (RPE65) to 11-*cis*-retinol (7). Finally, 11-*cis*-retinol is oxidized by RDHs in the RPE to 11-*cis*-retinal, which is returned to photoreceptor cells to complete the cycle.

The primary RDH responsible for regenerating 11-*cis*-retinal from 11-*cis*-retinol in RPE cells is RDH5. RDH5 also belongs to the SDR family of proteins, which are highly expressed in RPE cells (8). SDR family members are membrane-bound enzymes with ~250 amino acids. Missense mutations in RDH5 have been linked to human fundus albipunctatus, characterized by stationary night blindness, delayed dark adaption, accumulation of white spots in the retina, and occasional cone dystrophy (9). $Rdh5^{-/-}$ mice do not display age-related retinal degeneration and have normal dark adaption kinetics under standard conditions (10). However, under intense lighting (10,000 lux for

* This work was supported by National Institutes of Health Grants R01EY022658 (to A. M.), R24EY021126 (to K. P.), and P30EY011373, Research to Prevent Blindness Foundation, Foundation Fighting Blindness, Midwest Eye Banks, and Ohio Lions Eye Research Foundation. The authors declare that they have no conflicts of interest with the contents of this article.
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[2] The abbreviations used are: SDR, short-chain dehydrogenase/reductase; RPE, retinal pigmented epithelium; Rpe65, retinal pigment epithelium-specific 65-kDa protein; *Rdh10* cKO, *Rdh10* retinal pigmented epithelium-specific knockout; *cDKO*, RPE specific knockout of *Rdh10* and global knockout of *Rdh5*; RDH, retinol dehydrogenase; qRT-PCR, quantitative RT-PCR; ONL, outer nuclear layer; Rgr, retinal G-protein-coupled receptor protein; CRALBP, Cellular retinaldehyde-binding protein; LRAT, lecithin retinol acyltransferase; ADH, alcohol dehydrogenase.

3 min), *Rdh5*$^{-/-}$ mice display delayed dark adaption kinetics. *Rdh5*$^{-/-}$ mice can still produce 11-*cis*-retinal, albeit at a slower rate than WT mice. Haeseleer *et al.* (11) identified RDH11 together with other RDHs in the eye. RDH11 is expressed ubiquitously in many tissues, including RPE cells, and can facilitate the conversion of 11-*cis*-retinol to 11-*cis*-retinal. *Rdh5*$^{-/-}$ *Rdh11*$^{-/-}$ mice still produce 11-*cis*-retinal in the RPE, suggesting that Rdh11 plays a minor but complementary role in 11-*cis*-retinal regeneration (12). These observations suggest that additional enzyme(s) in the RPE catalyze 11-*cis*-retinal production.

In recent years, *Rdh10* was cloned from retinal cDNA and found to be associated with microsomal membranes (13). RDH10 is highly conserved in humans, mice, and cattle, and is predominantly expressed in RPE cells with lower expression levels found in Müller cells, heart, lungs, kidney, and liver (14). *Rdh10* is essential for retinoic acid biosynthesis during embryogenesis, as mice harboring a mutation in *Rdh10* show early embryonic lethality (15). *Rdh10* encodes a 341-amino acid protein with two hydrophobic domains, one at the N terminus (residues 2–23) and the other at the C terminus (residues 293–329), that associate with membranes (16). RDH10 physically interacts with CRALBP and RPE65 and co-localizes with RPE65 and CRALBP in cell culture model systems (17). The specific substrate and cofactor specificities of RDH10 have yet to be clarified. Wu *et al.* (13) reported that RDH10 is an efficient all-*trans*-RDH with $NADP^+$ as its preferred cofactor. However, Belyaeva *et al.* (18) demonstrated that RDH10 is an efficient 11-*cis*-RDH with NAD^+ as its preferred cofactor. The *in vivo* role of RDH10 in the visual cycle has not been reported.

In this study, we examined the physiological role of RDH10 in the visual cycle. Biochemical roles of RDH10 were investigated to establish its cofactor specificity. We also developed an RPE-specific *Rdh10* knock-out mouse model to study the role of Rdh10 in the visual cycle. Our data provide evidence that Rdh10 plays a complementary role in regenerating 11-*cis*-retinal in mice.

Experimental Procedures

Animals—RPE-specific *Rdh10* knock-out genotype (*rtTA-cre*$^{+/-}$, *Rdh10*$^{f/f}$ (*Rdh10* cKO)) mice were generated by crossing *Rdh10*$^{f/f}$ mice (19) with *rtTA-cre*$^{+/-}$ mice (20). Doxycycline (100 μg/g of body weight) was administered intraperitoneally in two consecutive daily doses to 1-month-old mice to activate *cre* for RPE-specific deletion of *Rdh10*. Double deletion of *Rdh5* and *Rdh10* (*cDKO*, global knock-out of *Rdh5* and RPE-specific knock-out of *Rdh10*) was elicited by crossing *Rdh10* cKO mice with *Rdh5*$^{-/-}$ mice. All genetically modified mice in this study have mixed background with 129S6 and C57BL/6 mice. Moreover, these mice were free of the *Crb1/rd8* mutation and had a leucine variation at amino acid 450 of Rpe65. WT (129S6) mice with Leu-450 of Rpe65 were purchased from Taconic (Hudson, NY). Genotyping of *Rdh10*$^{f/f}$ mice was accomplished with a previous protocol and primer sets (19). *rtTA-cre*$^{+/-}$ mice also were genotyped by a former protocol (20). Littermates of *Rdh10*$^{f/f}$ mutant mice with doxycycline treatment and WT mice were used as controls. Mice were fed a regular chow diet, Prolab®5P76 Isopro®RMH 3000 (LabDiet, St. Louis, MO) containing 29 IU/g of vitamin A. Some mice used to examine the effects of vitamin A deficiency were maintained on a vitamin A-deficient diet (AIN-93G growing rodent diet, catalogue number D13110GC) (Research Diets, Inc., New Brunswick, NJ) from 21 days to 6 months of age. All experimental animals were housed in the animal care facility at the School of Medicine, Case Western Reserve University in a 12-h light (~10 lux)/12-h dark cyclic environment. Dim red light transmitted through a Kodak No. 1 safelight filter (transmission >560 nm) was used in the dark for all animal experiments and procedures done after prior approval by the Case Western Reserve University Animal Care Committees, in agreement with guidelines set by the American Veterinary Medical Association Panel on Euthanasia and the Association of Research for Vision and Ophthalmology.

