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## Cyp27c1 red-shifts the spectral sensitivity of photoreceptors by converting vitamin A<sub>1</sub> into A<sub>2</sub>

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### Summary

Some vertebrate species have evolved means of extending their visual sensitivity beyond the range of human vision. One mechanism of enhancing sensitivity to long-wavelength light is to replace the 11-*cis* retinal chromophore in photopigments with 11-*cis* 3,4-didehydroretinal. Despite over a century of research on this topic, the enzymatic basis of this perceptual switch remains unknown. Here, we show that a cytochrome P450 family member, Cyp27c1, mediates this switch by converting vitamin A<sub>1</sub> (the precursor of 11-*cis* retinal) into vitamin A<sub>2</sub> (the precursor of 11-*cis* 3,4-didehydroretinal). Knockout of *cyp27c1* in zebrafish abrogates production of vitamin A<sub>2</sub>, eliminating the animal's ability to red-shift its photoreceptor spectral sensitivity, and reducing its ability to see and respond to near-infrared light. Thus, the expression of a single enzyme mediates

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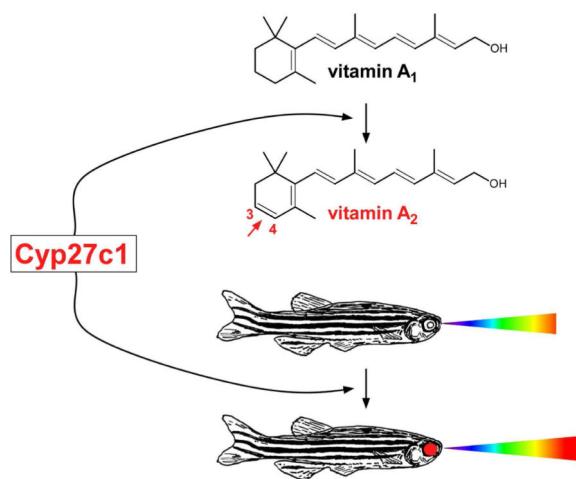
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#### Author Contributions

J.M.E. and J.C.C. conceived and coordinated the study. J.M.E. conducted expression profiling, genetic analysis, *in situ* hybridization and antibody staining, Western blotting, generation and maintenance of transgenic lines, and behavioral assays. J.R.A. and S.L.J. were involved in initial thyroid hormone treatment experiments and enzyme assays. S.E.T. consulted on the design of the behavioral assay and conducted analysis of behavioral data. M.J.H. and N.W.R. provided software for analysis of behavioral data as well as other support. M.B.T. produced the anti-Cyp27c1 antibody. J.M.E. and M.B.T. performed retinoid analyses. S.S. and V.J.K. performed single-cell suction electrode recordings and analyzed the resultant data. R.F., V.M.K., L.D.N., K.M.J., Y.X., and F.P.G. conducted *in vitro* biochemical assays and analyzed the resultant data. J.M.E. and J.C.C. wrote, and all authors edited and approved the manuscript.

dynamic spectral tuning of the entire visual system by controlling the balance of vitamin A<sub>1</sub> and A<sub>2</sub> in the eye.

## Graphical Abstract



## Introduction

The visual pigments of vertebrate rod and cone photoreceptors consist of a G protein-coupled receptor, the opsin, and a covalently bound chromophore, most commonly 11-*cis* retinal [1]. Vision begins when a photon of light induces a *cis* to *trans* isomerization of the chromophore, thereby altering the conformation of the opsin and, in turn, activating the phototransduction cascade [1, 2]. The spectral sensitivity of a visual pigment is determined by the structure of the chromophore and the electrostatic and steric interactions between the chromophore and the amino acid side chains within the binding cleft of the opsin [3, 4].

