Duplication and Divergence of Zebrafish CRALBP Genes Uncovers Novel Role for RPE- and Müller-CRALBP in Cone Vision

Ross Collery,1 Sarab McLoughlin,1 Victor Vendrell,1 Jennifer Finnegan,1 John W. Crabb,2 John C. Saari,5 and Breandaén N. Kennedy1

PURPOSE. During vertebrate phototransduction 11-cis-retinal is isomerized to all-trans-retinal. Light sensitivity is restored by recombination of apo-opsin with 11-cis-retinal to regenerate visual pigments. The conversion of all-trans retinal back to 11-cis-retinal is known as the visual cycle. Within the retina, cellular retinal-binding protein (CRALBP) is abundantly expressed in the retinal pigment epithelium (RPE) and Müller glia. CRALBP expressed in the RPE is known to facilitate the rate of the rod visual cycle. Recent evidence suggests a role for Müller glia in an alternate cone visual cycle. In this study, the role of RPE- and Müller-CRALBP in cone vision was characterized.

METHODS. The CRALBP orthologues rlbp1a and rlbp1b were identified in zebrafish by bioinformatic methods. The spatial and developmental expression of rlbp1a and rlbp1b was determined by in situ hybridization and immunohistochemistry. Depletion of the expression of the corresponding Cralbp a and Cralbp b proteins was achieved by microinjection of antisense morpholinos. Functional analysis was evaluated in 5-day post fertilization (dpf) larvae using the optokinetic response assay.

RESULTS. The zebrafish genome contains two CRALBP orthologues, rlbp1a and rlbp1b. These genes have functionally diverged, exhibiting differential expression at 5 dpf in RPE and Müller glia, respectively. Depletion of CRALBP in the RPE or Müller glia results in abnormal cone visual behavior.

CONCLUSIONS. The results suggest that cone photoreceptors incorporate 11-cis-retinoids derived from the rod and cone visual cycles into their visual pigments and that Müller-CRALBP participates in the cone visual cycle. (Invest Ophthalmol Vis Sci. 2008;49:3812–3820) DOI:10.1167/iovs.08-01957

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The visual system transforms light impulses detected by photoreceptors in the retina into images of the external environment. Rod photoreceptors function during dim night-light conditions, whereas vision in daylight is mediated by cone photoreceptors. Cones can adapt to 10 log units of illumination, and subtypes of cones have distinct spectral sensitivities enabling daylight color vision.1,2

In vertebrate photoreceptors, light is sensed by photopigments that consist of an opsin G-protein-coupled receptor and the light-sensitive chromophore 11-cis-retinal (for review, see Ref. 3). Different opsin proteins are present in each rod and cone, resulting in different spectral sensitivities. In rods, light photosomizes 11-cis-retinal in the photopigment to all-trans-retinal. This process induces a conformational change in rhodopsin, activating the phototransduction cascade. This cascade sends signals via the optic nerve to the visual cortex of the brain, where impulses are interpreted as a visual image. The process of regeneration of the rod photopigment is known as the rod visual cycle and requires regeneration of 11-cis-retinal from all-trans-retinal (for reviews see Refs. 4, 5). The process involves chemical modification and shuttling of retinoid intermediates within rods and the surrounding retinal pigment epithelium.

Cellular retinaldehyde-binding protein (CRALBP) is a cytoplasmic protein, abundantly expressed in the retinal pigment epithelium (RPE) and Müller glia of the retina and in the pineal gland.6–8 Structurally, human CRALBP is a ∼36-kDa monomeric protein, proposed to adopt an “open” or “closed” conformation, depending on whether it is carrying an endogenous ligand.9 CRALBP interacts structurally and functionally with 11-cis-retinol dehydrogenase (RDH5), an enzyme of the visual cycle in RPE.10 CRALBP is a member of the CRAL TRIO family of proteins that share a lipid-binding domain derived from the yeast sec14 protein.11 Residues 120-313 comprise the ligand-binding pocket within which retinoid-interacting residues, including W165, Y179, F197, C198, M208, Q210, M222, V223, M225, and W244, have been identified.9,12–15 The C terminus of CRALBP binds to the PDZ-domains of ezrin-radixin-moesin (ERM)–binding phosphoprotein50/sodium hydrogen exchanger regulatory factor-1 (EBP50/NHERF-1), which in turn binds to ezrin and actin, proteins localized to the apical processes of the retinal pigment epithelium and Müller-glia cells.16,17 The (N/D)TA(L/F) minimum binding motif at the CRALBP C terminus is found in multiple CRALBP orthologues and is proposed to bind CRALBP to apical processes of RPE cells and apical microvilli of Müller cells.

