Effects of deficiency in the \textit{RLBP1}-encoded visual cycle protein CRALBP on visual dysfunction in humans and mice

Jose Ronaldo Lima de Carvalho Jr\textsuperscript{1,2}, Hye Jin Kim\textsuperscript{1}, Keiko Ueda\textsuperscript{1}, Jin Zhao\textsuperscript{1}, Aaron P. Owji\textsuperscript{3}, Tingting Yang\textsuperscript{1}, Stephen H. Tsang\textsuperscript{1,2,4}, and Janet R. Sparrow\textsuperscript{1,4 §}

From the \textsuperscript{1}Department of Ophthalmology, Columbia University Irving Medical Center, New York, NY, United States; \textsuperscript{2}Department of Ophthalmology, Jonas Children’s Vision Care, Columbia University Irving Medical Center, New York, NY, United States; \textsuperscript{3}Department of Pharmacology, Columbia University Irving Medical Center, New York, NY, United States; \textsuperscript{4}Department of Pathology and Cell Biology, Columbia University Irving Medical Center, New York, NY, United States

Running title: \textit{RLBP1}/CRALBP Gene Deficiency

§To whom correspondence should be addressed: Janet R. Sparrow, Department of Ophthalmology, Columbia University, 635 W. 165th Street New York, NY 10032 USA; jrs88@columbia.edu; Tel. (212)-305-9944.

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Mutations in \textit{retinaldehyde binding protein 1} (\textit{RLBP1}), encoding the visual cycle protein \textit{cellular retinaldehyde-binding protein} (CRALBP), cause an autosomal recessive form of retinal degeneration. By binding to 11-cis-retinoid, CRALBP augments the isomerase activity of retinoid isomerohydrolase RPE65 (RPE65) and facilitates 11-cis-retinol oxidation to 11-cis-retinal. CRALBP also maintains the 11-cis configuration and protects against unwanted retinaldehyde activity. Studying a sibling pair that are compound heterozygous for mutations in \textit{RLBP1}/CRALBP, here we expand the phenotype of affected individuals, elucidate a previously unreported phenotype in \textit{RLBP1}/CRALBP carriers, and demonstrate consistencies between the affected individuals and \textit{Rlbp}/\textit{Cralbp}\textsuperscript{-/-} mice. In the \textit{RLBP1}/CRALBP-affected individuals, non-recordable rod-specific electroretinogram traces recovered after prolonged dark adaptation. In ultrawide-field fundus images, we observed radially arranged puncta typical of \textit{RLBP1}/CRALBP-associated disease. Spectral domain optical coherence tomography (SD-OCT) revealed hyperreflective aberrations within photoreceptor-associated bands. In short-wavelength fundus autofluorescence (SW-AF) images, speckled hyperautofluorescence and mottling indicated macular involvement. In both the affected individuals and their asymptomatic carrier parents, reduced SW-AF intensities, measured as quantitative fundus autofluorescence (qAF), indicated chronic impairment in 11-cis-retinal availability and provided information on mutation severity. Hypertension of SD-OCT signal into the choroid together with decreased near-infrared AF (NIR-AF) provided evidence for retinal pigment epithelial cell (RPE) involvement. In \textit{Rlbp}/\textit{Cralbp}\textsuperscript{-/-} mice, reduced 11-cis-retinal levels, qAF and NIR-AF intensities, and photoreceptor loss were consistent with the clinical presentation of the affected siblings. These findings indicate that \textit{RLBP1} mutations are associated with progressive disease involving RPE atrophy and photoreceptor cell degeneration. In asymptomatic carriers, qAF disclosed previously undetected visual cycle deficiency.

Vision is initiated by the absorption of a photon of light by the opsin chromophore 11-cis-retinaldehyde in the outer segments of rod and cone photoreceptor cells. This photon capture causes isomerization of 11-cis-retinaldehyde to the bleached product all-trans-retinaldehyde. For continued light
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detection, a supply of 11-cis-retinal is provided to rod and cone photoreceptor cells by a multi-step enzyme pathway, the visual cycle (retinoid cycle), that reconverts all-trans-retinal to light sensitive 11-cis-retinal. Cellular retinaldehyde-binding protein (CRALBP), a 36-kDA aqueous soluble carrier, participates in this process (1). CRALBP is expressed in abundance by both retinal pigment epithelial (RPE) and Muller cells of retina and is important for rod- and cone-driven vision (2).

In humans, mutations in retinaldehyde-binding protein 1 (RLBP1), the gene encoding CRALBP, cause autosomal recessive retinal diseases with phenotypes described as retinitis punctata albescens (RPA), autosomal recessive retinitis pigmentosa (arRP), Bothnia dystrophy and Newfoundland rod–cone dystrophy (2-9). The variable presentation likely reflects, at least in part, the effect of particular mutations on protein structure and function (8). The RLBP1/CRALBP phenotype is characterized by varying degrees of early onset delayed dark adaptation, abnormal electroretinographic (ERG) responses, loss of peripheral vision, and progressive macular atrophy (10,11). Also characteristic of CRALBP-deficiency is the presence of white dot-like aberrations within the fundus (11,12); these puncta are visible in color fundus and short-wavelength fundus autofluorescence (SW-AF) images (11,12). RPE atrophy is accompanied by hypertransmission of spectral domain optical coherence tomography (SD-OCT) signal into the choroid (10). It is estimated that RLBP1-associated disease accounts for 0.5% of all RP cases (13), a condition which has a prevalence of 1 in 4000 in developed countries (14).

CRALBP in RPE cells participates in supplying 11-cis-retinal to both rods and cones while cones also rely on 11-cis chromophore present in Muller cells (15-17). CRALBP performs multiple functions in the visual cycle. First, by noncovalently binding 11-cis-retinol as it is produced by the isomerase RPE65, CRALBP reduces product inhibition of the isomerase activity (18,19) thereby ensuring efficient production of 11-cis-retinol. CRALBP can bind both 11-cis-retinol and 11-cis-retinal (16) thereby facilitating the oxidation of protein bound 11-cis-retinol to 11-cis-retinal, catalyzed by retinol dehydrogenase 5 (RDH5) (16,20). Accordingly, delayed dark adaptation is exhibited by human patients carrying a mutation of the RLBP1/CRALBP gene and by Cralbp–/– mice (1,2,9). Additionally, the hydrophobic pocket of CRALBP imposes a conformational strain on 11-cis-retinal and thus maintains the isomeric state of 11-cis-retinal even in the presence of light (21-24). As a retinaldehyde carrier CRALBP also protects against unwanted aldehyde toxicity (16).