Typical rod photopigments have a maximum absorbance ( $\lambda_{\max}$ ) of around 500 nm [5]. However, it was first noted in the 19<sup>th</sup> century that certain freshwater fishes have rod photopigments whose  $\lambda_{\max}$  values are red-shifted by 20-30 nm relative to those of marine fishes and terrestrial vertebrates [6, 7]. The spectral sensitivity of cone photoreceptors is also red-shifted in freshwater species, in some cases by as much as 60 nm [8]. This red-shift is caused by replacement of the chromophore, 11-*cis* retinal with 11-*cis* 3,4-didehydroretinal, which contains an additional conjugated double bond within its  $\beta$ -ionone ring (Figure 1A) [9]. Rod photopigments containing 11-*cis* retinal are referred to as ‘rhodopsins’ on account of the rose-colored appearance of the unbleached pigment, whereas those containing 11-*cis* 3,4-didehydroretinal are called ‘porphyropsins’ on account of their purple color [7, 9, 10]. Therefore, the change in chromophore from 11-*cis* retinal to 11-*cis* 3,4-didehydroretinal is referred to as the ‘rhodopsin-to-porphyropsin’ switch.

This switch is believed to result in a better match between the sensitivity of the visual system and the spectral distribution of light in fresh water, which is often red-shifted relative to marine and terrestrial environments [9-11]. 3,4-didehydroretinoids have been identified in the eyes of the vast majority of freshwater fish and amphibian species that have been

examined [11, 12]. This observation suggests that thousands of vertebrate species may use chromophore switching to tune the spectral sensitivity of their visual systems. In addition, in many species of lampreys, fish, and amphibians, the visual system is dynamically tuned by altering the balance of 11-*cis* retinal and 11-*cis* 3,4-didehydroretinal in the retina when the animal moves between different light environments [11, 13-16]. For example, the ratio of 11-*cis* 3,4-didehydroretinal to 11-*cis* retinal increases when species such as salmon or sea lamprey migrate from the open ocean into inland freshwater environments (Figure 1B) [13, 15]. Conversely, many amphibians use 11-*cis* 3,4-didehydroretinal during the freshwater tadpole stage, but then switch to 11-*cis* retinal upon metamorphosis into a terrestrial adult (Figure 1B) [11, 14, 16]. Although several environmental factors, including changes in temperature or season, may affect the balance of the two chromophores [11, 17], there is clearly a strong correlation between the red-shifted light environment of freshwater habitats and the use of the red-shifted chromophore, 11-*cis* 3,4-didehydroretinal [10, 11, 13, 15].

Despite many decades of research on the rhodopsin-porphyropsin system, the enzyme that mediates this switch has been unknown. In this study, we employed transcriptome profiling of distantly related fish and amphibian models to identify the enzyme responsible for converting retinol into 3,4-didehydroretinol (henceforth referred to as vitamin A<sub>1</sub> and vitamin A<sub>2</sub>, respectively). We confirmed that this enzyme is required for the red-shift in photoreceptor sensitivity that occurs upon chromophore exchange *in vivo* and demonstrated that this change in sensitivity enhances the fish's ability to see and respond to near-infrared light.

## Results

### Animal Models of the Vitamin A<sub>1</sub>/A<sub>2</sub> Switch

To identify the enzyme responsible for converting vitamin A<sub>1</sub> into vitamin A<sub>2</sub>, we used zebrafish (*Danio rerio*) and American bullfrog (*Lithobates catesbeianus*) models. Zebrafish photoreceptors predominantly contain vitamin A<sub>1</sub>-based visual pigments under normal laboratory conditions, but treatment with thyroid hormone (TH) induces a conversion to vitamin A<sub>2</sub>-based pigments [18]. To corroborate this result, we treated zebrafish with TH and then analyzed the retinoid content of retinas and retinal pigment epithelium (RPE) by high-performance liquid chromatography (HPLC). We found a nearly complete conversion of vitamin A<sub>1</sub> into vitamin A<sub>2</sub> in the TH-treated animals (Figure 1C-F).