CRALBP contains a high-affinity binding site for 11-cis-retinol or 11-cis-retinal, vitamin A metabolites uniquely associated with sensing light.18 RPE-CRALBP facilitates 11-cis-retinal regeneration during the rod visual cycle. The functional requirement of CRALBP in retinal Müller cells and in the pineal gland is unknown. Mutations in the single human CRALBP gene, RLBP1, can lead to forms of blindness that reflect retinitis punctata albescens, a photoreceptor degeneration accompanied by subretinal, white-to-yellow punctate deposits and de-
layed rod and cone resensitization.19–22 These mutations can tighten or abolish CRALBP ligand binding.10,21 *Rlbp1*−/− (knockout) mice have delayed rhodopsin regeneration and dark adaptation after illumination coupled with diminished 11-cis-retinal production.23 However, photoreceptor degeneration characteristic of missense mutations in human CRALBP are not recapitulated in the *Rlbp1*−/− mouse.

RPE-CRALBP is an established facilitator of 11-cis-retinal regeneration during the rod visual cycle, accelerating the isomerization of all-trans- to 11-cis-retinol.24–26 Recently, evidence of a novel pathway that regenerates cone photopigments, the cone visual cycle, has accumulated.27–30 This cycle appears to involve a novel biochemical pathway for regenerating 11-cis-retinal involving enzymatic modification and shuttling of intermediates within cones and surrounding Müller glial cells.29 The expression of CRALBP in Müller cells and the binding of CRALBP to 11-cis-retinoids suggest that Müller-CRALBP plays a key role in the cone visual cycle.

The function of Müller-CRALBP has not been selectively assessed. Although patients with missense CRALBP mutations and CRALBP knockout mice display abnormal cone responses, the phenotype could arise from defective RPE- and/or Müller-CRALBP, as there is a single mammalian gene.19,23 Approaches to dissecting the role(s) of retinal CRALBP include generating models with selective loss of RPE- or Müller-CRALBP. This loss can be achieved in mice by using conditional knockout approaches, but is time-consuming and expensive and requires the maintenance of several transgenic lines. An alternative is the zebrafish, which has abundant cones and is amenable to genetic manipulation.

In the current study, we demonstrated that zebrafish contain two CRALBP ohnologues and that these duplicated genes have diverged such that zebrafish *rlbp1a/Cralbp a* is predominately expressed in the RPE and zebrafish *rlbp1b/Cralbp b* is predominantly expressed in Müller glia. Using antisense morpholino technology for selective knockdown of RPE-CRALBP or Müller-CRALBP, we demonstrated that depletions of either pool results in abnormal cone-mediated vision. This finding suggests that RPE- and Müller-CRALBP function in the cone visual cycle.

### MATERIALS AND METHODS

#### Animal Breeding and Maintenance

Zebrafish were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. They were maintained and raised under standard conditions at 28.5°C on a 14-hour light:10-hour dark cycle. Wild-type Tübingen zebrafish embryos were obtained by natural spawning and raised in embryo medium.31 Larvae used for in situ hybridization were raised in embryo medium supplemented with 0.003% phenylthiourea (Sigma-Aldrich) to inhibit pigmentation. Larvae were staged according to their age in days post fertilization (dpf).

#### Sequence Analysis

Human CRALBP protein sequence (P12271) was used as a probe to search Ensembl, build version 7 (http://www.ensembl.org/) for orthologous sequences in multiple species using the BLAST algorithm. Two zebrafish ohnologues, *rlbp1a* and *rlbp1b* (NP_991253; NP_956999), were identified, along with orthologues from chimpanzee (*Pan troglodytes*, XP_510580), cow (*Bos taurus*, NP_776876), mouse (*Mus musculus*, NP_065624), rat (*Rattus norvegicus*, NP_001099744), chicken (*Gallus gallus*, NP_001019865), African clawed toad (*Xenopus laevis*, NP_001005455), and cavefish (*Pufferfish*; *Fugu rubripes*; SIFRURP000000129795, SIFRURP000001647753), and cichlids (two ohnologues; *Tetraodon nigroviridis*; CAG01050, CAP99866). Protein alignments and phylogenetic tree-building were performed in CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) provided in the public domain by European Bioinformatics Institute, European Molecular Biology Laboratory, Heidelberg, Germany) with default settings. The results were annotated in image-analysis software (Illustrator, ver. 11; Adobe Systems, San Jose, CA).