Here we report findings in two siblings with early-onset retinal disorders who were compound heterozygous for two pathogenic mutations in the RLBP1 gene. Previously unreported features of RLBP1/CRALBP disease in the affected patients are presented and replication in Cralbp–/– mice is demonstrated. Importantly, to the best of our knowledge, this is the first work to show a phenotype in asymptomatic human carriers of RLBP1/CRALBP mutations.

Results

RLBP1/CRALBP-associated disease in affected human patients

A family with two clinically affected female siblings (Fig. 1), a 15-year-old (II-1) and a 13-year-old (II-2), presented to the Department of Ophthalmology at Columbia University Irving Medical Center with a complaint of photophobia and poor night vision over the prior year. The younger brother (II-3) and the parents (I-1 and I-2) were asymptomatic. Whole exome sequencing of DNA obtained from peripheral blood revealed that both children were compound heterozygous for the missense mutation c.25C>T:p.Arg9Cys and c.286_297:p.Phe96_99 deletion in the RLBP1 gene and diagnosis of RPA was made. These mutations have been reported previously (11,25). Concurrent genetic analyses of the parents revealed that the father is heterozygous.

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for the c.25C>T:p.Arg9Cys mutation and the mother is heterozygous for the c.286_297:p.Phe96_99del variant (Fig. 1). Clinical and genetic characteristics of the patients and parents are summarized in Table 1.

**Full-field electroretinogram (ffERG)**

ffERGs were performed in both scotopic and photopic states. Standard dark-adapted (30 min) rod-specific ERGs were extinguished in both daughters (II-1 and II-2; Fig. 2, top rows). Light-adapted 30-Hz flicker and single-flash were within the normal range (Fig. 2, middle rows). Interestingly, after prolonged dark-adaptation (>8h overnight eye-patch), the rod responses measured by ERG increased to the normal range (Fig. 2, bottom rows). The young brother (II-3) had normal ERG results (not shown).

**Fundus imaging**

In ultrawide-field (UWF) pseudo-color fundus images (200°), the patients exhibited radially arranged white spots that were more visible in peripheral retina (Fig. 3A and B). The white spots were also more pronounced in the older sibling. UWF-autofluorescence/green (UWF-AF/green) images using 532 nm excitation provided images that were largely grainy but the optic disk and retinal vessels were visible (Fig. 3C and D). The fovea was detected as a small dark zone with surrounding speckled brightness. The horizontally oriented oval-shaped central zone of increased brightness that in healthy eyes envelopes the macula and optic disk is typical of UWF-AF/green fundus images (26) was less pronounced.

In both siblings, SW-AF (blue) images acquired with 488 nm excitation presented with typical central hypoautofluorescence but contrasting foveal darkness was not evident (Fig. 3E and G). Outside the zone of reduced autofluorescence was a ring of speckled autofluorescence that at greater eccentricity transitioned into faintly autofluorescent macular spots and mottling.

Near-infrared autofluorescence (NIR-AF) fundus images obtained from both children presented with the central elevated intensity that is characteristic of healthy eyes and that is attributable to increased melanin optical density (Fig. 3F and H). Hypoautofluorescent dots surrounded by areas of apparently normal fluorescence were present in the parafoveal region.

In SD-OCT scans, hyperreflective aberrations were observed in II-1 and II-2 (Fig. 3I and K, J and L). These changes were characterized by undulations and discontinuities of the ellipsoid zone (yellow arrows), hyperreflective irregularities in the interdigitation zone (white arrows) and foci of hypertransmission into the choroid (white asterisks). These abnormalities were observed in both siblings and were more pronounced in non-foveal macula. In both patients the external limiting membrane (ELM) was continuous (Fig. 3K and L).

**Quantitation of fundus images in affected patients and carrier parents**

SW-AF originates from bisretinoid by-products of the visual cycle. To assess SW-AF intensities, the quantitative fundus autofluorescence (qAF) approach was used to measure intensities in the two patients and their parents. In the two RLBPI/CRALBP-affected siblings, qAF intensities within the concentric segments positioned at an eccentricity of 7°-9° were not measurable. This profound and uniform reduction in qAF was evident in qAF color-coded images (Fig. 3M and N) when comparison was made to a healthy control eye of similar age (Fig. 3O).

Importantly, although the carrier parents did not present with visual symptoms and SW-AF, NIR-AF and SD-OCT images were unremarkable (Fig. 4, A-F), in both parents color-coded qAF images revealed lower SW-AF intensities (Fig. 4G and H) than in an age-similar healthy eye (Fig. 4I). The qAF value calculated as the mean of the 8 concentric segments was below the 95% confidence interval of healthy eyes (Fig. 5A). qAF was also lower in the
mother who carried the c.286_297:p.Phe96_99 deletion than in the father who was a heterozygous carrier of the c.25C>T:p.Arg9Cys mutation.

Melanin-derived NIR-AF (27-30) intensities originating primarily in RPE were measured along a horizontal axis through the fovea and were compared with the measurements obtained from a group of 19 healthy subjects that served as control. Interestingly, intensities in the NIR-AF profiles were decreased in both the probands and heterozygous parents (Fig. 5 B); this reduction is indicative of RPE involvement in the disease processes. It is important to note that as with qAF, the reduction in the central NIR-AF signal was more pronounced in the carrier (mother) bearing the c.286_297:p.Phe96_99 deletion than in the carrier (father) harboring the missense mutation c.25C>T:p.Arg9Cys carrier. The affected siblings were compound heterozygous and exhibited a difference in the NIR-AF profiles. Although younger, the 13 year old sibling presented with a greater decrease in NIR-AF. This finding deserves further investigation.