Quantitative RT-PCR (qRT-PCR)—Total RNA was extracted with a RNase mini kit (Qiagen, Valencia, CA), and cDNA was synthesized using a quantitative cDNA Kit (Qiagen). Real-time PCR amplification was done with SYBR Green Master (Roche Applied Science). Gapdh gene expression was used to normalize the relative expression of genes. Primer sequences for mice were: mRdh10-F1, 5′-gtgacgtggggaagagagag-3′ and mRdh10-R1, 5′-tggaagaaaggccttagtgg-3′; mRdh5-F, 5′-ttctttcgaacccctgtgac-3′ and mRdh5-R, 5′-tgggtcacagatcaggttca-3′; mRdh11-F, 5′-actgcagacggctttgagat-3 and mRdh11-R, 5′-ttatggaagtggatcctgcc-3′; mCralbp-F, 5′-gaacttcaagggcttcacca-3′ and mCralbp-R, 5′-ccgtgaacaaagaccctctg-3′; mSDRpe65-F, 5′-ccagattcttacccatctga-3′ and mSDRpe65-R, 5′-agtccatggaaggtcacagg-3′; mLrat-F, 5′-tatggctctcggatcagtcc-3′ and mLratR, 5′-cagattgcaggaagggtcat-3′; mZo1-F, 5′-aaagcagagaggacttgtcagc-3′ and mZo1-R, 5′-gctcctctcttgccaacttttct-3′; mGapdh-F, 5′-gtgttcctacccccaatgtg-3′ and mGapdh-R, 5′-ggagacaacctggtcctcag-3′; mSDRhod-F, 5′-cttcctgatctgctggcttc-3′ and mSDRhod-R, 5′-acagtctctggccaggctta-3′; mMcone2L, 5′-gagattcaagaagctgcgcc-3′ and mMcone2R, 5′-tgtcccagaacgaagtagcc-3′; mScone-F, 5′-atggtcaacaatcggaacca-3′ and mScone-R, 5′-accatctccagaatgcaagc-3′.

Histology—Retinal histology and immunohistochemistry were performed as described previously (21). Briefly, eye cups were dissected and fixed in 4% paraformaldehyde overnight. Then tissue was subjected to a sucrose gradient followed by embedding in a 20% sucrose/OCT (1:1) compound and freezing. Retinal sections were prepared at a 12-μm thickness for immunohistochemistry. Peanut agglutinin was used for cone staining. Biotinylated peanut agglutinin was purchased from Vector Laboratories (Burlingame, CA) (catalogue number B-1075) (dilution 1:400). Secondary antibody streptavidin Alexa Fluor 488 conjugate was obtained from Life Technologies (catalogue number S-11223) (dilution 1:500).

Immunoblots—SDS-PAGE analyses were carried out with 10% polyacrylamide gels followed by transfer onto PVDF membranes blocked with 0.05% BSA in PBS solution. Membranes were incubated with primary antibody overnight followed by secondary antibody treatment for 1 h at a dilution of 1:5000. Antibody binding was detected with 5-bromo-4-chloro-3-indolyl-phosphate (NBT) and nitro blue tetrazolium (BCIP) solutions from Promega Corp. (Madison, WI). Rabbit polyclonal antibody against RDH10 was purchased from Abcam, Inc. (catalogue number ab80891)(Kendall Square, MA)(dilution 1:200).

Mouse monoclonal antibody for β-actin was purchased from Santa Cruz Biotechnology, Inc. (Paso Robles, CA) (catalogue number sc-81178)(dilution 1:1000).

Retinoid Analyses—Eyes were extracted from dark-adapted and light-exposed mice, and retinoids were extracted as described previously (21, 22). Briefly, eyes were homogenized in a glass tissue grinder in 1.2 ml of buffer (50 mM MOPS, 10 mM NH_2OH, and 50% aqueous ethanol, pH 7.0). The reaction was incubated at room temperature for 20 min and then stopped by adding 1 ml of ice-cold ethanol. Retinoids were extracted from the aqueous phase twice, once with 4 ml and then with 1 ml of hexane. The hexane layer was separated and dried under vacuum after centrifugation and then dissolved in 0.3 ml of hexane. Retinoid extracts were separated by normal phase HPLC (Ultrasphere-Si, 4.6 μm 3 × 250 mm; Beckman Coulter) with 10% ethyl acetate and 90% hexane at a flow rate of 1.4 ml/min. Retinoid amounts were measured with Agilent ChemStation software. Dark adaption kinetics of retinoids were quantified after exposing 24-h dark-adapted mice to bright light (10,000 lux for 3 min) followed by dark adaptation for different time periods (21, 22).

RDH10 Overexpression in Sf9 Cells—*RDH10* cDNA was PCR-amplified from total cDNA of human primary RPE cells by using primer BamRDH10F, 5′-ggccccgggggatccgatgaacatcgtggtggagttcttc-3′, and XhoRDH10R, 5′-atacaaaactcgagttagattccatttttttgcttcattattgtttgtggcttgc-3′, isolated from the total cDNA of human primary RPE cells to introduce the BamHI and XhoI restriction sites, respectively. *RDH10* cDNA was cloned in the BamHI and XhoI sites of the pFastBacHT$^{\Delta 6His}$ vector (23). The final construct of RDH10 in pFastBacHT$^{\Delta 6His}$ encoded RDH10 without any His-tagged protein. Five clones underwent sequencing, and one was used for transforming DH10B cells to obtain a recombinant bacmid. The recombinant bacmid was transfected to Sf9 cells to express RDH10 (11). Microsomal preparations of RDH10 were isolated from Sf9 cells for enzymatic assays. The RDH10 microsomal fraction was isolated by ultracentrifugation (105,000 × *g*, 2 h). and stored at −80 °C in storage buffer (100 mM sodium phosphate, pH 7.4, 1 mM DTT, and 20% glycerol). Reactions were carried out with RDH10 protein in a buffer containing 100 mM sodium phosphate, pH 7.4, 1 mM cofactor (NAD^+, $NADP^+$, NADH, or NADPH), and 0.5% BSA (W/V). Seventy μg of RDH10 protein were used for reactions, incubated at 37 °C for required periods, and stopped by 2 volumes of ice-cold methanol. Retinoids were extracted twice with hexane and, after drying in a SpeedVac, were resuspended in 0.3 ml of hexane for further analysis by HPLC. Retinoids were separated in 10% ethyl acetate and 90% hexane.

Statistical Analyses—Data representing the means ± S.D. for the results of at least five independent experiments were compared by the one-way analysis of variance test with $p < 0.05$ considered statistically significant.