#### WholeMount In Situ Hybridization

PCR primers amplified full-length cDNAs of *rlbp1a* and *rlbp1b* and introduced 5′ clamps and restriction sites to facilitate cloning (in italic) *(rlbp1a)*: 5′-ctcctccagacgaaggtgctgg-3′; *(rlbp1b)*: 5′-cgcgaatcgcagaggaatgctgg-3′; and *(rlbp1b)*: 5′-cgcgaatcgcagaggaatgctgg-3′. This cycle can be achieved in mice by using conditional knockout approaches, but is time-consuming and expensive and requires the maintenance of several transgenic lines. An alternative is the zebrafish, which has abundant cones and is amenable to genetic manipulation.

In the current study, we demonstrated that zebrafish contain two CRALBP ohnologues and that these duplicated genes have diverged such that zebrafish *rlbp1a/Cralbp a* is predominately expressed in the RPE and zebrafish *rlbp1b/Cralbp b* is predominantly expressed in Müller glia. Using antisense morpholino technology for selective knockdown of RPE-CRALBP or Müller-CRALBP, we demonstrated that depletions of either pool results in abnormal cone-mediated vision. This finding suggests that RPE- and Müller-CRALBP function in the cone visual cycle.

#### Western Blot Analysis and Immunohistochemistry

Adult zebrafish were dark-adapted before dissection of whole eyes, RPE, and neuroretina. Tissues were homogenized and boiled in 1× SDS-PAGE electrophoresis buffer (16 mM Tris [pH 6.8]), 8% glycerol, 0.6% SDS, 270 mM β-mercaptoethanol, and 0.003% bromophenol blue. Samples were separated on a 15% SDS-polyacrylamide gel for 2 hours before overnight transfer to a nitrocellulose membrane. CRALBP isoforms were immunodetected by using a polyclonal antibody to bovine CRALBP (UW55; kind gift of one of the authors; JCS) followed by HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich) and chemiluminescence reagents (ECL; GE Healthcare, Piscataway, NJ).

Adult and larval zebrafish eyes were fixed with 4% formaldehyde (Sigma-Aldrich) and cryoprotected by a sucrose series before embedding in OCT (Sakura Fintek, Torrance, CA). Twelve-micrometer sections were cut and thaw-mounted onto charged slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). The sections were rehydrated, blocked in 2% (vol/vol) normal goat serum, 1% bovine serum albumin, and 1% Triton X-100 in PBS before incubating overnight at 4°C with polyclonal anti-CRALBP antibody. Polyclonal blue opsin (kind gift of David Hyde, Department of Biological Sciences, University of Notre Dame, Notre Dame, IN) and monoclonal zpr-1 antibodies probed for cone-specific markers. Slides were rinsed in PBS before incubating with Cy5 or Cy3-conjugated goat anti-rabbit/mouse secondary antibody as appropriate (Jackson Immunoresearch Europe Ltd., Newmarket, UK). After rinsing in PBS and counterstaining with 300 nM DAPI, slides were mounted (Vectorshield; Vector Laboratories, Burlingame, CA). Sections were examined with a laser scanning confocal microscope (LSM 510 Meta; Carl Zeiss Meditec, Inc.; Dublin, CA). Photographs were oriented on computer (Photoshop ver. 5 software; Adobe Systems).

#### Morpholino Knockdown

Morpholino oligonucleotides targeting ATG translational start sites of *rlbp1a* and *rlbp1b* were designed by Gene Tools (Gene Tools LLC, Philomath, OR) from cDNA sequences *(rlbp1a)* start blocking MO, TCTCACTTACCCAGCATTGCCCT; *(rlbp1b)* start blocking MO, CGAATACTTCCAGTACAGCATAG). Morpholino oligonucleotides were resuspended in nuclelease-free water and injected into wild-type,
one- to two-cell zebrafish embryos along with 0.01% phenol red tracer dye.

