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

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* Running title: RDH10 is involved in 11-cis-retinal regeneration

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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.

ABSTRACT

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 knockout of Rdh10 in RPE cells
(Rdh10 cKO) displayed delayed 11-cis-retinal regeneration and dark adaption after bright
light illumination. Retinal function measured by ERG 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 when compared to Rdh5-/- mice. Normal retinal morphology was observed in 6-
month-old Rdh10 cKO and cDKO mice suggesting 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 photoisomerised to...
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1all-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) family, retinol dehydrogenase 12 (RDH12) and RDH8, present respectively in the inner and outer segments of the retina (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 then isomerized and hydrolyzed by retinal pigment epithelium-specific protein 65kDa (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 approximately 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 display delayed dark adaption kinetics. Rdh5−/− mice can still produce 11-cis-retinal, albeit at a slower rate than WT mice. Haeselee F. et al. 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 (11). 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 a 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 B.X. et al. reported that RDH10 is an efficient all-trans-RDH with NADP+ as its preferred cofactor (13). However, Belyaeva O.D. et al. demonstrated that RDH10 is an efficient 11-cis-RDH with NAD+ as its preferred cofactor (18). 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 knockout mouse model to study the role of Rdh10 in the visual cycle. Our data provide evidence that Rdh10 plays a

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1Abbreviations used are: RPE, retinal pigmented epithelium; Rdh10 cKO, Rdh10 retinal pigmented epithelium specific knockout; cDKO, RPE specific knockout of Rdh10 and global knockout of Rdh5; ERG, electroretinogram; qRT-PCR, quantitative RT-PCR; RDH, retinol dehydrogenase; Rpe65, retinal pigment epithelium-specific 65-kDa protein; ONL, outer nuclear layer.
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complementary role in regenerating 11-cis-retinal in mice.

**EXPERIMENTAL PROCEDURES**

**Animals**- RPE-specific Rdh10 knockout genotype (rtTA-cre+/cKO, Rdh10 ($\delta$) (Rdh10 cKO)) mice were generated by crossing Rdh10/$\delta$ mice (19) with rtTA-cre+/cKO mice (20). Doxycycline (100 µg/g 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 knockout of Rdh5 and RPE-specific knockout 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 Leu450 of Rpe65 were purchased from Taconic (Hudson, NY). Genotyping of Rdh10 cKO mice was accomplished with a previous protocol and primer sets (19). rtTA-cre+/cKO mice also were genotyped by a former protocol (20). Littermates of Rdh10 cKO 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, catalog 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, Pleasanton, CA). Gapdh gene expression was used to normalize the relative expression of genes. Primer sequences for mice were: mRdh10-F1, 5’-gtgaacttggaagagagag-3’ and mRdh10-R1, 5’-tggaagaagagccctgtaag-3’; mRdh5-F, 5’-tttttctacacctctgtagac-3’ and mRdh5-R, 5’-tggctcacagatcggtagct-3’; mRdh11-F, 5’-tttgctagagatcaggttca-3’; mRdh7-F, 5’-actgcagacggctttgagat-3 and mRdh11-R, 5’-ttatggaatgggtgctagcc-3’; mCrabp, 5’-gaacgcaaagccttagtgg-3’; mRdh5-F, 5’-ccagttacagatcaggttca-3’ and mRdh5-R, 5’-ccgtgaagaagacccctgtaag-3’; mSDRpe65-F, 5’-ccagttacagatcaggttca-3’ and mSDRpe65-R, 5’-agtcctagGGgtgctagcc-3’; mLrat-F, 5’-tggcttcctgatggctagcc-3’ and mLrat-R, 5’-caggtgacagaggggtcat-3’; mZo1-F, 5’-aaagcagagaggactgtcagc-3’ and mZo1-R, 5’-ggtctctetccactgtaa-3’; mSDRhod-F, 5’-agtcctagGGgtgctagcc-3’ and mSDRhod-R, 5’-ggagacaactctgctcag-3’; mSDRhod-F, 5’-ggtctctgatgtgctgctac-3’ and mSDRhod-R, 5’-acagtctgtgagcctag-3’; mSDRhod-F, 5’-ggtctctgatgtgctgctac-3’ and mSDRhod-R, 5’-acagtctgtgagcctag-3’; mMcone2R, 5’-ttatggaatgggtgctagcc-3’ and mMcone2R, 5’-ttatggaatgggtgctagcc-3’; mScone-F, 5’-tttggctagagatcaggttca-3’ and mScone-R, 5’-tttggctagagatcaggttca-3’.