Next, we analyzed a specialized pattern of regional chromophore localization within the American bullfrog retina. Most amphibians employ A<sub>2</sub>-based visual pigments during the aquatic phase of their life cycle, switching to predominantly A<sub>1</sub>-based pigments after metamorphosis [11, 14, 16]. However, American bullfrogs retain A<sub>2</sub>-based pigments in the dorsal third of their retinas and RPE, even as adults (Figure 1G) [10]. Since adult bullfrogs spend a considerable amount of time with their eyes just above the water's surface [19], the presence of vitamin A<sub>2</sub>-based pigments in the dorsal retina has been speculated to facilitate downward vision into the murky, red-shifted aquatic environment [10]. We dissected dorsal and ventral RPE from adult American bullfrogs, extracted retinoids, and analyzed them by HPLC. Whereas the ventral RPE contained exclusively vitamin A<sub>1</sub> and its derivatives, the dorsal RPE contained a mixture of vitamin A<sub>1</sub> and A<sub>2</sub> (Figure 1H-I). Thus, both TH-treated

zebrafish and American bullfrog represent suitable model systems for analyzing the mechanistic basis of the rhodopsinporphyropsin switch.

### Identifying a Candidate Enzyme

The RPE plays a critical role in regenerating 11-*cis* retinal and is the locus of vitamin A<sub>1</sub> to A<sub>2</sub> conversion in some species [10, 20, 21]. Therefore, to identify candidate genes that might encode the vitamin A<sub>1</sub> 3,4-dehydrogenase, we profiled zebrafish and bullfrog RPE by RNA-seq. We compared the transcriptomes of RPE from TH-treated zebrafish with that of vehicle-treated controls, identifying a total of 35 TH-upregulated genes (Figure 2A, Table S1). We also compared dorsal and ventral RPE from adult bullfrogs, identifying a total of 40 dorsally enriched genes (Figure 2B, Table S2). In both analyses, one candidate gene stood out as being among the most highly upregulated and enriched, the cytochrome P450 family member, *cyp27c1* (Figure 2A,B). Members of the P450 family are involved in the metabolism of a variety of xenobiotics and endogenous small molecules including retinoids, although a functional role for *cyp27c1* has not previously been reported [22-25]. Thus, Cyp27c1 is an excellent candidate for the vitamin A<sub>1</sub> 3,4-dehydrogenase.

To determine whether the presence of *cyp27c1* correlates with the distribution of vitamin A<sub>2</sub> and its derivatives, we analyzed *cyp27c1* expression in zebrafish and bullfrog eyes. We first confirmed that the *cyp27c1* transcript is significantly increased in TH-treated zebrafish RPE and dorsal bullfrog RPE by quantitative real-time PCR (Figure 2C). We then determined the cellular expression pattern of *cyp27c1*, by performing *in situ* hybridization on eyes from TH-treated *albino* zebrafish and vehicle controls [26]. The *albino* strain was used to improve visualization of staining in the RPE, and we confirmed that it undergoes a TH-dependent switch to vitamin A<sub>2</sub> similar to wild-type strains (data not shown). The *cyp27c1* antisense probe localized exclusively to the RPE after TH-treatment, with no signal observed in the vehicle-treated control eyes (Figure 2D). In contrast, the RPE-specific transcript, *rpe65a*, was detected in both TH- and vehicle-treated eyes, confirming the presence of RPE in the sections (Figure 2D). Taken together, these results indicate a specific upregulation of *cyp27c1* in the RPE of TH-treated fish, and are consistent with a previous report suggesting that the RPE is the primary locus of vitamin A<sub>2</sub> production in some species [10].

To assay the expression levels of Cyp27c1 protein in zebrafish and bullfrog RPE, we raised a polyclonal anti-Cyp27c1 antibody in rabbit, and confirmed its activity against Cyp27c1 produced in HEK-293 cell culture (Figure S1). We then used this antibody in a Western blot to confirm the induction of Cyp27c1 protein in TH-treated zebrafish RPE and its enrichment in dorsal bullfrog RPE (Figure 2E). In addition, using immunohistochemistry, we determined that Cyp27c1 protein is localized specifically to the RPE of TH-treated, but not control *albino* zebrafish, in a pattern consistent with the *cyp27c1* transcript (Figure 2D,F). Overall, the distribution of both the *cyp27c1* transcript and protein correspond precisely to the pattern of vitamin A<sub>2</sub> production in zebrafish and bullfrog.