Cralbp<sup>−/−</sup> mice: UPLC measurement of retinoids

CRALBP deficiency does not block the functioning of the visual cycle but is considered to modulate its kinetics (2). Thus to test the efficiency of CRALBP activity, we measured retinoid levels in dark-adapted mice (Fig. 6A and B). To enable extraction efficiency the nucleophile hydroxylamine was used to promote 11-cis-retinal release from the stable retinal oxime derivatives (anti and syn) (λ max, 367 nm) (31). Measurements of 11-cis-retinal in 2 month old dark-adapted wild-type mice were similar to values previously reported (32,33). Meanwhile in Cralbp<sup>−/−</sup> mice, 11-cis-retinal measured as picomoles per eye was significantly lower than in wild-type mice (p < 0.001, ANOVA and Sidak’s multiple comparison test) (Fig. 6C). However, expressed as a fraction of total retinoid there was no significant difference (Fig. 6D), as was previously reported (1).

Amounts of 11-cis-retinol were also lower in the Cralbp<sup>−/−</sup> mice (Fig. 6E).

We also monitored the recovery of retinoid levels following visual pigment bleaching (white light, 8200-900 lux, 3 mins) in dark adapted Rlbp1/Cralbp<sup>−/−</sup> and wild-type mice. With 1 and 2 hours in the dark after photobleaching, there was no evidence of recovery of 11-cis-retinal levels in the Rlbp1/Cralbp<sup>−/−</sup> mice and 11-cis-retinal was significantly lower than in WT mice (Fig. 6F). Conversely, all-trans-retinyl ester amounts were significantly higher at 1 and 2 hours after the photobleach (Fig. 6G). As a percent of dark adapted pre-bleach levels, all-trans-retinal was higher immediately after photobleaching (data not shown).

**Fundus imaging in mice**

In the Cralbp<sup>−/−</sup> and wild-type mice, we imaged in vivo fundus autofluorescence using instrumentation similar to that employed clinically. SW-AF (488 nm) images were uniformly low in intensity and due to poor contrast blood vessels were muted (Fig. 7A). The brightness of the internal fluorescent reference in the qAF images (488 nm) attested to the long exposure time used to generate the fundus AF in these mice. Puncta were not observed in the fundus.

Measurement of SW-AF by qAF revealed that fundus intensities were significantly lower in the Cralbp<sup>−/−</sup> mice as compared to the agouti 129S1/SvImJ mice from age 6 to 18 months (Fig. 7B). qAF in the Cralbp<sup>−/−</sup> mice also declined at age 9 month and was significantly lower at 18 months (p <0.05).

NIR-AF intensities in Cralbp<sup>−/−</sup> mice at 6 and 9 months of age were similar to that of agouti wild-type at age 2, 6 and 9 months (Fig. 7C). Levels then dropped significantly at 12 and 18 months of age. In Cralbp<sup>−/−</sup> mice NIR-AF intensities at 12 and 18 months were significantly lower than in wild-type (Fig. 7C).

**High performance liquid chromatography (HPLC)**

Since SW-AF was reduced in the Cralbp<sup>−/−</sup> mice we also measured the bisretinoid
lipofuscin fluorophores that form as a by-product of the visual cycle and that serve as the source of this SW-AF emission. A representative HPLC chromatogram in Figure 8 illustrates the detection of bisretinoids in an agouti wild-type mouse (2 eyes/sample). With identification using UV-visible absorbance spectra (monitoring at 430 nm) and retention times determined from authentic compound, elution peaks were assigned to the bisretinoids A2E ($\lambda_{\text{max}} = 431, 332$), iso-A2E ($\lambda_{\text{max}} = 426, 339$) A2GPE (A2- glycerophosphoethanolamine) ($\lambda_{\text{max}} = 448, 351$) and A2-DHP-PE (A2-dihydropyridinephosphatidylethanolamin) ($\lambda_{\text{max}} = 487, 335$) (Fig. 8A). Conversely, even in an extract from 10 eyes pooled from Cralbp+/− mice, A2E and iso-A2E were consistently less abundant than in a sample of 2 eyes from wild-type mouse (Fig. 8A).

Since ultra-performance liquid chromatography (UPLC) provides excellent sensitivity and resolution with smaller sample sizes than HPLC, we used UPLC for further quantitative analysis. A2GPE increased with age in both the Cralbp+/− and wild-type mice (Fig. 8B), but at all ages A2GPE was also significantly lower in Cralbp+/− than wild-type.

RPE65 is the isomerohydrolase responsible for converting all-trans-retinyl-ester to 11-cis-retinol within RPE cells (34-36) and RLBPI/CRALBP-associated disease is considered to share clinical features with RPE65-related dystrophy (37,38). Rpe65 null mutant (Rpe65−/−) mice do not generate 11-cis-retinal chromophore (39). A comparison of 6 month old Cralbp+/− and Rpe65−/− mice (Fig. 8C) revealed that whereas A2-GPE was reduced in Cralbp+/− mice, an A2-GPE peak was undetectable in the Rpe65−/− mice even in a sample of 10 eyes.

**Analysis of outer nuclear layer (ONL) in Cralbp−/− mice**

Since retinoids are also required to sustain the viability of photoreceptor cells (1,16) we examined photoreceptor cell numbers by counting nuclei in the ONL (Fig. 9). The numbers of photoreceptor cell nuclei spanning the width of the outer nuclear layer in the wild-type mice at age 8-9 months was 10.0 (± 0.38, SD) (Fig. 9A); this value was typical of the 9-11 range reported in the literature (40-47). In the Cralbp+/− mice at 8-9 months of age the mean number of nuclei was reduced to 8.6 (± 0.48, SD) and at age 12 months a further reduction to 7.32 (± 0.44, SD) (p < 0.05; two-way ANOVA and Sidak’s multiple comparisons test) occurred (Fig. 9A and B).