**Histology**- Retinal histology and immunohistochemistry were performed as previously described (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. PNA was used for cone staining. Biotinylated peanut agglutinin (PNA) was purchased from Vectorlabs (Burlingame, CA) (Catalog no. B-1075) (dilution 1:400). Secondary antibody Streptavidin Alexa 488 conjugate was obtained from Life technologies (Grand island, NY) (Catalog no. 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. (catalog no. ab80891)(Kendall Square, MA)(dilution 1:200). Mouse monoclonal antibody for β-actin was purchased from Santa Cruz Biotechnology, Inc. (Paso Robles, CA) (catalog no. sc-81178)(dilution 1:1000).

Retinoid analyses- Eyes were extracted from dark-adapted and light-exposed mice and retinoids were extracted as previously described (21,22). Briefly, eyes were homogenized in a glass tissue grinder in 1.2 ml of buffer (50 mM MOPS, 10 mM NH₂OH 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 3x250 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).

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 mostly in the microsomal fraction of Sf9 cells infected with recombinant RDH10, but not in uninfected Sf9 cells (Fig. 1A). 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 then was 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 were then used to evaluate RDH10 enzymatic activity. Analyses by HPLC showed that in presence of NAD+, RDH10 enzymatic activity. Analyses by HPLC 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. 1B, C and 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, even though its in vivo role there is yet unknown. Indeed, global knockout 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 knockout in mice, RPE-specific deletion of 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 then was evaluated in the RPE two weeks afterwards (Fig. 2A). The relative Rdh10 mRNA amount was reduced more than 80% in Rdh10 cKO mice as compared to control Rdh10$^{f/f}$ mice when checked by qRT-PCR (Fig. 2B). Immunoblotting analyses indicated an 85% decreased Rdh10 expression in Rdh10 cKO mice relative to that in control Rdh10$^{f/f}$ mice (Fig. 2C). Loss of Rdh10 expression in the RPE of Rdh10 cKO mice was further confirmed by immunohistochemistry (Fig. 2D). 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. Since 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-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. 3A). Also there was no change in 11/13cis-retinyl ester content (Fig. 3B) and all-trans-retinyl ester content (Fig. 3C) 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 knockout mice (cDKO) by breeding Rdh10 cKO with Rdh5$^{f/f}$ mice, employing Rdh5$^{f/f}$ and Rdh5$^{f/f}$Rdh11$^{f/f}$ mice for comparison (10,12). Amounts of retinoids were measured 24 h after dark adaption. No significant differences were observed in 11-cis-retinal content between dark-adapted cDKO, Rdh5$^{f/f}$Rdh11$^{f/f}$, Rdh5$^{f/f}$ and WT mice (Fig. 3A). 11/13-cis-retinyl esters were found elevated in cDKO, Rdh5$^{f/f}$Rdh11$^{f/f}$ and Rdh5$^{f/f}$Rdh11$^{f/f}$ mice as compared with WT mice (Fig. 3B). The amounts of 11/13-cis-retinyl esters in cDKO mice were significantly (p < 0.005) increased as compared with those in Rdh5$^{f/f}$ mice and matched the amounts found in Rdh5$^{f/f}$Rdh11$^{f/f}$ mice. Reduced amounts of all-trans-retinyl esters were found in Rdh5$^{f/f}$ mice as compared with WT mice (Fig. 3C). But the amounts of 11/13-cis-retinyl esters in cDKO mice were significantly (p < 0.005) increased as compared with those in Rdh5$^{f/f}$ mice and matched the amounts found in Rdh5$^{f/f}$Rdh11$^{f/f}$ mice. Reduced amounts of all-trans-retinyl esters were found in Rdh5$^{f/f}$ mice as compared with WT mice (Fig. 3C). These results support a complementary role for RDH10 in the visual cycle and reveal a possible redundancy of RDHs in the RPE.