### Cyp27c1 Generates Vitamin A<sub>2</sub> *in vitro*

To test if Cyp27c1 is sufficient to produce vitamin A<sub>2</sub>, we expressed Cyp27c1 in HEK-293 cells, which were then incubated with vitamin A<sub>1</sub> for 24 hours and subjected to HPLC to

assess retinoid content. Cells transfected with an empty vector contained only the vitamin A<sub>1</sub> substrate, whereas cells transfected with the Cyp27c1 expression construct contained vitamin A<sub>2</sub> in addition to vitamin A<sub>1</sub> (Figure 3A). To further characterize enzyme kinetics, we expressed and purified Cyp27c1 and determined its catalytic efficiency with three different retinoid substrates: all-*trans* retinol, retinal, and retinoic acid. Cyp27c1 can act on all three substrates but most efficiently catalyzes the conversion of retinol (vitamin A<sub>1</sub>) to 3,4-didehydroretinol (vitamin A<sub>2</sub>; Figure 3B, Figure S2). The catalytic efficiency of Cyp27c1 with vitamin A<sub>1</sub> ( $k_{\text{cat}}/K_m = 1.9 \pm 0.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ ) is among the highest reported for animal cytochrome P450 family members [27-30]. In addition, only human CYP21A2 (2.8 s<sup>-1</sup>) and CYP7A1 (2.5-6 s<sup>-1</sup>) have been shown to have  $k_{\text{cat}}$  values this high ( $k_{\text{cat}} = 3.8 \text{ s}^{-1}$  for zebrafish Cyp27c1, Figure 2S) [28-30]. These results confirm that Cyp27c1 has the requisite biochemical activity to mediate the rhodopsin-porphyrpsin switch.

### Cyp27c1 Drives Vitamin A<sub>2</sub> Production *in vivo*

If Cyp27c1 mediates the rhodopsin-porphyrpsin switch, zebrafish lacking this enzyme should not produce vitamin A<sub>2</sub> in response to TH treatment. To test this hypothesis, we generated several mutant alleles of *cyp27c1* by using transcription activator-like effector nucleases (TALENs) to introduce small insertions/deletions within the first and fourth exons [31]. We obtained multiple alleles carrying frameshift mutations predicted to result in premature stop codons (Figure 4A; Figure S3). Two independent lines were crossed to produce transheterozygous mutant fish (*cyp27c1*<sup>1/2</sup>), which survived to adulthood without overt developmental abnormalities. Upon treatment with TH, the mutant fish failed to produce Cyp27c1 protein, in contrast to their wild-type siblings (Figure 4B). We then used HPLC to assess retinoid content in the eyes of TH-treated *cyp27c1*<sup>1/2</sup> mutants and their wild-type siblings. While a conversion to vitamin A<sub>2</sub> was observed in TH-treated wild-type fish, TH-treated *cyp27c1*<sup>1/2</sup> mutants failed to produce any vitamin A<sub>2</sub> (Figure 4C), indicating that Cyp27c1 is necessary for vitamin A<sub>2</sub> production *in vivo*. We also observed loss of vitamin A<sub>2</sub> production in fish carrying two additional heteroallelic combinations, *cyp27c1*<sup>1/3</sup> and *cyp27c1*<sup>4/5</sup> (Figure S3), indicating that this effect is attributable to the loss of Cyp27c1 rather than off-target mutations caused by the TALENs.

As TH-treated *cyp27c1*<sup>1/2</sup> fish fail to produce vitamin A<sub>2</sub> (the precursor of 11-*cis* 3,4-didehydroretinal), their photoreceptors should also fail to undergo a red-shift in sensitivity. To test this hypothesis, we used single-cell suction electrode recording to measure the sensitivity of individual red cones from *cyp27c1*<sup>1/2</sup> mutants and wild-type siblings, treated with either TH or vehicle control. We measured red cones because they undergo the largest red-shift upon switching to 11-*cis* 3,4-didehydroretinal [18]. The flash sensitivity of individual red cones was determined from the amplitudes of their electrical responses to a series of dim flashes of 560, 600, 660, and 700 nm light, and fitted with a model that incorporates contributions from both vitamin A<sub>1</sub>- and A<sub>2</sub>-based pigments [8]. No significant difference was observed between the sensitivities of vehicle-treated *cyp27c1*<sup>1/2</sup> and wild-type red cones at any of the wavelengths tested, and both wild-type and mutant red cones had maximal sensitivity ( $\lambda_{\text{max}}$ ) at 561 nm, in close agreement with a previous estimate of  $\lambda_{\text{max}} = 565 \text{ nm}$ , determined by MSP (Figure 4D,E) [18]. In addition, knockout of *cyp27c1* did not affect the dark current or the kinetics of the flash responses of the red cones,