**Discussion**

We have presented the clinical and molecular analyses of two siblings compound heterozygous for disease-causing mutations in the RLBPI gene, their carrier parents and Cralbp+/− mice. By employing multiple imaging modalities, we observed that the dot-like puncta characteristic of color fundus images in RLBPI-disease (11,12) were prominent and radially arranged in peripheral retina when viewed by UWF imaging. These puncta were also autofluorescent in SW-AF images and hypofluorescent in NIR-AF images. The SD-OCT scans presented foci of abnormal outer retinal reflectivity together with hypertransmission of SD-OCT signal into the choroid. Full-field ERG recordings revealed that rod responses in the affected siblings were severely depressed. Nevertheless, to our knowledge this is the first report to show that after prolonged dark-adaptation the patients with RPA recovered their rod-specific ERG responses. This finding together with reduced levels of 11-cis-retinal and 11-cis-retinol in the Cralbp+/− mice confirmed that a deficiency in CRALBP does not block the enzymatic production of 11-cis-retinoid but 11-cis retinal is regenerated at a slower rate. The reduction in SW-AF intensity measured as qAF in the affected adolescents, the two carriers and Cralbp+/− mice is corroborated by HPLC quantitation of bisretinoids in the mice and results from reduced availability of visual chromophore.
We gained insight into the structural correlates of the white spots. The hypopigmentation evinced as white dots in pseudocolor fundus images and as reduced melanin signal (27-30) in NIR-AF images indicated a change in the RPE monolayer while the hypertransmission of SD-OCT signal posterior to the RPE was indicative of RPE atrophy at these positions. We have previously observed that outside the macula of GPR143/OA1 carriers, pigmented and non-pigmented RPE cells segregate into radial arrays (29) not unlike the pattern of radially arranged dots observed in the UWF pseudocolor images (Fig. 3A and B) of the current study. This similarity suggests that the pattern of dot-like lesions is established by RPE cells. Perhaps the reduction in melanin signal recorded by NIR-AF (27-30) reflects a remodeling of the RPE monolayer to fill gaps left by RPE loss. The involvement of RPE in the atrophy associated with RLBP1/CRALBP1-disease is indicated in other studies by the hyperfluorescence observed in fluorescein angiograms (9) and by the same hypertransmission of SD-OCT signal into the choroid at sites of atrophy (10). It has also been noted that the dot-like fundus lesions progress to RPE atrophy (6). Here the reduced melanin-derived NIR-AF signal was not restricted to the RLBP1/CRALBP-affected and Cralbp<sup>-/-</sup> mice, instead this phenotype was also exhibited by the heterozygous carrier parents.

In the SD-OCT scans presented here the abnormal foci of hyperreflectivity in photoreceptor-attributable bands was limited to the IZ and EZ bands perhaps because of the younger ages of the affected siblings. In previous reports, however, these focal lesions incorporated EZ, ELM and extended into the ONL (48) thus replacing photoreceptor cell-attributable bands. The degenerative changes in photoreceptor cells indicated by these SD-OCT findings are corroborated by reduced ONL area in the Cralbp<sup>-/-</sup> mice. The reduction in qAF in RLBP1/CRALBP-affected patients and Cralbp<sup>-/-</sup> mice and in HPLC quantified bisretinoid in Cralbp<sup>-/-</sup> mice is attributable to chronic deficiency in the 11-cis-retinal chromophore. Indeed we observed attenuated qAF even in the asymptomatic carrier parents carrying a single mutant allele. This finding indicates that a reduction of approximately half of the gene product reduces bisretinoid. Since bisretinoid lipofuscin accumulation underlies retinal diseases such as recessive Stargardt disease, various strategies aimed at reducing bisretinoid formation have been investigated in recent years (49-59). Here we show for the first time that slowing of the visual cycle by a deficiency in CRALBP activity associated with biallelic mutations (patients) or even a mutation in one allele (parent carriers) also confers a decrease in bisretinoid lipofuscin. Moreover, our findings suggest that quantitation of autofluorescence may be used as predictor of mutation severity as it showed a difference between the missense and deletion variants in the carriers.

Several findings in Cralbp<sup>-/-</sup> mice replicated the disease features observed in the RLBP1/CRALBP-affected patients. Consistent with the abnormal ERG responses in the patients, 11-cis-retinal was significantly lower in Cralbp<sup>-/-</sup> mice than in the wild-type. Measurement of SW-AF by qAF showed that fundus intensities were significantly lower in the Cralbp<sup>-/-</sup> mice in keeping with the reduced qAF recorded in the patients. Moreover, HPLC quantitation of bisretinoid visual cycle by-products, the source of SW-AF, were also reduced in the mutant mice. NIR-AF intensities originating primarily in RPE cells were found to be reduced in the mice at 12 and 18 months of age and correspondingly, were reduced in the affected patients. Consistent with the central atrophy observed in RPA (10,11) and with the SD-OCT evidence of structural aberration in photoreceptor cells observed here, we found that the numbers of photoreceptor cell nuclei spanning the width of ONL was decreased by 30% at age 12 months in Cralbp<sup>-/-</sup> versus wild-type mice.

The CRALBP Phe96-99del mutation results in the loss of four residues, three of which are hydrophobic (F96, L97, and F99) and one which is charged (R98); all are located on
helix α4 of the N-terminal all-helical alpha domain. While this region is approximately 25 Å away from the ligand binding pocket of CRALBP, insights into potential structure-function relationship can be gained from the R234W mutant crystal structure (23). The conformation of R234 is essential for normal activity of CRALBP. Disruption of the R234 conformation in the R234W mutant crystal structure resulted in a cascade of side chain conformational changes that ultimately affected the 11-cis-retinal binding site and resulted in a 5-fold decrease in enzymatic activity.

The long-range effects of this single mutation at position R234 (Fig. 10, blue residue) and the resulting structural rearrangements of three hydrophobic residues (Fig. 10, light pink) underscore the importance of this region for maintaining normal protein function. Deletion of residues 96-99 could alter the conformation of R234 by preventing its interaction with E51, with which it makes direct contact in the native crystal structure (C-O to N=O distance = 2.7 Å); this would also affect the retinal binding pocket. Furthermore, F99 interacts with P232 (Fig. 10, black) and loss of F99 could alter the conformation of the nearby R234 by disruption of this interaction. These potential structural rearrangements within the Phe96-99del mutant provide a theoretical basis for decreased enzymatic activity through long-range disruption of the 11-cis-retinal binding site, similar to that seen in the R234W crystal structure.