Optokinetic Response Assay

The optokinetic response assay was performed essentially as previously described.35 This assay uses eye movement to calculate visual response to a moving stimulus. Briefly, 5 dpf larval zebrafish were placed in a Petri dish containing 9% methylcellulose to immobilize the larvae, while allowing respiration to continue. A drum containing alternating black-and-white stripes (18° per stripe) was rotated at a speed of 20 rpm. Larvae were recorded during 5 seconds of no rotation, 25 seconds of clockwise rotation, 5 seconds of no rotation, and 25 seconds of anticlockwise rotation. The number of saccades per minute was quantified with a microscope equipped with a digital camera (model SZX16 microscope with a model DP71 digital camera and Cell F software; Olympus, Tokyo, Japan).

RT-PCR of the 5'UTR

Total RNA was extracted from adult zebrafish eyes (TRIzol; Invitrogen) and resuspended in nuclelease-free water. The concentration and purity of RNA were measured with a spectrophotometer (NanoDrop Technologies, Wilmington, DE), and contaminating genomic DNA was removed using RQ1 DNase (Promega). RNA was stored at −80°C until used. Reverse transcription was performed on 1 µg total RNA with an RT-PCR system (Thermocorpus; Invitrogen) at 50°C, after priming with random hexamers. Synthesized cDNA was stored at −20°C until used. cDNA was used in standard PCR reactions with 1 µL cDNA per 25 µL PCR reaction, in standard PCR conditions, with extension times ad-
justed to 1 minute per kilobase of target amplicon. Primers were designed complementary to expressed sequence tags (ESTs) identified after BLAST analysis of EST databases with full-length \textit{rlbp1a} mRNA sequence as the probe (EST: AGENCOURT_21412034, GenBank Acc: CN177714) (\textit{rlbp1a} -ex(--3)-F1, GAGCTGTCATTCTGGGGTC; \textit{rlbp1a} ex(-3)-F1, GGATGGCTCCAGAGCTGTCA; \textit{rlbp1a} -ex(--2)-F1, GAGAAACACCTCAACAGCAATG; \textit{rlbp1a} -ex(--1)-F1, GAGGTCGCGTACACATGAGTCG; \textit{rlbp1a} -ex(+1)-R1, GCTTCAATCTCAGCAACG).

**RESULTS**

Two Cralbp Ohnologues in Zebrafish

Using the human CRALBP protein sequence as a probe, we identified two CRALBP ohnologues in the zebrafish genome. The teleost genome has duplicated since its radiation from other vertebrates, accounting for the presence of two CRALBP genes in zebrafish \textit{(Danio rerio)}, cavefish \textit{(Tetraodon nigroviridis)}, and pufferfish \textit{(Fugu rubripes)} \cite{36} (Fig. 1A). \textit{rlbp1a} and \textit{rlbp1b} are located on zebrafish chromosomes 25 and 7, respectively (Fig. 1B). The encoded proteins, zebrafish Cralbp a and Cralbp b, have \textit{81\%} protein identity to each other, and predicted sizes of \textit{35.5} and \textit{35.7} kDa, respectively (Fig. 1C). Of the 10 residues specifically implicated in the ligand-binding pocket of human CRALBP, 8 are evolutionarily conserved in zebrafish. In addition, residues R150 and R233, asso-

**TABLE 1. Lengths of \textit{rlbp1a} and \textit{rlbp1b} Coding Exons and Introns**

<table>
<thead>
<tr>
<th></th>
<th>\textit{rlbp1a}</th>
<th>\textit{rlbp1b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>44 bp (12 bp)</td>
<td>43 bp (9 bp)</td>
</tr>
<tr>
<td>2</td>
<td>129 bp</td>
<td>129 bp</td>
</tr>
<tr>
<td>3</td>
<td>205 bp</td>
<td>205 bp</td>
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<tr>
<td>4</td>
<td>179 bp</td>
<td>179 bp</td>
</tr>
<tr>
<td>5</td>
<td>159 bp</td>
<td>159 bp</td>
</tr>
<tr>
<td>6</td>
<td>111 bp</td>
<td>111 bp</td>
</tr>
<tr>
<td>7</td>
<td>533 bp (129 bp)</td>
<td>624 bp (147 bp)</td>
</tr>
<tr>
<td>Intron</td>
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<td></td>
</tr>
<tr>
<td>1–2</td>
<td>356 bp</td>
<td>268 bp</td>
</tr>
<tr>
<td>3–4</td>
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<td>768 bp</td>
</tr>
<tr>
<td>5–6</td>
<td>3157 bp</td>
<td>3157 bp</td>
</tr>
<tr>
<td>6–7</td>
<td>4697 bp</td>
<td>1529 bp</td>
</tr>
</tbody>
</table>