indicating that phototransduction remained intact (Figure 4E). In contrast, TH treatment induced a 57 nm red-shift in the  $\lambda_{\max}$  of wild-type fish to 618 nm, whereas the  $\lambda_{\max}$  of TH-treated *cyp27c1*<sup>1/2</sup> mutant fish was unchanged (Figure 4D,E). Thus, knockout of *cyp27c1* completely eliminates the red-shift in red cone spectral sensitivity induced by TH treatment.

### Behavioral Impact of Vitamin A<sub>2</sub>

It has long been speculated that the switch from vitamin A<sub>1</sub> to vitamin A<sub>2</sub>-based photopigments could be an adaptive response to changes in the animal's light environment, permitting the animal to adjust its spectral sensitivity to more closely match the spectral quality of its surroundings [10, 11, 13, 15]. The *cyp27c1* mutant zebrafish present an opportunity to test the relationship between vitamin A<sub>2</sub> and behavioral responsiveness to long-wavelength light. We therefore developed an assay to measure changes in swimming behavior in response to light of various wavelengths (Figure 5A-C). When exposed to a directional light source, adult zebrafish display a positive phototactic response (Movies S1 and S2) [32]. We hypothesized that TH-treated wild-type fish would have a stronger phototactic response to near-infrared light than *cyp27c1*<sup>1/3</sup> mutants due to the vitamin A<sub>2</sub>-induced red-shift in spectral sensitivity.

We compared the behavior of dark-adapted, vehicle- and TH-treated *cyp27c1*<sup>1/3</sup> and wild-type siblings in response to 590 nm and 770 nm light (Figure 5; see Figure S4 for LED characterization). Fish with vitamin A<sub>1</sub>- and A<sub>2</sub>-based photopigments are predicted to perceive 590 nm light similarly, based on the sensitivity curves of red cones (Figure 5B). As expected, in both the vehicle and TH treatments, *cyp27c1*<sup>1/3</sup> mutants and wild-type fish exhibited positive phototaxis toward a 590 nm light source (Figure 5D). This finding indicates that normal visually-mediated behaviors are intact and functional in *cyp27c1*<sup>1/3</sup> mutants. In contrast, upon exposure to 770 nm light, only wild-type, TH-treated fish demonstrated a positive phototactic response, while *cyp27c1*<sup>1/3</sup> mutants and vehicle control fish behaved as if they were in the dark (Figure 5E). These data indicate that the red-shift in photoreceptor spectral sensitivity caused by switching to vitamin A<sub>2</sub>-based photopigments improves the animal's ability to detect and respond to near-infrared light. This increase in long-wavelength sensitivity may provide a selective advantage under some environmental conditions, and may thus have contributed to the evolution of the rhodopsin-porphyrin system.

### Discussion

We have shown that the action of a single enzyme, Cyp27c1, is necessary and sufficient for the production of vitamin A<sub>2</sub> and its derivatives, and for the resultant red-shift in photoreceptor spectral sensitivity. Furthermore, we have demonstrated that this change in sensitivity at the cellular level can be translated into altered behavioral responsiveness to long-wavelength light. These findings assign a biochemical function to a previously orphan member of the cytochrome P450 family of enzymes. In addition, the generation of *cyp27c1* mutants offers an opportunity to study the behavioral consequences of switching from vitamin A<sub>1</sub>- to vitamin A<sub>2</sub>-based photopigments.