Results

Retinol Dehydrogenase Activity of RDH10 Expressed in Sf9 Cells—To examine retinol dehydrogenase activity of RDH10, human RDH10 cloned from human primary RPE cells was expressed in Sf9 cells. Immunoblotting analysis with polyclonal human RDH10 antibody then detected expression of RDH10

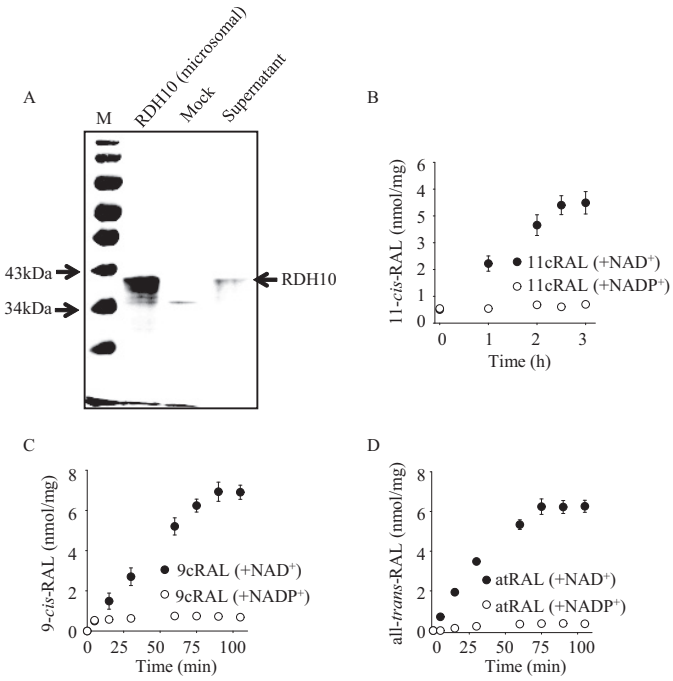

FIGURE 1. **Retinol dehydrogenase activity of human RDH10 protein.** *A*, Sf9 cells were infected with recombinant (RDH10) and wild-type (mock) baculovirus vector. Microsomal extracts were obtained, and immunoblotting was performed with RDH10 antibody. RDH10 expression is indicated with an *arrow*. *B–D*, enzymatic activity was measured with 70 μg of a RDH10 microsomal preparation in the presence of either 1 mM NAD^+ or 1 mM $NADP^+$ as a cofactor. Production of 11-*cis*-retinal (*11-cis-RAL*) was examined after the addition of 45 μM 11-*cis*-retinol to the reaction at 37 °C (*B*). 11cRAL, 11-*cis*-retinal. Similarly, production of 9-*cis*-retinal (*9cRAL*) (*C*) or all-*trans*-retinal (*atRAL*) (*D*) was examined after the addition of 45 μM 9-*cis*-retinol or all-*trans*-retinol to the reaction at 37 °C. NAD^+ was strictly preferred by RDH10 in these enzymatic reactions.

mostly in the microsomal fraction of Sf9 cells infected with recombinant RDH10, but not in uninfected Sf9 cells (Fig. 1*A*). To determine the cofactor preference in this enzyme, microsomal fractions with RDH10 expression (70 μg) were incubated with 1 mM NAD^+/NADH or $NADP^+$/NADPH in reaction buffer containing 100 mM sodium phosphate, pH 7.4, 0.5% BSA, and 1 mM DTT. The reaction was initiated by adding the retinol substrate, and the retinal product was then measured. NAD^+ was found to be the preferred cofactor for the dehydrogenase activity of RDH10. This enzyme preparation exhibited no detectable activity in the presence of $NADP^+$. Different *cis*- and *trans*-retinol substrates then were used to evaluate RDH10 enzymatic activity. Analyses by HPLC showed that in presence of NAD^+, RDH10 efficiently converted 11-*cis*-retinol to 11-*cis*-retinal at 24 pmol/min/mg. Moreover, the oxidation rates of all-*trans*-retinol and 9-*cis*-retinol were 3-fold (124 pmol/min/mg) and 2-fold (81 pmol/min/mg) higher than that of 11-*cis*-retinol (Fig. 1, *B–D*, Table 1). These data indicate that RDH10 can oxidize 11-*cis*-retinol to 11-*cis*-retinal and prefers NAD^+ as cofactor for this reaction.

Loss of Rdh10 in the RPE of Rdh10 cKO Mice—RDH10 is highly expressed in the RPE, although its *in vivo* role there is yet unknown. Indeed, global knock-out of *Rdh10* in mice is embryonically lethal (15). To understand the role of RDH10 in vision, we bred RPE-specific *Rdh10*-deficient mice. To assure such selective *Rdh10* knock-out in mice, RPE-specific deletion of

TABLE 1
Specific activity of RDH10 with differing substrates and cofactors

Substrate	Cofactor	Specific activity
		pmol/min/mg
11-*cis*-Retinol	NAD^+	24 ± 1.7
11-*cis*-Retinol	$NADP^+$	<0
9-*cis*-Retinol	NAD^+	81 ± 2
9-*cis*-Retinol	$NADP^+$	<0
All-*trans*-retinol	NAD^+	124 ± 15
All-*trans*-retinol	$NADP^+$	<0

Rdh10 was accomplished with the help of cre protein expressed exclusively in the RPE by the rtTA system after doxycycline treatment (20). Doxycycline was given intraperitoneally to *rtTA-cre*$^{+/-}$, *Rdh10*$^{f/f}$ mice at 1 month of age. *Rdh10* gene expression was then evaluated in the RPE 2 weeks afterward (Fig. 2*A*). The relative *Rdh10* mRNA amount was reduced more than 80% in *Rdh10* cKO mice as compared with control *Rdh10*$^{f/f}$ mice when checked by qRT-PCR (Fig. 2*B*). Immunoblotting analyses indicated an 85% decreased Rdh10 expression in *Rdh10* cKO mice relative to that in control *Rdh10*$^{f/f}$ mice (Fig. 2*C*). Loss of Rdh10 expression in the RPE of *Rdh10* cKO mice was further confirmed by immunohistochemistry (Fig. 2*D*). *Rdh10* cKO mice failed to exhibit abnormalities in their behavior, breeding, or life span.