It is also possible that loss of the three hydrophobic residues F96, L97, and F99, and the charged R98 in the Phe96-99del mutation disrupt the structure and function through a different, unknown mechanism. For example the absence of these residues could perturb nearby residues that make contacts critical for stabilizing the retinal binding site or catalytic residues. Position 9 is in the disordered N-terminus region of the crystal structure, so no structure-function relationship can be assessed for this region. Nevertheless, the R9C mutation could affect the pH dependence of enzymatic activity and this Arg9 may be important in other conformational states.

What is the mechanism by which CRALBP deficiency causes RPE cell atrophy? The protein CRALBP in RPE cells serves as both an acceptor of 11-cis-retinol in the isomerization step of the visual cycle and as a facilitator of the oxidation of 11-cis-retinol to 11-cis-retinaldehyde (16,60,61). By chaperoning 11-cis-retinal it also protects against damage from free aldehyde. Whether the loss of the retinaldehyde-carrier function of CRALBP in the presence of regenerated 11-cis-retinal is permissive for unwanted aldehyde toxicity, remains to be determined in our ongoing studies.

The current qAF findings are of additional interest. It was noted previously that Cralbp−/− mice are protected from light damage (1) and it was suggested that Cralbp deficiency reduces the quantity of the mediator of light-mediated retinopathy (1). Based on age- and genotype-associations we have previously presented evidence implicating bisretinoids as the initiators of light damage (62). The reductions in bisretinoid and retinoid reported here likely reveal the mechanism by which RLBP1/CRALBP deficiency protects against light damage.

For the most part, treatments for arRP are not available. However, gene therapy currently exists for patients carrying RPE65 mutations (63-65) that share some clinical features with RLBP1/CRALBP-disease. Moreover, subretinal injection of an adeno-associated vector driving the expression of human CRALBP in Cralbp−/− mice improved both cone and rod electroretinographic responses (66,67).

In summary, with deficiency in CRALBP, the cone and rod visual cycles fail to provide normal levels of visual chromophore. The latter deficiency manifests in RLBP1/CRALBP-affected patients as non-recordable rod ERG responses that recover after prolonged dark adaptation. The chronically diminished 11-cis-retinal is realized as pronounced declines in bisretinoid visual cycle by-products, the source of SW-AF, in RLBP1/CRALBP-affected patients and with
lesser declines in symptomless RLBP1/CRALBP-carriers. The reductions in qAF and NIR-AF intensities in the affected patients and in their heterozygous parents have not previously been reported. Indeed these decreases in qAF and NIR-AF are indicative of RPE changes in heterozygous carriers and signify a phenotype not previously recognized in humans heterozygous for RLBP1/CRALBP mutations.

Experimental procedures

*Human patients.* The patients presented to the Department of Ophthalmology, Columbia University Medical Center. Each patient had a complete ophthalmic examination that included best-corrected visual acuity, tonometry and anterior segment, media and fundus examination. Pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine before imaging and fundus examination. The patients provided written informed consent under a protocol approved by the Institutional Review Board of Columbia University. The study was approved by the Institutional Review Board at Columbia University and adhered to tenets established by the Declaration of Helsinki.

*Electroretinography.* ffERG were performed in the patients according to the standards from the International Society for Clinical Electrophysiology (ISCEV) using the Diagnosys Espion Electrophysiology system (Diagnosys LLC, Lowell, MA) and Dawson Trick Litzkow (DTL) fiber electrodes in both scotopic and photopic states.

*Fundus Imaging.* Horizontal SD-OCT scans (9 X 9 mm scans; 870 nm; 7 microM axial resolution) through the macula were acquired by cSLO (confocal scanning laser ophthalmoscopy; Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany) in high-resolution mode with averaging of 100 single scans. The scans were registered automatically to a simultaneously acquired infrared reflectance (IR-R) (820 nm) fundus image. Nomenclatures used to identify reflectivity bands in SD-OCT images were as published (68).

SW-AF images (488 nm excitation; emission 500 to 680 nm; 30° × 30° field) were obtained using a cSLO (confocal scanning laser ophthalmoscopy; Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany). For qAF, images were acquired and analyzed as described previously (69-72) using a Spectralis HRA+OCT equipped with an internal fluorescent reference for correction of variable laser power and differences in detector sensitivity. The images were acquired in high-speed mode (8.9 frames/s), as a minimum of 12 frames (video format) and saved in non-normalized mode. A minimum of 6 frames per video was required for the analysis. Two sessions of three videos each were performed per eye. An averaged non-normalized image was generated from each video and the two best images from each session were analyzed (4 averaged images per eye).

Mean gray levels were determined in eight circular segments, positioned 7 to 9° from the fovea. Vessels in the sampling area, which would decrease the qAF level, were accounted for by the software algorithm. qAF values were calculated after gray levels were calibrated to gray levels in the reference; and after accounting for the zero-gray level of the laser, refractive error, image magnification and age-adjusted lens transmission (73). For each eye, a qAF value was computed as the mean of the qAF values of the segments (qAF₈). The healthy control group (74) was composed of 87 subjects of white ethnic background with an age range of 5 to 58 years old. Color-coded qAF maps were computed based on pixel-wise transformation of qAF values (WaveMetrics, Lake Oswego, OR).

NIR-AF (790 nm excitation, >830 nm emission, 30 x 30° field) images were obtained with a Heidelberg Retina Angiograph 2 scanning laser ophthalmoscope (HRA2-cSLO, Heidelberg Engineering, Heidelberg, Germany) using the indocyanine-green angiography mode. To obtain high-quality images, the eye-tracking function was utilized, and 100 single frames were averaged and saved in normalized mode. Semi-
quantitative analysis of NIR-AF imaging was also performed. With a sensitivity of 96 (within a range of 51-100%) and the eye-tracking function, 100 single frames were averaged to obtain high-quality images saved in non-normalized mode. ImageJ (Microsoft Java 1.1.4; http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used to analyze and plot the NIR-AF signal. Nineteen subjects (mean age 35.96 years) without a history of eye disease served as the healthy-eye group. These individuals self-identified as Caucasian (11), African American (3), Asian (3) and Hispanic (2).

UWF pseudocolor images were acquired using the Optos 200 Tx imaging system (Optomap Daytona, United Kingdom, Scotland; lasers: red, 633 nm; and green, 532 nm) with red and green false-color display. UWF-AF/green were also acquired (532 nm excitation; 570-780 emission; 200° field).