**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 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. 4A) failing 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 Rdh10f/f mice used as WT controls for this experiment.

To further probe the delay in 11-cis-retinal kinetics, we determined if intense light exposure (2000 cd/m²) for 3 min would affect the a-wave recovery of Rdh10 cKO mice with Rdh10f/f mice used as controls. Mice were kept 24 h in dark prior to this experiment. After light exposure, a-wave recovery, normalized to the a-wave before illumination, was analyzed by ERG every 10 min up to 1 h in the dark. A significant delay (p < 0.05) in a-wave recovery was observed in Rdh10 cKO mice as compared with controls (Fig. 4B). 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). Since experiments measuring retinoid kinetics in Rdh10 cKO mice revealed delayed 11-cis-retinal recovery (Fig. 4A, 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 Rdh10f/f mice at 6 months of age, the ONL thickness of Rdh10 cKO mice was found comparable to that of Rdh10f/f mice (Fig. 5A). Moreover, retinal thickness as a measure of global retinal morphology was also similar in these mice (Fig. 5B). 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 (PNA) which stains S-opsin and M-opsin cones. Transcript levels of S-opsin, M-opsin and rhodopsin also were comparable among Rdh10 cKO and Rdh10f/f mice (Fig. 5C). Moreover, Rdh10 cKO mice had no change in cone density relative to control mice at this age (Fig. 5D). 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. 4A, 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 Rdh10f/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. 6A, B), suggesting a redundancy in RDH genes. 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 Rdh10f/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 compared to Rdh10f/f mice at 6 months of age (Fig. 7A). 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. 7B). 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-regulated in Rdh5<sup>-/-</sup> mice at both 6 and 12 months age, but Rdh11 expression was not (Fig. 7C, 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<sup>-/-</sup> 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. Though double knockout 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 over-expressing RDH10 protein. RDH10 was found to be a strictly NAD<sup>+</sup>-specific enzyme with dual specificity for trans- and cis-retinol, consistent with earlier findings (18). In cells, the concentration of NAD<sup>+</sup> is higher than that of NADP<sup>+</sup> which could cause Rdh10 to evolve its strict specificity towards NAD<sup>+</sup> (29,30). Homology modeling by Belyaeva O.D. et al. proposed a steric hindrance for NADP<sup>+</sup> but not NAD<sup>+</sup> in the cofactor-binding pocket of Rdh10 (18). Though 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 hydrofase pathway regenerates 11-cis-retinal in the dark through the Rpe65 enzyme (34,35). But 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-retinol 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<sup>-/-</sup> mice displayed increased amounts of 11/13-cis-retinyl esters when compared with Rdh10<sup>+/+</sup> control mice. One hypothesis for this finding is that Rdh5 interacts with Rpe65 to modulate its expression (37). In Rdh5<sup>-/-</sup> 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-retinol content in Rdh10 cKO, Rdh5<sup>-/-</sup> mice is the cause of the delayed
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recovery of visual function measured by ERG that correlated quite well with their delayed dark adaptation. This recovery was slower in Rdh10 cKO mice than in Rdh10\(^{wt}\) mice used here as controls instead of WT mice, but moderate relative to the severe delay noted in Rdh5\(^{-/-}\) mice. This indicates a role for 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). Even though 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 and 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), short-chain dehydrogenase/reductases (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 5 classes (40,41). The expressed sequence tag (EST) 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 indentified in humans, rats, and mice which 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. Short chain dehydrogenase/reductase (SDR family) 9 (Dhrs9) is a dual substrate specific enzyme which 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.
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Author contributions: B.S., A.M. and K.P. conceived and designed the experiments. B.S., W.Y., L.P., and V.P. conducted experiments and acquired data. B.S., W.Y., L.P., and 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.