Loss of Rdh10 in the RPE Affects Retinoid Content of Dark-adapted Mice—Retinoid recycling is the primary function of the visual cycle, abnormalities of which impair vision. One crucial step in this cycle is the regeneration of 11-*cis*-retinal known to be catalyzed by RDH5 and RDH11. Because RDH10 also participates in converting 11-*cis*-retinol to 11-*cis*-retinal *in vitro* (Fig. 1 and Table 1), we examined retinoid flow in *Rdh10*-deficient mice. To study the role of Rdh10 in retinoid recycling between the retina and the RPE, retinoids were measured in mice reared in a 12-h dark and 12-h light cycle after 24 h of dark adaption. No significant differences were observed in 11-*cis*-retinal content between dark-adapted *Rdh10* cKO and control *Rdh10*$^{f/f}$ mice (Fig. 3*A*). Also there was no change in 11/13-*cis*-retinyl ester content (Fig. 3*B*) and all-*trans*-retinyl ester content (Fig. 3*C*) in *Rdh10* cKO mice as compared with *Rdh10*$^{f/f}$ mice. To further examine the role of Rdh10 relative to Rdh5, a well established enzyme involved in visual cycle processing in the RPE (10), we then generated double knock-out mice (*cDKO*) by breeding *Rdh10* cKO with *Rdh5*$^{-/-}$ mice, employing *Rdh5*$^{-/-}$ and *Rdh5*$^{-/-}$*Rdh11*$^{-/-}$ mice for comparison (10, 12). Amounts of retinoids were measured 24 h after dark adaptation. No significant differences were observed in 11-*cis*-retinal content between dark-adapted *cDKO*, *Rdh5*$^{-/-}$*Rdh11*$^{-/-}$, *Rdh5*$^{-/-}$, and WT mice (Fig. 3*A*). 11/13-*cis*-Retinyl esters were found elevated in *cDKO*, *Rdh5*$^{-/-}$*Rdh11*$^{-/-}$, and *Rdh5*$^{-/-}$ mice as compared with WT mice (Fig. 3*B*). However, the amounts of 11/13-*cis*-retinyl esters in *cDKO* mice were significantly ($p <$ 0.005) increased as compared with those in *Rdh5*$^{-/-}$ mice and matched the amounts found in *Rdh5*$^{-/-}$*Rdh11*$^{-/-}$ mice. Reduced amounts of all-*trans*-retinyl esters were found in *Rdh5*$^{-/-}$ mice as compared with WT mice (Fig. 3*C*). These results support a complementary role of RDH10 in the visual cycle and reveal a possible redundancy of RDHs in the RPE.

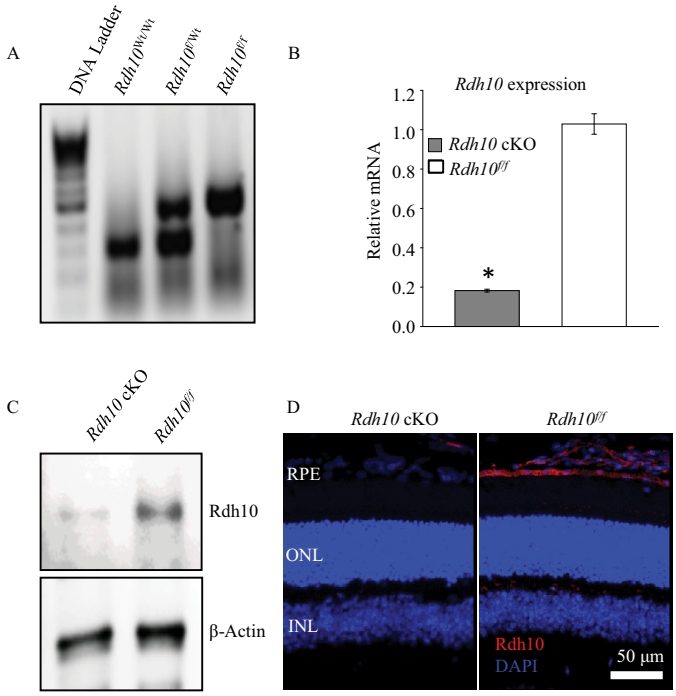

FIGURE 2. **Loss of *Rdh10* expression in the RPE of *rtTA-cre*$^{+/-}$, *Rdh10*$^{f/f}$ (*Rdh10* cKO) mice.** *A*, agarose gel showing PCR products from the wild-type gene and the flox element flanking the *Rdh10* gene in wild-type (*Rdh10*$^{wt/wt}$), heterozygous (*Rdh10*$^{wt/f}$), and homozygous (*Rdh10*$^{f/f}$) mice. *B*, qRT-PCR was performed for *Rdh10* with *Rdh10* cKO and *Rdh10*$^{f/f}$ isolated mouse RPE tissue. Relative expression levels are presented. *Error bars* indicate S.D. ($n = 5$). *, $p <$ 0.005 *Rdh10* cKO *versus* *Rdh10*$^{f/f}$. Data were normalized against the housekeeping gene, Gapdh. *C*, expression levels of Rdh10 in *Rdh10* cKO and *Rdh10*$^{f/f}$ mice were determined by immunoblotting. RPE tissues were isolated from *Rdh10* cKO and *Rdh10*$^{f/f}$ mice, and cell lysates were prepared with Nonidet P-40 lysis buffer. Reduced expression of Rdh10 protein was seen in *Rdh10* cKO mice as compared with *Rdh10*$^{f/f}$ mice. *D*, immunohistochemistry was performed with retinas from *Rdh10* cKO and *Rdh10*$^{f/f}$ mice using Rdh10 polyclonal antibody. Loss of expression of Rdh10 in the RPE can be seen in the retina of a *Rdh10* cKO mouse. *INL*, inner nuclear layer. *Scale bar*, 50 μm.

Delayed Dark Adaption Kinetics in Rdh10 cKO Mice—Delayed dark adaption is due to slow regeneration of 11-*cis*-retinal in the RPE. To examine whether *Rdh10* cKO mice evidence delayed dark adaption kinetics, we evaluated 11-*cis*-retinal production after various periods of dark adaptation after light exposure. Mice were illuminated for 3 min with 10,000 lux light that bleached 95% of chromophore and then were kept in the dark for 1, 2, 3, 6, or 24 h to examine their rates of 11-*cis*-retinal production. Here *Rdh10* cKO mice exhibited a delay in 11-*cis*-retinoid production as compared with controls (Fig. 4*A*), taking to regenerate this chromophore completely for 6 h as compared with 2 h for controls. However, the delay of chromophore regeneration in *Rdh5*$^{-/-}$ mice was even more severe, taking 6 h to produce 11-*cis*-retinal to the same full extent as in *Rdh10*$^{f/f}$ mice used as WT controls for this experiment.