For analysis, all images were registered and aligned using i2k Retina software (Dual Align LLC, Clifton Park, NY, USA). For illustrative purposes, some nonaligned images were used in figures.

**Mice.** Cralbp\(^{-/-}\) mice (Rpe65-Leu450; agouti) were obtained as a gift from Dr. Vladimir J Kefalov, and were bred in-house. 129S1/SvlmJ mice (Rpe65-Leu450; agouti) and Rpe65\(^{-/-}\) mice (Rpe65\(^{Rd12}\); black) were purchased from Jackson Laboratories, Bar Harbor Maine. Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University and complied with guidelines set forth by the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were free of the Crb1/Rd8 mutation.

**Analysis of retinoid in mice eyes.** For UPLC quantitation of retinoids, frozen mouse eyecups (1 eye/sample) were homogenized and derivatized with O-ethylhydroxylamine (100 mM; 1 mL, on ice) in DPBS (pH 6.5, without CaCl\(_2\) and MgCl\(_2\)) using glass homogenizers. After vortexing, samples were allowed to stand for 15 minutes at 4°C. All-trans-retinyl acetate was added to each sample as an internal standard. Methanol (1 mL) was added and the samples were vortexed and extracted with hexane (3 mL, 3 times). After centrifugation (1500g for 5 minutes at 4°C), the hexane phase was dried under argon gas and then resuspended in 20 μL of acetonitrile. For separation, a Waters Acquity UPLC-PDA system (Waters, Milford, MA) was used with a CSH C18 column (1.7 μm, 2.1 × 100 mm) and gradients of water (A) and acetonitrile (B) with 0.1% of formic acid as follows: starting at 60% B; holding for 5 min; a linear increase to 70% B over 55 min; a linear increase to 100% B over 10 min; holding for 20 min. The flow rate was 0.3–0.5 mL/min. RAL (O-ethyl) oximes were monitored at 360 nm and all-trans-retinol and all-trans-retinyl palmitate were monitored at 320 nm. UV absorbance peaks were identified by comparison with external standards of synthesized 11-cis-retinal (O-ethyl) oxime, all-trans-retinal (O-ethyl) oxime, all-trans-retinol and all-trans-retinyl palmitate. Molar quantities per eye were calculated based on standard solutions with concentrations determined spectrophotometrically. Peak areas were calculated using Waters Empower Software and results were analyzed in Excel (Microsoft, Redmond WA).

**qAF in mice.** Mice were anaesthetized, pupils were dilated, the cornea was lubricated and mice were positioned as previously described (75-77). SW-AF images (488 nm, 55° wide-field lens; 0.98 mm detection pupil) were captured with a cSLO (Spectralis HRA; Heidelberg Engineering, Heidelberg, Germany) with laser power set at ~280 μW and sensitivity at 100-105, after visual pigment was bleached for 20 seconds. Nine successive frames were acquired in SW-AF high-speed mode and were saved as non-normalized images. Mean gray levels (GL) in the SW-AF images were measured in 8 predefined segments and qAF at 488 nm excitation was calculated by normalization to the GL of the reference after subtraction of zero light (GL\(_0\)) and inclusion of a reference calibration factor.
A mean of 100 frames were also obtained at 790 nm excitation (NIR-AF) with high resolution ART mode (Automatic Real-Time) and resized with Photoshop CS4 (Adobe Systems, San Jose, CA) to 768 X 768 pixels (equivalent to high-speed mode). Intensity at 790 nm was calculated by subtracting the minimal GL of optic nerve head measured by ImageJ (a public domain, Java-based image processing program developed at the National Institutes of Health).

**Measurement ONL.** Mouse eyes were marked superiorly with tattoo ink (Ketchum Manufacturing Inc., Ottawa, Canada), fixed in Alcoholic Z-fix (4% paraformaldehyde, 20% isopropanol, 2% trichloroacetic acid and 2% zinc chloride) and paraffin sections (5 microns) were stained with H&E. Using digitized images of three sections traversing the optic nerve head (ONH), nuclei in rows spanning the width of the ONL were counted at 200 micron intervals superior and inferior to the ONH along the vertical meridian (0.2 to 1.6 or 2 mm) and ONL area was calculated using an measurement interval of 0.2 mm multiplied by the sum of ONL thicknesses in superior and inferior hemiretina (ONH to 2.0 mm).

**Bisretinoid analysis in mice.** Mouse eyecups (4-10 eyes/sample) were homogenized, extracted in chloroform/methanol, concentrated as previously described (78,79). When indicated mouse eyes were freeze-dried before extraction. For UPLC quantitation, murine eyecup samples were re-dissolved in ethanol after extraction and injected (10 µL) into a Waters Acquity UPLC-MS system (Waters, Milford, MA) using an Acquity BEH Phenyl Column (1.7 µm, 2.1 x 100 mm; Waters, Milford, MA). Eluents were water-acetonitrile (1:1) with 0.1% formic acid (A) and isopropanol-acetonitrile (9:1) with 0.1% formic acid (B). Separation was achieved using the following elution gradients: 0-50 min, 100-55%; 50-110 min, 55-35%. Flow rate was 0.2 mL/min. UV absorbance peaks were identified by comparison with external standards of synthesized and purified A2E and A2GPE. Identification was confirmed by mass to charge ratio (m/z). Molar quantities per eye were calculated based on standard solutions with concentrations determined spectrophotometrically. Peak areas were calculated using Waters Empower Software and results were analyzed in Excel (Microsoft, Redmond WA).

For analysis by HPLC (Alliance System, Waters Corp.), samples were re-dissolved in CHCl₃/MeOH=1:1 after extraction as above and injected (30 µL) as previously described (80). Absorbance peaks were identified using synthesized authentic compounds, and molar quantities per eye were calculated by comparison with synthesized standards. A2E and isoA2E were measured separately and summed.