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Figure legend

**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 NADP⁺ as a cofactor. Production of 11-cis-retinal was examined after the addition of 45 μM 11-cis-retinol to the reaction at 37 °C. (B). Similarly, production of 9-cis-retinal (C) or all-trans-retinal (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.

**Figure 2.** Loss of Rdh10 expression in the RPE of rtTA-cre⁺⁻, Rdh10⁰⁰⁰ (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⁺⁺), heterozygous (Rdh10⁺⁻) and homozygous (Rdh10⁰⁰) mice. (B) qRT-PCR was performed for Rdh10 with Rdh10 cKO and Rdh10⁰⁰ isolated mouse RPE tissue. Relative expression levels are presented. Error bars indicate SDs. (n=5). *p < 0.005 Rdh10 cKO versus Rdh10⁰⁰. Data were normalized against the housekeeping gene, Gapdh. (C) Expression levels of Rdh10 in Rdh10 cKO and Rdh10⁰⁰ mice were determined by immunoblotting. RPE tissues were isolated from Rdh10 cKO and Rdh10⁰⁰ 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 Rdh100/0 mice. (D) Immunohistochemistry was performed with retinas from Rdh10 cKO and Rdh100/0 mice using Rdh10 polyclonal antibody. Loss of expression of Rdh10 in the RPE can be seen in the retina of a Rdh10 cKO mouse. Scale bar 50 μm.

**Figure 3. Retinoid content in dark-adapted mice lacking retinal dehydrogenases in the eye.** Mice (6-weeks-old) were kept in the dark for 24 h and their 11-cis-retinal (A), 11/13-cis-retinyl esters (B) and all-trans-retinyl esters (C) were quantified by HPLC. Bars indicate SDs, n=5. *p < 0.005.

**Figure 4. 11-cis-Retinal and a-wave recovery kinetics in dark-adapted Rdh10 cKO mice.** (A) Rdh10 cKO, Rdh5-/- and Rdh100/0 mice were dark adapted for 24 h followed by a 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) ERG evaluation of a-wave recovery was performed after exposing dark adapted Rdh10 cKO, Rdh5-/- and Rdh100/0 mice to light at 2000 cd/m² for 3 min. Bars indicate SDs. (n=5). *p < 0.05 Rdh10 cKO , Rdh5-/- vs Rdh100/0 mice.

**Figure 5. Analysis of retinal histology and rod and cone opsin expression in Rdh10 cKO and Rdh100/0 mice.** (A) Cryosections of Rdh10 cKO and Rdh100/0 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 Rdh100/0 retinas (n=5). (B) Paraffin sections of retinas from Rdh10 cKO and Rdh100/0 6-month-old mice were compared. (C) qRT-PCR was performed in Rdh10 cKO and Rdh100/0 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, Rdh100/0 and WT 6-month-old mouse retinas were stained with 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 Rdh100/0 mice.** (A) ONL thicknesses of cDKO, Rdh5-/- and Rdh100/0 6-month-old mice were measured; there was no significant difference in ONL thickness among these retinas (n=5). (B) When paraffin sections of retinas from cDKO, Rdh5-/- and Rdh100/0 6-month-old mice were compared, there were no significant changes in retinal morphology among them. Scale bar 50 μm.

**Figure 7. RPE specific gene expression in Rdh10 cKO and Rdh5-/- mice.** (A) The RPE of Rdh10 cKO and Rdh100/0 mice (6-month-old) and (B) (12-month-old) were isolated and qRT-PCR was performed to quantify RPE-specific gene expression. (C) The RPE of Rdh5-/- mice (6-month-old) and (D) (12-month-old) were isolated and Rdh5, Rdh10 and Rdh11 expression was evaluated by qRT-PCR. Error bars indicate SDs. (n=5). Data were normalized with the housekeeping gene, Gapdh. A significant difference is indicated with a star (*). *(p < 0.05, n=5)
RDH10 is involved in 11-cis-retinal regeneration

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 is involved in 11-cis-retinal regeneration

Figure 1

A) Western blot analysis showing the protein bands at 43kDa and 34kDa. M: molecular weight markers, Mock: mock sample, Supernatant: supernatant sample. Arrows indicate the position of RDH10.