The length of each exon is shown, and the length of the coding regions in partially coding exons is shown in parentheses.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Expression of \textit{rlbp1a} and \textit{rlbp1b} in the pineal during larval development. In situ hybridization shows \textit{rlbp1a} and \textit{rlbp1b} expression in the pineal at 1 dpf (A, E) and increasing at 3 to 5 dpf (B, C, F, G). Expression of \textit{rlbp1b} remains strong in the pineal at 7 dpf (H). (F, Inset) The pineal with the parapineal showed expression to the left (blue bracket). \textit{Rlb1a} and \textit{rlbp1b} did not show diurnal or circadian expression (I–N). Dorsal images of 3 dpf larvae harvested in light (I, J, K) and dark (L, M, N) phases and probed for \textit{rlbp1a} and \textit{rlbp1b} and \textit{aanat2} transcripts. Arrows: the pineal.}
\end{figure}

\textit{Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933241/ on 06/11/2018}
ciated with inherited forms of blindness, are conserved across all orthologues.\textsuperscript{19–21} Whereas Cralbp\textsubscript{b} contains the C-terminal DTAL sequence associated with binding to the PDZ-domains of EBP50/NHERF-1,\textsuperscript{16,37} Cralbp\textsubscript{a} does not, suggesting altered function or subcellular localization (Supplementary Fig. S1, online at http://www.iovs.org/cgi/content/full/49/9/3812/DC1). We note that human proteins named CRALBP-like 1\textsuperscript{38} and CRALBP-like 2 (NP_001010852) have been reported; however, as these proteins have only \(\sim\)41\% and \(\sim\)43\% protein identity to human CRALBP, respectively, they were not considered true CRALBP orthologues.

**Exon–Intron Structure of the Zebrafish CRALBP Genes**

The coding regions of \(rlbp1a\) and \(rlbp1b\) comprise seven exons and six introns (Fig. 1 and Supplementary Figs. S2, S3). The size of the coding exons is highly conserved, although the intron sizes have changed considerably (Table 1). Searches of ESTs reveal three putative noncoding exons (exon \(-5, -2,\) and \(-1\)) in the 5' UTR of \(rlbp1a\) but not of \(rlbp1b\). Alignments with genomic sequence demonstrate these exons to extend \(\sim\)12 kb upstream of the \(rlbp1a\) translational start site with introns of 1375, 9116, and 1301 nt, respectively (Supplementary Fig. S4). In agreement with the bioinformatic analyses, PCR using primers extending from these noncoding exons to exon 1 demonstrate they are incorporated into transcripts in adult eye mRNA. It is unclear whether these noncoding exons represent alternative promoter start sites or alternative splice forms.

**Expression of \(rlbp1a\) and \(rlbp1b\) in Larval Pineal and Eye**

The spatial and temporal expression of \(rlbp1a/Cralbp\textsubscript{a} and \(rlbp1b/Cralbp\textsubscript{b} transcripts was determined by wholemount in situ hybridization. Both \(rlbp1a\) and \(rlbp1b\) were expressed in the developing eye and pineal gland from 1 dpf and were strongly expressed in those organs from 3 to 5 dpf (Figs. 2, 3). At 7 dpf, \(rlbp1a\) was expressed in the eye, but was not detected in the pineal gland, whereas \(rlbp1b\) was expressed in both the eye and pineal. Approximately 50\% of larvae examined showed parapineal expression of \(rlbp1b\). The parapineal gland is located ventrolateral to the pineal and is thought to regulate asymmetric brain development.\textsuperscript{39} To determine whether expression of either zebrafish CRALBP gene exhibited circadian or diurnal regulation, we performed wholemount in situ hybridization on embryos collected during mid-light and mid-dark time points (Fig. 2). No
obvious change in the pineal expression level of \textit{rlbp1a} or \textit{rlbp1b} was observed during light and dark phases. Larvae probed for \textit{aanat2} showed the expected cyclic dark-phase upregulation and light-phase downregulation.\textsuperscript{40} Thus, neither Cralbp\textsubscript{a} nor Cralbp\textsubscript{b} displayed evidence of circadian or diurnal expression profiles in the pineal.
Expression of Zebrafish CRALBP Isoforms in Müller Glia and RPE