Our work raises a number of intriguing questions about the role of Cyp27c1 *in vivo*. For example, the preferred substrate(s) of Cyp27c1 *in vivo* is currently unknown. A number of potential substrates are present in the RPE, including all-*trans* retinol, all-*trans* retinyl esters, 11-*cis* retinol, 11-*cis* retinal, or even 11-*cis* retinyl esters [20, 33]. Our cell-free assays suggest that all-*trans* retinol could be the *in vivo* substrate, but future studies will be needed to measure the activity of Cyp27c1 on 11-*cis* retinoids and characterize the retinoids metabolized by Cyp27c1 *in vivo*. Interestingly, whereas some enzymes involved in the visual cycle (LRAT, RPE65, 11cRDH) are localized to the smooth endoplasmic reticulum, prediction algorithms suggest that Cyp27c1 may be located in mitochondria, as are Cyp27a1 and Cyp27b1 [20, 34, 35]. The subcellular localization of Cyp27c1 and the pathways by which retinoids might be trafficked between cellular organelles also remain open questions.

The ability of TH to induce the expression of *cyp27c1* raises the possibility that endogenous TH might play a general role in the regulation of responsiveness to long-wavelength light under natural conditions. For instance, TH is thought to play a key role in coordinating the physiologic changes that salmonids undergo during migration, suggesting that TH may mediate the switch between vitamin A<sub>1</sub>- and A<sub>2</sub>-based photopigments in these animals [36, 37]. In addition, the salmon genome contains multiple paralogous red and green cone opsin genes, and TH treatment induces a shift in the expression of these opsins in favor of the red-shifted paralogs [37]. Lastly, both zebrafish and mice require thyroid hormone receptor β2 for normal expression of long wavelength-sensitive cone opsin [38, 39]. These findings suggest that TH signaling might represent an ancient pathway for the regulation of sensitivity to long-wavelength light, both at the level of chromophore modification and opsin gene expression.

Despite the widespread use of the rhodopsin-porphyrin system amongst cold-blooded vertebrates, vitamin A<sub>2</sub>-based photopigments have never been documented in the eyes of birds or mammals. One possible explanation for this absence is that A<sub>2</sub>-based chromophores are less thermally stable than those based on A<sub>1</sub>. Thus, increased thermal noise may limit the usefulness of A<sub>2</sub> chromophores in the visual systems of warm-blooded species. Nevertheless, orthologs of *cyp27c1* are present in nearly all sequenced avian genomes as well as most mammalian genomes, including the human genome. The function of *cyp27c1* in these species is currently unknown, but several studies suggest diverse roles for this gene outside of the eye. For example, 3,4-didehydroretinoic acid (a derivative of vitamin A<sub>2</sub>) has been reported to have biological activity similar to retinoic acid in chicken embryos, and is the predominant form of 'retinoic acid' in the developing chick [40, 41]. In addition, the presence of 3,4-didehydroretinoids has been documented in normal human skin (and at elevated levels in hyperkeratotic lesions and some skin neoplasms), suggesting a potential role for CYP27C1 in retinoid biosynthesis in this organ [42, 43]. Future studies will be needed to address the role of *cyp27c1* in these contexts.

Further work may explore the extent of Cyp27c1 expression and function in the visual systems of different species. A recent study demonstrated that the ratio of vitamin A<sub>1</sub> to A<sub>2</sub> can vary between rods and cones in the same retina [44]. This finding raises the possibility that Cyp27c1 might be differentially expressed between these two cell types in some species. Alternatively, these species might express Cyp27c1 in Müller glia, a cell type that

supports cone, but not rod, pigment regeneration [21]. It is also worth noting that vitamin A<sub>2</sub>-based chromophores are known to be used in some invertebrate species. This fact suggests that functional homologs of Cyp27c1 could be widespread in the animal kingdom.

The present study also has important implications for the field of optogenetics. Optogenetic actuators are microbial opsins that use retinal as a chromophore and can modulate the electrical activity of neurons in response to light [45]. Actuators with red-shifted excitation spectra are highly desirable, because longer wavelength light penetrates more deeply into tissue and red-shifting reduces spectral overlap with co-expressed sensors. Accordingly, several research groups have created red-shifted optogenetic actuators either by bioprospecting for novel actuators with red-shifted excitation spectra or by generating red-shifted variants of known actuators by protein engineering [46-49]. It was recently shown that the sensitivity of optogenetic actuators can be red-shifted *in vitro* when the chromophore, 3,4-didehydroretinal, is substituted for retinal [50]. This finding suggests that co-expression of Cyp27c1 could be used to red-shift optogenetic actuators into the near infrared in selected neuronal populations *in vivo*.