To further probe the delay in 11-*cis*-retinal kinetics, we determined whether intense light exposure (2000 cd/m^2) for 3 min would affect the a-wave recovery of *Rdh10* cKO mice with *Rd10*$^{f/f}$ mice used as controls. Mice were kept 24 h in the dark prior to this experiment. After light exposure, a-wave recovery, normalized to the a-wave before illumination, was analyzed by electroretinogram every 10 min up to 1 h in the dark. A significant delay ($p < 0.05$) in a-wave recovery was observed in *Rdh10*

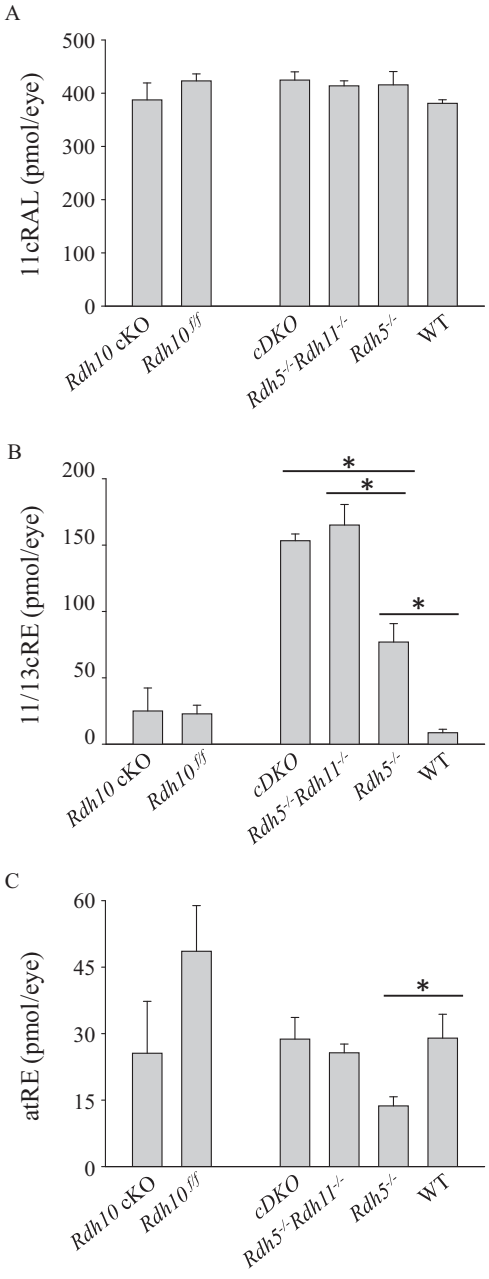

FIGURE 3. **Retinoid content in dark-adapted mice lacking retinal dehydrogenases in the eye.** *A–C*, mice (6 weeks old) were kept in the dark for 24 h, and their 11-*cis*-retinal (*11cRAL*) (*A*), 11/13-*cis*-retinyl esters (*11/13cRE*) (*B*), and all-*trans*-retinyl esters (*atRE*) (*C*) were quantified by HPLC. *Error bars* indicate S.D., $n = 5$. *, $p < 0.005$.

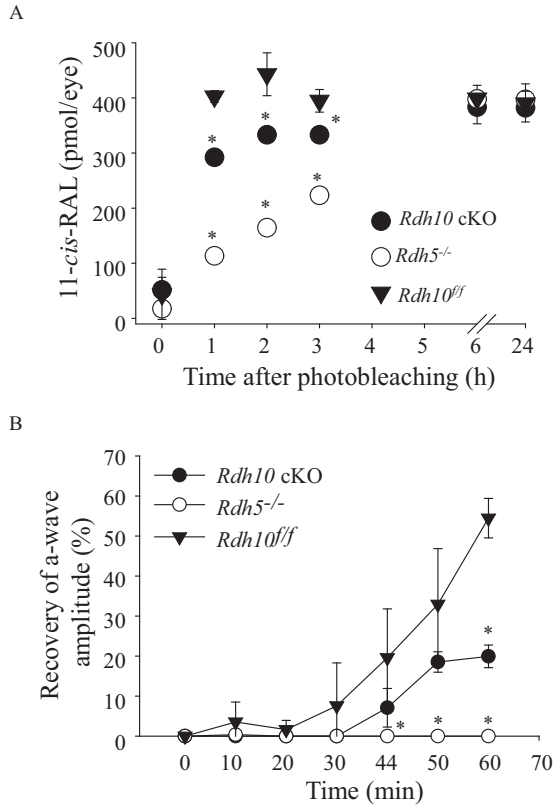

FIGURE 4. **11-*cis*-Retinal (*11-cis-RAL*) and a-wave recovery kinetics in dark-adapted *Rdh10* cKO mice.** *A*, *Rdh10* cKO, $Rdh5^{-/-}$ and $Rdh10^{f/f}$ mice were dark-adapted for 24 h followed by 10,000 lux light exposure for 3 min. Eyes were collected for retinoid analysis at various time points after light exposure, and retinoids were quantified by HPLC. *B*, electroretinogram evaluation of a-wave recovery was performed after exposing dark-adapted *Rdh10* cKO, $Rdh5^{-/-}$, and $Rdh10^{f/f}$ mice to light at 2000 cd/m^2 for 3 min. *Error bars* indicate S.D. ($n = 5$).*, $p < 0.05$ *Rdh10* cKO, $Rdh5^{-/-}$ *versus* $Rdh10^{f/f}$ mice.

cKO mice as compared with controls (Fig. 4*B*). These observations suggest that Rdh10 participates in 11-*cis*-retinal regeneration.

Presence of Normal Retinal Morphology in Rdh10 cKO and cDKO Mice—Impairment of the visual cycle leads to retinal degeneration in both humans and mice (24–27). Because experiments measuring retinoid kinetics in *Rdh10* cKO mice revealed delayed 11-*cis*-retinal recovery (Fig. 4, *A* and *B*), retinal morphology was examined by light microscopy and immunohistochemistry. When thicknesses of the outer nuclear layer (ONL) were measured to assess photoreceptor viability in *Rdh10* cKO mice and control $Rdh10^{f/f}$ mice at 6 months of age, the ONL thickness of *Rdh10* cKO mice was found comparable with that of $Rdh10^{f/f}$ mice (Fig. 5*A*). Moreover, retinal thickness as a measure of global retinal morphology was also similar in these mice (Fig. 5*B*). At the microscopic level, no morphological changes within the retina were observed in 6-month-old *Rdh10* cKO mice. Cone numbers in retinas of these *Rdh10* cKO mice were further examined by qRT-PCR and peanut agglutinin, which stains S-opsin and M-opsin cones. Transcript levels of S-opsin, M-opsin, and rhodopsin were also comparable among *Rdh10* cKO and $Rdh10^{f/f}$ mice (Fig. 5*C*). Moreover, *Rdh10* cKO mice had no change in cone density relative to control mice at this age (Fig. 5*D*). Normal retinal morphology and normal amounts of 11-*cis*-retinal in dark-adapted eyes were also demonstrated in *Rdh10* cKO mice fed vitamin A-deficient diets from 21 days to 6 months.