B) Graph showing the nmoles of 11-cis-RAL per mg protein over time (hour) with '11cRAL (+NAD^+)' and '11cRAL (+NADP^+)' plotted.

C) Graph showing the nmoles of 9-cis-RAL per mg protein over time (min) with '9cRAL (+NAD^+)' and '9cRAL (+NADP^+)' plotted.

D) Graph showing the nmoles of all-trans-RAL per mg protein over time (min) with 'atRAL (+NAD^+)' and 'atRAL (+NADP^+)' plotted.
RDH10 is involved in 11-cis-retinal regeneration

Figure 2

A

DNA Ladder
Rdh10\textsuperscript{f/f}
Rdh10\textsuperscript{cKO}
Rdh10\textsuperscript{f/f}

B

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2b.png}
\caption{Relative mRNA expression of Rdh10.}
\end{figure}

C

Rdh10\textsuperscript{cKO}
Rdh10\textsuperscript{f/f}

D

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2d.png}
\caption{Immunostaining for Rdh10 and \(\beta\)-Actin.}
\end{figure}
Figure 3

RDH10 is involved in 11-cis-retinal regeneration

A

11cRAL (pmol/eye)

Rdh10 cKO  |  Rdh10 Δ/Δ  |  cDKO  |  Rdh5 Δ/Rdh11 Δ  |  Rdh5 Δ  |  WT

B

11/13cRE (pmol/eye)

Rdh10 cKO  |  Rdh10 Δ/Δ  |  cDKO  |  Rdh5 Δ/Rdh11 Δ  |  Rdh5 Δ  |  WT

C

atRE (pmol/eye)

Rdh10 cKO  |  Rdh10 Δ/Δ  |  cDKO  |  Rdh5 Δ/Rdh11 Δ  |  Rdh5 Δ  |  WT

* Denotes statistically significant differences.
RDH10 is involved in 11-cis-retinal regeneration

Figure 4

A

B

11-cis-RAL (pmol/eye)

Recovery of a-wave amplitude (%)

Time after photobleaching (hour)

Time (min)

Rdh10 cKO
Rdh5-/-
Rdh10f/f

Rdh10 cKO
Rdh5-/-
Rdh10f/f
RDH10 is involved in 11-cis-retinal regeneration

Figure 5

A

- RDH10 is involved in 11-cis-retinal regeneration

B

- RDH10 is involved in 11-cis-retinal regeneration

C

- RDH10 is involved in 11-cis-retinal regeneration

D

- RDH10 is involved in 11-cis-retinal regeneration
Figure 6

RDH10 is involved in 11-cis-retinal regeneration
RDH10 is involved in 11-cis-retinal regeneration

Figure 7

A  6-month-old

|       | Rdh10 cKO | Rdh10f/f |
|-------|-----------|----------|
| Rdh5  |           |          |
| Rdh11 |           |          |
| Crelp |           |          |
| Rpe65 |           |          |
| Lrat  |           |          |
| Zb-1  |           |          |

B  12-month-old

|       | Rdh10 cKO | Rdh10f/f |
|-------|-----------|----------|
| Rdh5  |           |          |
| Rdh11 |           |          |
| Crelp |           |          |
| Rpe65 |           |          |
| Lrat  |           |          |
| Zb-1  |           |          |

C  6-month-old

|       | Rh5/−− | Rh10f/f |
|-------|--------|--------|
| Rdh5  |        |        |
| Rdh10 |        |        |
| Rdh11 |        |        |

D  12-month-old

|       | Rh5/−− | Rh10f/f |
|-------|--------|--------|
| Rdh5  |        |        |
| Rdh10 |        |        |
| Rdh11 |        |        |
Conditional Ablation of Retinol Dehydrogenase 10 in the Retinal Pigmented Epithelium Causes Delayed Dark Adaption in Mice
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