**Structural analysis.** Structural figures were made in PyMOL using CRALBP crystal structure (PDB 3HY5).

**Statistical analysis.** P values were calculated using PRISM 8 (GraphPad Software).

**Data Availability.** All of the data are in the manuscript.
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Author contributions: JRLC and HJK contributed equally to the work. JRLC: conducted experiments, designed experiments, acquired data, wrote manuscript; HJK: designed experiments; conducted experiments; acquired data, wrote manuscript; KU: conducted experiments, acquired data; JZ: conducted experiments, acquired data; AO: conducted experiments, designed experiments; TY: conducted experiments, designed experiments, wrote manuscript; SHT: designed studies, analyzed data; JRS: designed studies, analyzed data; wrote and revised manuscript.

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FOOTNOTES
The abbreviations used are: CRALBP, cellular retinaldehyde-binding protein; cSLO, confocal laser scanning ophthalmoscopy; ffERG, full-field electroretinography; NIR-AF, near-infrared fundus autofluorescence; ONL, outer nuclear layer; RDH5, retinol dehydrogenase 5; RLBP1, retinaldehyde-binding protein 1; RPA, retinitis punctata albescens; RPE, retinal pigment epithelium; SD-OCT, spectral domain-optical coherence tomography; SW-AF, short-wavelength fundus autofluorescence.

Table 1: Clinical and genetic characteristics of the family members

| Patient | Gender | Age | Clinical symptoms | BCVA (logMAR) | qAF8 | Genetics |
|---------|--------|-----|-------------------|---------------|------|----------|
|         |        |     |                   | OD | OS | OD | OS |
| I-1     | M      | 43  | No symptoms       | 0.0 | 0.0 | 183 | 155 |
|         |        |     |                   | RLBP1+/− | c.25C>T:p.Arg9Cys |
| I-2     | F      | 42  | No symptoms       | 0.0 | 0.0 | 114 | 118 |
|         |        |     |                   | RLBP1+/− | c.286_297:p.Phe96_99del |
| II-1    | F      | 15  | Photophobia, poor night vision | 0.06 | 0.06 | - | - |
|         |        |     |                   | RLBP1+/− | c.25C>T:p.Arg9Cys & c.286_297:p.Phe96_99del |
| II-2    | F      | 13  | Photophobia, poor night vision | 0.06 | 0.24 | - | - |
|         |        |     |                   | RLBP1+/− | c.25C>T:p.Arg9Cys & c.286_297:p.Phe96_99del |
| II-3    | M      | 7   | No symptoms       | 0.02 | 0.02 | N/A | N/A |