RDH5 is a primary enzyme for 11-*cis*-retinal recycling (10), and our results indicate a complementary role of Rdh10 in recycling chromophore (Fig. 4, *A* and *B*). To understand the role of RDH5 and RDH10 together in retinal physiology, retinas of *cDKO* mice were examined by measuring their ONL thickness and viewing their morphology with $Rdh5^{-/-}$ and $Rdh10^{f/f}$ mice used as controls. No obvious changes in ONL thickness and retinal morphology of *cDKO* mice were detected as compared with control mice at 6 months of age (Fig. 6, *A* and *B*), suggesting a redundancy in RDH genes.

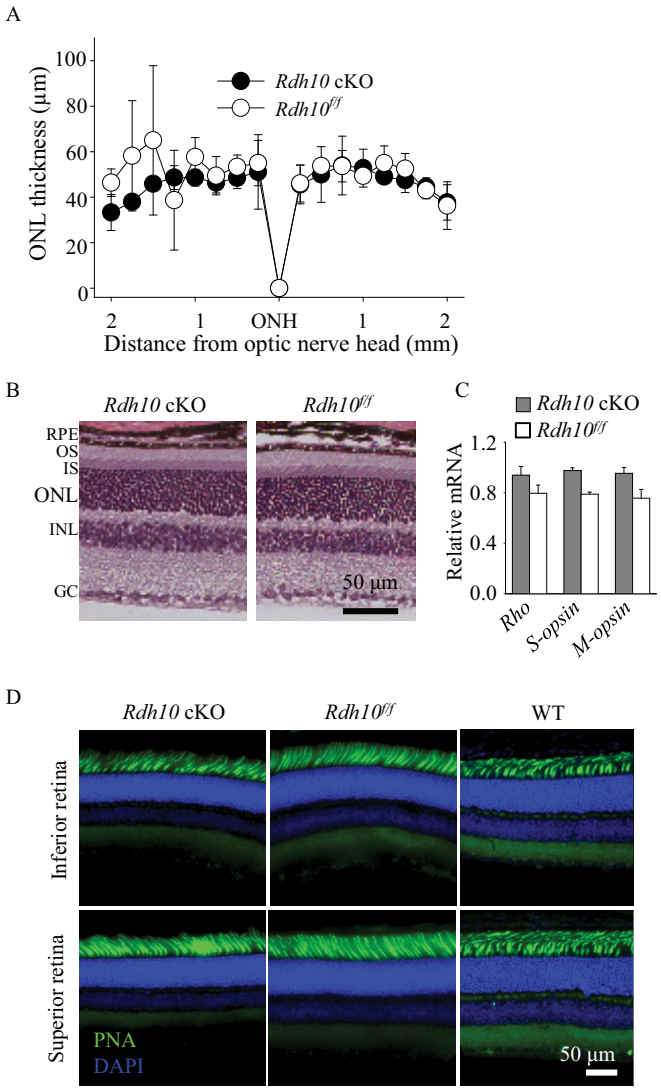

FIGURE 5. **Analysis of retinal histology and rod and cone opsin expression in *Rdh10* cKO and *Rdh10*$^{f/f}$ mice.** *A*, cryosections of *Rdh10* cKO and *Rdh10*$^{f/f}$ retinas from 6-month-old mice were stained with DAPI, and ONL thicknesses were measured. There was no significant difference in ONL thickness between *Rdh10* cKO and *Rdh10*$^{f/f}$ retinas ($n = 5$). *B*, paraffin sections of retinas from *Rdh10* cKO and *Rdh10*$^{f/f}$ 6-month-old mice were compared. *OS*, outer segment; *IS*, inner segment; *INL*, inner nuclear layer; *GC*, ganglion cell layer. *C*, qRT-PCR was performed in *Rdh10* cKO and *Rdh10*$^{f/f}$ mice after RNA was isolated from their retinas. Relative expression levels were determined after comparison with the housekeeping gene, Gapdh. There was no significant difference ($n = 5$). *D*, *Rdh10* cKO, *Rdh10*$^{f/f}$, and WT 6-month-old mouse retinas were stained with peanut agglutinin (*PNA*) (*green*) for cone sheaths and DAPI (*blue*) for nuclei. No significant changes in cones were noted. *Scale bar*, 50 μm.

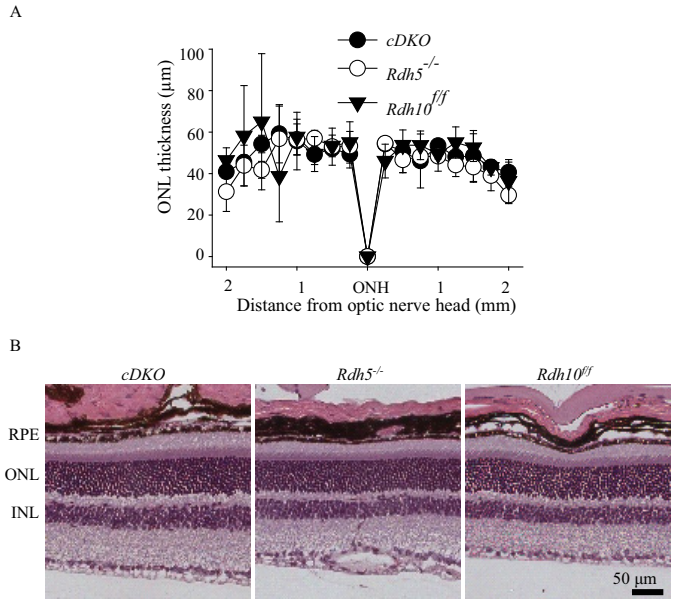

FIGURE 6. **Analysis of retinal histology in *cDKO*, *Rdh5*$^{-/-}$, and *Rdh10*$^{f/f}$ mice.** *A*, ONL thicknesses of *cDKO*, *Rdh5*$^{-/-}$, and *Rdh10*$^{f/f}$ 6-month-old mice were measured; there was no significant difference in ONL thickness among these retinas ($n = 5$). *ONH*, optic nerve head. *B*, when paraffin sections of retinas from cDKO, *Rdh5*$^{-/-}$, and *Rdh10*$^{f/f}$ 6-month-old mice were compared, there were no significant changes in retinal morphology among them. *INL*, inner nuclear layer. *Scale bar*, 50 μm.