Analysis of CRALBP protein expression in zebrafish was performed with a polyclonal antibody raised against recombinant bovine CRALBP that does not distinguish between zebrafish Cralbp a and Cralbp b isoforms (Fig. 3). Consistent with other species, immunohistochemical staining of retinal sections confirmed extensive expression of zebrafish Cralbp a/Cralbp b in the RPE and Müller glia (Fig. 3A). Expression was strongest toward the ganglion cell layer where the Müller end feet are located (Fig. 3A). Müller glial expression was verified by colocalization with GFP expressed under the control of a promoter for glial fibrillary acidic protein (GFAP), an established Müller marker (Fig. 3B). Western blot analyses provided the first indication that the RPE and Müller glia express unique CRALBP isoforms. The analysis revealed distinct bands of ~33 to 35 kDa in protein extracted from the entire retina. The slower mobility isoform was specific to the RPE, and the faster mobility isoform specific to the neuroretina (Fig. 3E).

As duplicated genes often diverge in function, we hypothesized that the zebrafish CRALBP ohnologues evolve distinct expression profiles in the retina. In situ hybridizations with gene-specific probes indicated that at 5 dpf rlbp1a was predominantly expressed in the RPE and rlbp1b in Müller glia (Figs. 4A–F). Retinal cryosections demonstrated robust expression of rlbp1b in Müller cells (Fig. 4E), which had projections spanning the retina and nuclei in the inner nuclear layer. rlbp1a and rlbp1b were also expressed at the interface between the retina and the olfactory placodes (Figs. 4B, 4E) and rlbp1b was expressed in the ciliary epithelium, as shown in other species.

The results conflicted with the Western blot data as Cralbp b is predicted to have a slower mobility than Cralbp a. Many proteins, including proteins found in the retina, do not segregate at the predicted size on Western blots, which may be due to posttranslational modification. To confirm the in situ findings at the protein level, we microinjected antisense morpholinos, for selective translational block of each zebrafish CRALBP isoform. No change in gross cone morphology or expression of cone- or rod-specific markers was observed after specific knockdown of either Cralbp a or Cralbp b (Figs. 4G–O and data not shown). Thus, the zebrafish genome contains duplicated CRALBP genes that, at 5 dpf, exhibit exclusive patterns of expression within the RPE (rlbp1a) or Müller glia (rlbp1b).

**CRALBP labeling in the RPE, but not in Müller glia, whereas Cralbp b knockdown resulted in a specific depletion of CRALBP labeling in Müller glia but not in the RPE (Figs. 4G–I). No change in gross cone morphology or expression of cone- or rod-specific markers was observed after specific knockdown of either Cralbp a or Cralbp b.**

Thus, the zebrafish genome contains duplicated CRALBP genes that, at 5 dpf, exhibit exclusive patterns of expression within the RPE (rlbp1a) or Müller glia (rlbp1b).

**Müller- and RPE-CRALBP in Cone Vision**

At 5 dpf, zebrafish vision is mediated by cone photoreceptors, and the rod photoreceptors, though present, do not contribute to visual function. The ability to deplete RPE-CRALBP or Müller-CRALBP selectively at 5 dpf enabled us to assess their contribution to cone vision by using the optokinetic response assay. In this assay, larval fish are immobilized in a viscous medium that allows free rotation of the eyes, and a drum lined with alternating black and white stripes rotates about the larvae. A saccade, or change in eye angle greater than 20°, is detected as a response to this moving stimulus is easily observed, and the number of saccades as a function of time is used as a quantitative test for visual response. Wild-type and control morpholino-injected larvae responded with ~25 to 30 saccades per minute of drum rotation (Fig. 5). Knockdown of RPE-CRALBP or Müller-CRALBP resulted in a statistically significant reduction in saccade response, showing that zebrafish CRALBP expressed in RPE and Müller glia are independently essential for normal cone vision.