BCVA: best corrected visual acuity; logMAR: logarithm of the minimum angle of resolution; OD: right eye; OS: left eye; qAF8: average quantitative autofluorescence of the 8 segments of the ring at an eccentricity of approximately 7º to 9º from the fovea.
Figure 1. **Autosomal recessive inheritance patterns.** Affected female siblings (II-1; II-2; green filled circles) compound heterozygous for the missense mutation c.25C>T:p.Arg9Cys and c.286_297:p.Phe96_99 deletion in the *RLBP1* gene. Two generations (I, II) are shown. Male, *square*; female, *circle*. Retinitis punctata albescens patients, *green filled symbol*; carriers, *dot in symbol*; unknown, *unfilled symbol*.
Figure 2. Rod-cone dysfunction in full-field electroretinography (ffERG). Patients II-1 and II-2. Rod-specific ERG with 0.01cd.s/m² stimulus following standard 30 min dark-adaptation was absent. Maximum ERG (3.0cd.s/m²) had a diminished response. Photopic ERG was within the normal range. After prolonged dark-adaptation (>8h overnight eye-patch), rod-specific ERG and maximum ERG responses recovered. Normal control is shown in the right column. DA, dark-adapted; LA, light-adapted.
Figure 3. Multimodal images in siblings deficient in CRALBP. Images of II-1 (left) and II-2 (right) both of whom are compound heterozygous for mutations in the \textit{RLBP1} gene. (A, B) Ultrawide-field (UWF) color fundus photographs. Note the radially arranged white dots more visible in the peripheral retina. (C, D) Green-UWF-autofluorescence. Fovea was detected as a small dark zone with surrounding speckled brightness. (E, G) Short wavelength fundus autofluorescence (SW-AF). A ring of speckled hyperautofluorescence transitioned into faintly autofluorescent macular spots and mottling at greater eccentricity. (F, H) Near-infrared fundus autofluorescence (NIR-AF). The fovea exhibits the characteristic elevated NIR-AF intensity. Hypoautofluorescent dots surrounded by areas of normal fluorescence in the parafoveal region can be appreciated. (I, J and K, L) Spectral-domain optical coherence tomography (SD-OCT). Note undulations of the ellipsoid zone (yellow arrows), hyperreflective irregularities in the interdigitation zone (white arrows) and foci of hypertransmission into the choroid (white asterisks). K and L represent magnified images of the area indicated by the short white lines in I and J. (M, N, O) Color-coded quantitative fundus autofluorescence images of the patients II-1 (M) and II-2 (N) had undetectable levels of AF except for a parafoveal ring. A healthy control eye 11 year-old subject is shown (O).
Figure 4. Fundus images of the parents who are carriers of mutations in RLBPI/CRALBP. Subjects I-1 (left) and I-2 (right). Subject I-1 is heterozygous for c.25C>T mutation in the RLBPI gene and subject I-2 is heterozygous for the c.286_297del variant in the RLBPI gene. C.289_297:p.Phe96_99 deletion carrier has less autofluorescence than missense mutation c.25C>T:p.Arg9Cys carrier. (A, C) Short wavelength fundus autofluorescence (SW-AF). Images acquired in normalized mode do not reveal differences in gray level intensities. (B, D) Near-infrared fundus autofluorescence (NIR-AF). The fovea exhibits typical elevated intensity; contrast with AF in periphery appears to be increased. (E, F) Spectral-domain optical coherence tomography (SD-OCT). Reflectivity layers appear normal. (G, H, I). Color-coded quantitative fundus autofluorescence (qAF) images of the carriers I-1 (G), I-2 (H) and an age-matched healthy eye (I). Reduced qAF characterizes the symptomless carriers.
Figure 5. Quantitation of fundus autofluorescence. (A) Short-wavelength (488 nm) fundus autofluorescence measured as quantitative fundus autofluorescence (qAF) at an eccentricity of 7°-9° and plotted as a function of age. Mean qAFₙ of parents (blue, I-1; and green I-2) are plotted together with mean (solid black line) and 95% confidence levels (dashed lines) acquired from eyes of healthy subjects. Note that qAF intensities at the same location in the affected siblings were not measurable. (B) Semi-quantitative near-infrared autofluorescence (NIR-AF) intensity profiles plotted as a function of temporal-to-nasal (left to right) distance (mm) along a horizontal line through the fovea (0). Gray lines represent mean (solid lines) and 95% confidence intervals (dashed lines) of healthy control eyes. Subjects are represented as follows: blue line, I-1; green line, I-2; red line, II-1; orange line II-2.
Figure 6. Retinoid levels in dark-adapted agouti Cralbp\(^{-/-}\) and wild-type (WT) mice. Age 2-4 months. (A, B) Representative UPLC chromatograms demonstrating separation of hexane extracted retinoids in eyes of agouti WT (A) and Cralbp\(^{-/-}\) mice (B). Monitoring at 340 nm. Age 4 months; 1 eye/sample. The numbers indicate the identity of peaks based on comparison with standards. 1, 11-cis-retinol; 2, all-trans-retinol; IS (internal standard), all-trans-retinyl acetate; 3, anti 11-cis-retinal-(O-ethyl) oxime; 4, anti all-trans-retinal-(O-ethyl) oxime; 3’, syn 11-cis-retinal-(O-ethyl) oxime; 4’, syn all-trans-retinal-(O-ethyl) oxime; 5, all-trans-retinyl palmitate; 6, 11-cis-retinyl palmitate. (C, D) Quantitation of retinoids as picomoles / eye (C). Presentation of data in C as percentage of total retinoid (D). All-trans-retinol (atROL), all-trans-retinyl palmitate (atRE), all-trans-retinal (atRAL). (E) Quantitation of 11-cis-retinol (11cisROL) and 11-cis-retinyl palmitate (11cisRE) as peak area/eye. (F, G) Levels of 11-cis-retinal (F) and all-trans-retinyl ester (G) in dark-adapted (DA) mice recovering from a photobleach for 1 and 2 hours in the dark. Mean ± SD, 10-12 samples (C-E); 4 samples (F-G); p values determined by ANOVA and Sidak’s multiple comparisons test.
Figure 7. Fundus images acquired from agouti Cralbp<sup>−/−</sup> and wild-type (WT) mice. (A) Infrared reflectance (IR; 820 nm), short wavelength fundus autofluorescence (SW-AF; 488 nm) and near-infrared autofluorescence (NIR-AF; 790 nm) images acquired from agouti Cralbp<sup>−/−</sup> and WT mice (age 6 months). (B) SW-AF intensities measured in agouti Cralbp<sup>−/−</sup> and WT mice with quantitative fundus autofluorescence (qAF) protocols and plotted as a function of age. Gray levels (GL) were measured 8.25-19.25° from the disc center and were normalized to GL in an internal fluorescent reference (rectangular area at top of image) to calculate qAF. Means of 3-8 mice; *, p < 0.05, ANOVA and Sidak’s multiple comparisons test. (C) NIR-AF intensities measured in agouti Cralbp<sup>−/−</sup> and WT mice. Mean ± SD of 3-8 mice; *, p < 0.05, ANOVA and Sidak’s multiple comparisons test.
Figure 8. Reduced bisretinoids in Cralbp<sup>-/-</sup> mice retinae. HPLC analysis of bisretinoids in wild-type (WT) and Cralbp<sup>-/-</sup> mice. (A) Representative reverse phase HPLC chromatograms with monitoring at 430 nm, illustrate the detection of the bisretinoids identified on the basis of UV-visible absorbance and retention times (Rt) recorded using authentic standards. A2E (Rt 36.5 min), isoA2E (Rt 37.8 min), A2GPE (Rt 38.4 min), A2-DHP-PE (Rt 45.1 min) and oxidized bisretinoid (1, Rt 39.7 min). Age 12 months. Insets: (top) UV-visible absorbance spectra of chromatographic peaks corresponding to bisretinoids, (inside) fluorescence at λ<sub>ex</sub> 430 nm/λ<sub>em</sub> 600 nm and UV-visible absorbance of peak 1. (B) Quantification of bisretinoid in Cralbp<sup>-/-</sup> and WT mice. (C) A2-GPE is not detectable in Rpe65<sup>-/-</sup> mouse eyecups analysed by UPLC. Eyecups from pigmented Cralbp<sup>-/-</sup> and WT mice were analyzed by UPLC with a BEH phenyl column and monitoring at 430 nm. Values are means ± SD of 3-7 independent samples. 2-4 eyes/sample. ***, p< 0.001, **, p< 0.01, *, p< 0.05, p-values determined by one-way ANOVA and Tukey’s multiple comparison test.
**Figure 9. Photoreceptor cell loss in Cralbp−/− mice.** (A) Numbers of nuclei per column extending the width of outer nuclear layer (ONL). Means ± SD; Cralbp−/−: 6 eyes (age 8-9 months), 5 eyes (age 12 months); Wild-type (WT): 10 eyes (age 8-9 months); 8 eyes (12 months). **, p < 0.01, ANOVA and Tukey’s multiple comparisons test. (B) Representative light micrographs of inferior hemiretina of Cralbp−/− and WT mice (age 12 months) within 500-1000 microns from optic nerve head.
Figure 10. Crystal structure of cellular retinaldehyde-binding protein (CRALBP). Two views of the CRALBP crystal structure (PDB 3HY5) with key residues colored as indicated. Residues in light pink (I198, F235, I238) underwent conformational change in the R234W mutant crystal structure (PDB 3HX3, not shown) to alter 11-cis-retinal binding affinity and decreased enzymatic activity. Residues lost in the Phe96-99del mutant are shown in orange (F96, L97, R98, and F99) and the interacting P232 is shown in black. 11-cis-retinal is depicted by orange spheres.
Effects of deficiency in the RLBP1-encoded visual cycle protein CRALBP on visual dysfunction in humans and mice
Jose Ronaldo Lima de Carvalho Jr., Hye Jin Kim, Keiko Ueda, Jin Zhao, Aaron P. Owji, Tingting Yang, Stephen H. Tsang and Janet R Sparrow

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