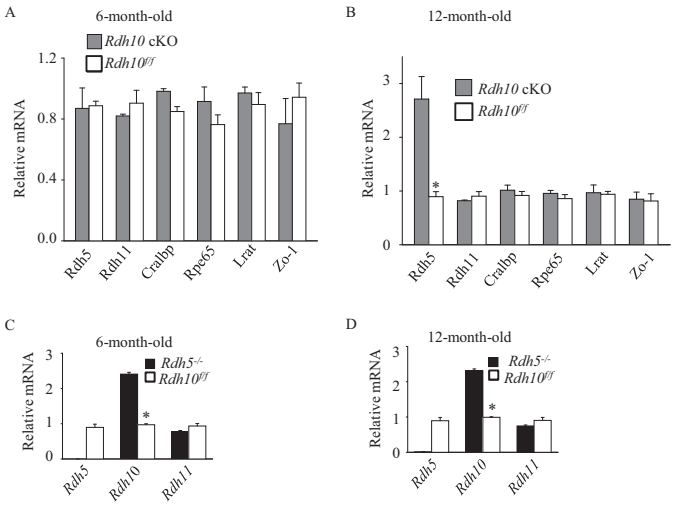

FIGURE 7. **RPE-specific gene expression in *Rdh10* cKO and *Rdh5*$^{-/-}$ mice.** *A* and *B*, the RPEs of 6-month-old (*A*) and 12-month-old (*B*) *Rdh10* cKO and *Rdh10*$^{f/f}$ mice were isolated, and qRT-PCR was performed to quantify RPE-specific gene expression. *C* and *D*, the RPEs of 6-month-old (*C*) and 12-month-old (*D*) *Rdh5*$^{-/-}$ mice were isolated, and *Rdh5*, *Rdh10*, and *Rdh11* expression was evaluated by qRT-PCR. *Error bars* indicate S.D. ($n = 5$). Data were normalized with the housekeeping gene, Gapdh. A significant difference is indicated with an asterisk (*, $p < 0.05$, $n = 5$).

The Retinal Pigmented Epithelium of 6-Month-old Rdh10 cKO Mice Displays No Pathology—To examine the effects of Rdh10 deletion in the RPE further, we evaluated RPE-specific gene expression by qRT-PCR. *Rdh10* cKO mice aged 6 or 12 months and age-matched *Rdh10*$^{f/f}$ mice were compared. RPE tissues of mice were dissected for RNA isolation followed by qRT-PCR. RPE-specific genes including *Rdh5*, *Rdh11*, *Cralbp*, *Rpe65*, *Lrat*, and *Zo-1* were analyzed. No changes of expression of these genes were detected in *Rdh10* cKO mice as compared with *Rdh10*$^{f/f}$ mice at 6 months of age (Fig. 7*A*). In contrast, however, at 12 months of age, *Rdh5* gene expression was found to be up-regulated in *Rdh10* cKO mice, whereas expression of the other genes remained unchanged (Fig. 7*B*). This observation indicates that compensatory mechanisms in the RPE can produce 11-*cis*-retinal.

Finally, to compare the roles of RDH10 and RDH11, expression of *Rdh10* and *Rdh11* was examined in *Rdh5*$^{-/-}$ mice. Because *Rdh5*$^{-/-}$ mice do not recapitulate a human retinal disease phenotype (9), we speculated that compensatory gene regulation is involved. Both *Rdh10* and *Rdh11* are expressed in the RPE. Of particular note is that *Rdh10* expression was up-regu-

lated in *Rdh5*$^{-/-}$ mice at both 6 months and 12 months age, but *Rdh11* expression was not (Fig. 7, *C* and *D*). This result clearly indicates that there is transcriptional regulation among the dehydrogenase genes, and also suggests that RDH10 is a more significant 11-*cis*-retinol dehydrogenase than RDH11 in the RPE.

Discussion

The interaction of 11-*cis*-retinal with visual pigment initiates the signal for visual perception, whereas retinoid recycling maintains it (28). Our data reveal that, apart from Rdh5 and Rdh11, Rdh10 also participates in the production of 11-*cis*-retinal in the RPE. Our 11-*cis*-retinal recovery kinetic experiments provide evidence that Rdh10 complements Rdh5 as the primary dehydrogenase in the mouse visual system. Similar to what is found in *Rdh5*$^{-/-}$ mice, normal retinal morphology was observed in *Rdh10* cKO mice. Lack of *RDH5* in humans causes fundus albipunctatus disease, whereas in mice, loss of either *Rdh5* or *Rdh10* does not induce a retinal disease phenotype, indicating a milder effect on visual function in mice. Although double knock-out of *Rdh10* and *Rdh5* in mice caused a severe delay in 11-*cis*-retinal recovery, complete 11-*cis*-retinal regeneration was noted after 24 h of dark adaption. This indicates that visual chromophore regeneration is provided by multiple enzymes acting in a compensatory fashion.

RDH10 Has 11-cis-Retinol Dehydrogenase Activity—We demonstrated the enzymatic activity of the microsomal fraction of Sf9 cells overexpressing RDH10 protein. RDH10 was found to be a strictly NAD^+-specific enzyme with dual specificity for *trans*- and *cis*-retinol, consistent with earlier findings (18). In cells, the concentration of NAD^+ is higher than that of $NADP^+$, which could cause Rdh10 to evolve its strict specificity toward NAD^+ (29, 30). Through homology modeling, Belyaeva *et al.* (18) proposed a steric hindrance for $NADP^+$ but not NAD^+ in the cofactor-binding pocket of Rdh10. Although RDH10 catalyzed the conversion of 11-*cis*-retinol to 11-*cis*-retinal, its specific activity with both all-*trans*-retinol and 9-*cis*-retinol was higher. This indicates that Rdh10 acts in some unidentified pathway of the visual cycle to regenerate chromophore. One such pathway could involve the retinal G-protein-coupled receptor protein (Rgr). Involved in the cellular transport of 11-*cis*-retinal, Rgr is a signaling receptor coupled to a G-protein localized to the intracellular compartments of RPE and Müller cells (31–33). The isomerase hydrolase pathway regenerates 11-*cis*-retinal in the dark through the Rpe65 enzyme (34, 35). However, under continuous lighting, Rgr can contribute to the regeneration of 11-*cis*-retinal from all-*trans*-retinal in the RPE (36). Rdh10 also could oxidize all-*trans*-retinol to all-*trans*-retinal, which would further prime Rgr for 11-*cis*-retinal production.