**DISCUSSION**

Although the complete functional profile of CRALBP is unknown, its physiological importance is underlined by genetic association with heritable forms of blindness.
known component of the rod visual cycle regeneration of 11-cis-retinal, where it functions as an acceptor of 11-cis-retinol and a substrate carrier for 11-cis-retinol dehydrogenase (RDH5). Insights into CRALBP function have been gathered from rod-dominant models. In the present study, using the cone-dominant zebrafish, we identified two CRALBP oohnologues and characterize their expression and function.

Both zebrafish CRALBP genes were expressed in the sensory pineal at early developmental stages, but by 7 dpf, only rlbp1b continued to exhibit pineal expression. CRALBP expression in the pineal was observed earlier than in the retina, consistent with the rapid organogenesis of the zebrafish pineal. Many phototransduction components, though expressed in mammalian and nonmammalian pineals, are probably evolutionarily relics, as entainment of circadian output from the mammalian pineal is controlled by light-sensitive ganglion cells in the retina. However, in zebrafish, signals from the eye are not necessary for circadian entrainment, and the pineal contains cone-like photoreceptors that are directly sensitive to light.

We found no evidence that either zebrafish CRALBP oohnologue is under diurnal or circadian regulation in the pineal. However, it is plausible that pineal CRALBP(s) regulate entrainment of circadian rhythms to new light–dark cycles. Further studies are needed to resolve the potential role for CRALBP in olfaction. Our data indicate expression of zebrafish CRALBP genes in olfactory placodes, consistent with a recent report implicating Pax6 as a regulator of CRALBP expression in the developing mouse brain and the established role of Pax6 in eye and olfactory system development.

In the retina, recent work in cone-dominant models has hypothesized a cone visual cycle involving Müller-CRALBP. Characterization of the cone visual cycle is warranted given that loss of cone vision results in debilitating blindness. CRALBP knockout mice have abnormal cone physiology, but this effect cannot be attributed solely to either RPE- or Müller-CRALBP, as expression is eliminated in both locations. The duplication and divergence of expression of zebrafish CRALBP oohnologues has enabled us to analyze the role of each oohnologue selectively. In our study, rlbp1b was predominately expressed in Müller glial cells and rlbp1a in the RPE. Knockdown of either RPE-CRALBP or Müller-CRALBP resulted in abnormal cone vision. Based on the known biochemistry of CRALBP, abnormal cone vision resulting from zebrafish Cralbp a/Cralbp b depletion probably resulted from impaired retinoid metabolism, culminating in delayed regeneration of 11-cis-retinal and diminished visual pigment assembly. In summary, our data (1) provide primary evidence confirming that Müller-CRALBP contributes to cone vision and (2) demonstrates a novel role for RPE-CRALBP in contributing to cone vision.

We propose a revised model of CRALBP function in the rod and cone visual cycles (Fig. 6). RPE-CRALBP facilitates provision of the 11-cis-retinal required by rods for visual pigment regeneration as previously described. Müller-CRALBP, and perhaps RPE-CRALBP, facilitate the provision of 11-cis-retinol to cones. Unlike rods, cones have an 11-cis-retinol dehydrogenase activity that oxidizes 11-cis-retinol to 11-cis-retinal. Furthermore, cones, but not rods, can regenerate visual pigments from 11-cis-retinol or 11-cis-retinal. Thus, we speculate that RPE-CRALBP facilitates the provision of 11-cis-retinol to cones. This suggests two cell sources and three pathways that provide 11-cis-retinoids to cones compared with one for rods. However, CRALBP purified from RPE has been found bound only to 11-cis-retinal, whereas CRALBP purified from the neuroretina is bound to 11-cis-retinol. Thus, further investigation and refinement of the model is warranted, as the model does not pinpoint a location in the neuroretina where CRALBP is bound to 11-cis-retinal.

A recent report provides evidence of the existence of a previously unannotated, noncoding exon in the human RBP1 gene. The data suggest the presence of alternative transcription start sites in the human CRALBP gene, although it remains to be determined whether in vivo these are uniquely or preferentially used during development or in specific cells. We also identify previously unidentified, noncoding exons in the zebrafish rlbp1a gene and confirm their presence in transcripts containing the rlbp1a coding sequence. Zebrafish represent an excellent model system with which to characterize the regulation of CRALBP genes in vivo because of their amenability to transient transgenesis, rapid development, and transparency.

In addition, the duplication and divergence of expression of zebrafish CRALBP genes provides a serendipitous model to distinguish the transcriptional regulators of RPE- and Müller-specific expression.

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References