Loss of Rdh10 Does Not Cause Retinal Degeneration in Mice—No obvious retinal structural abnormalities were found in *Rdh10* cKO mice. These mice also exhibited normal retinal function, suggesting a redundancy in the retinoid metabolic pathway. Retinoid regulation is the essential function of retinoid dehydrogenase enzymes. In dark-adapted mice, loss of Rdh10 in the RPE did not affect retinoid content. However, *cDKO* and *Rdh5*$^{-/-}$ mice displayed increased amounts of 11/13-*cis*-retinyl esters as compared with *Rdh10*$^{f/f}$ control mice. One hypothesis for this finding is that Rdh5 interacts with Rpe65 to modulate its expression (37). In *Rdh5*$^{-/-}$ mice, free Rpe65 accelerates isomerase activity, producing more 11-*cis*-retinol, which in turn is converted by Lrat to 11-*cis*-retinyl esters, and this results in the increased 11/13-*cis*-retinyl ester accumulation (24). All-*trans*-retinyl esters are aberrantly converted to 13-*cis*-retinol and then to 13-*cis*-retinyl esters by Lrat in the absence of Rdh5 (38). In contrast, there could be a decrease in Rpe65 activity in WT mice possessing Rdh5 activity but lacking accumulation of 11/13-*cis*-retinyl esters.

Intense illumination of 10,000 lux light for 3 min drastically decreases the amount of 11-*cis*-retinal in the eyes of mice. This loss of 11-*cis*-retinal content in *Rdh10* cKO and *Rdh5*$^{-/-}$ mice is the cause of the delayed recovery of visual function measured by electroretinogram that correlated quite well with their delayed dark adaption. This recovery was slower in *Rdh10* cKO mice than in *Rdh10*$^{f/f}$ mice used here as controls instead of WT mice, but moderate relative to the severe delay noted in *Rdh5*$^{-/-}$ mice. This indicates a role of Rdh10 in retinoid recycling. After 3 min of intense light exposure, 5–10% of chromophore still remained in the eyes of *Rdh10* cKO mice, similar to the intermediate range of 2–12% observed in patients with the *Rdh5* mutation (39). Recovery of ocular 11-*cis*-retinal was faster in *Rdh10* cKO than in *Rdh5*$^{-/-}$ mice, which again illustrates the complex interactions of retinol dehydrogenase enzymes. Our data demonstrate that Rdh5 is the principle enzyme and Rdh10 acts as a complimentary enzyme (10, 17). Although up-regulation of only *Rdh10* in *Rdh5*$^{-/-}$ mice suggests a more dominant role for Rdh10 than Rdh11, Rdh10 plays only a mild complementary role in 11-*cis*-retinal production (11).

Compensatory Regulation of 11-cis-Retinal—The normal retinal morphology of *Rdh10* cKO and *cDKO* mice, as well as their complete recovery of 11-*cis*-retinal after dark adaptation, indicates compensatory regulation of the many dehydrogenases responsible for 11-*cis*-retinal production. Interconversions of various retinol isomers, retinal, and retinoic acid to active retinoids are mediated by three distinct families of retinol dehydrogenases: alcohol dehydrogenases (ADHs), SDRs, and aldehyde dehydrogenases (ALDHs). The mouse genome only encodes class I, II, III, and IV alcohol dehydrogenase genes, whereas the human genome contains all five classes (40, 41). The expressed sequence tag in mice revealed the presence of Adh1 and Adh4 activity in the RPE. ADH1 and ADH4 catalyze the conversion of 11-*cis*-retinol to 11-*cis*-retinal (42, 43). In mammals, ADH is a cytosolic NAD^+-dependent enzyme (43). However, the cytosolic protein fraction of bovine RPE contains too little oxidative activity to explain 11-*cis*-retinal regeneration in *Rdh5*$^{-/-}$ mice. Instead, short-chain dehydrogenase enzymes catalyzing *trans*- or *cis*-retinol oxidation and containing about 250 residues are located in membrane fractions. There are at least 17 different SDR proteins identified in humans, rats, and mice that catalyze retinoid reactions (44). Most murine SDRs (CRAD1, CRAD2, CRAD3, RDH1) are expressed in the eye and require *cis*-retinoids as substrates. One or more of these could participate in regenerating 11-*cis*-retinal in *cDKO* mice (45–48). Possible alternative enzymes that utilize NAD^+ could be located in the

membrane fraction of *Rdh5*$^{-/-}$ mice (10, 49). Therefore, apart from Rdh10, another NAD$^+$-specific enzyme could produce chromophore. SDR family 9 (Dhrs9) is a dual substrate-specific enzyme that could be an additional candidate for this regeneration (50). In yet another interesting observation, we found increased expression of *Rdh5* in *Rdh10* cKO mice as well as the reverse. This indicates that chromophore production is due to up-regulation of remaining dehydrogenase genes that compensate for the deleted dehydrogenase function in the RPE. In contrast, dysfunction of Cralbp (26, 27), Rgr (33, 51), and Rdh11 (52) can cause retinal dystrophy in humans (53), whereas mouse models with deletions of these genes display normal retinal morphology. However, these mutated mice still demonstrate delayed dark adaption after intense light exposure. Here the *Rdh10* cKO mouse also displayed delayed dark adaption after intense light exposure despite having normal retinal morphology at 6 months of age. Results with the *Rdh10* cKO mouse also suggest a complex regulation of visual chromophore regeneration. Compensatory mechanisms of gene expression in *Rdh*-deleted mouse models open up the novel therapeutic possibility of augmenting the expression of an orthologous gene to prevent disease progression in inherited retinal diseases with RDH abnormalities (9, 23). Such diseases in humans include fundus albipunctatus and cone dystrophy caused by RDH5 mutations, and both Leber congenital amaurosis and retinitis pigmentosa due to RDH12 mutations.

Collectively, the present study reveals that RPE-specific deletion of *Rdh10* does not suffice to cause retinal degeneration in mice, and that RDH10 is a secondary enzyme in the production of 11-*cis*-retinal in the RPE.

Author Contributions—B. S., A. M., and K. P. conceived and designed the experiments. B. S., W. S., L. P., and V. P. conducted experiments and acquired data. B. S., W. S., L. P., V. P., Y. Z. L., M. D. G., K. P, and A. M. analyzed and interpreted the data. B. S., L. P., K. P., and A. M wrote the manuscript. All authors commented on the manuscript and approved the final version.

Acknowledgments—We thank Drs. Vladimir Kefalov (Washington University) and Leslie T. Webster, Jr., Tanu Parmar, and Eisuke Arai (Case Western Reserve University) for their comments.

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