## Experimental Procedures

See the Supplemental Experimental Procedures for detailed experimental procedures.

### Animals and Thyroid Hormone Treatment

All procedures were carried out in accordance with animal protocols approved by the Animal Studies Committee of Washington University. Adult zebrafish (>3 months old) were used for all experiments. The specific strains include: WT AB\* and SJD, *albino*, *cyp27c1*<sup>1</sup>, *cyp27c1*<sup>2</sup>, *cyp27c1*<sup>3</sup>, *cyp27c1*<sup>4</sup>, and *cyp27c1*<sup>5</sup>. The *cyp27c1* transgenic lines were generated using TALENs targeting exon 1 (1 - 4) and exon 4 (5). This strategy generated short indels that resulted in frameshift mutations leading to premature stop codons and severe truncation of the transcript. Thyroid hormone treatment consisted of a three-week incubation in either thyroid hormone (750 μL 400 μg/mL L-thyroxine in 0.1 M NaOH per liter of water for a final concentration of 300 μg/L) or vehicle control (750 μL of 0.1 M NaOH per liter of water). Bullfrog eyes were obtained post-mortem.

### High Performance Liquid Chromatography (HPLC)

Conditions for HPLC were selected to isolate retinal and retinol. Tissue from zebrafish and bullfrog was isolated, frozen on dry ice and stored at -80 °C prior to use. Retinoids were extracted in hexanes and dried under nitrogen. Retinal was then converted to retinol by treating the sample with NaBH<sub>4</sub> prior to drying under nitrogen and resuspension in acetonitrile. Reverse phase HPLC was conducted on an Agilent 1100 Series HPLC system equipped with a YMC carotenoid 5.0 μm column (4.6 mm × 250 mm, cat no. 924012, YMC America Inc., Allentown, PA). For the tissue samples, we injected 90 μL of sample and eluted isocratically with a mobile phase of acetonitrile:methanol:dichloromethane 70:15:15 (vol:vol:vol) at a flow rate of one mL min<sup>-1</sup> and a column temperature of 18°C.

HPLC was also conducted on HEK-293 cells, which had been transfected with Cyp27c1 or an empty vector and supplied with vitamin A<sub>1</sub> as a substrate (further described in the



Supplemental Experimental Procedures). Retinoids were extracted from the HEK-293 cells and their media as above and resuspended in mobile phase. These samples were eluted isocratically with a mobile phase of acetonitrile:water 80:20 (vol:vol) at a flow rate of one mL min<sup>-1</sup> and column temperature of 18°C. Retinol (vitamin A1) and 3,4-didehydroretinol (vitamin A2) were identified by comparison with authentic standards obtained from Sigma (R7632, St. Louis, MO) and Santa Cruz Biotechnology Inc. (sc-209587, Dallas, TX), respectively.

### RNA-seq and qRT-PCR

RNA was isolated from zebrafish and bullfrog RPE using an RNeasy kit. Zebrafish cDNA libraries were generated using NuGen Ovation RNA-Seq System v2, while bullfrog mRNA was enriched using the Illumina Ribo-Zero kit. Samples were assayed for quality and quantity and sequenced on an Illumina HiSeq 2500 using single-end 50 bp reads. Zebrafish reads were aligned to a curated zebrafish transcriptome assembly. The reads were aligned and differential expression analysis was conducted as described in the supplemental experimental procedures. qRT-PCR was conducted on the same samples used for RNA-seq, to confirm these results. Primer sequences and other details related to qRT-PCR are listed in the Supplemental Experimental Procedures.

### *In situ* Hybridization, Immunohistochemistry, and Western Blotting

For *in situ* hybridization (ISH) and immunohistochemistry, 12 µm frozen sections were collected at or adjacent to the level of the optic nerve head. The approach and probes used for ISH are described in the Supplemental Experimental Procedures. To generate an antibody for IHC and Western blot, we created a peptide fragment encompassing amino acids 293-482 of zebrafish Cyp27c1 which was used by PrimmBiotech (West Roxbury, MA) to immunize two rabbits and three rats PrimmBiotech. This antibody was used for IHC and Western blotting as described in the Supplemental Experimental Procedures.

### Enzyme Kinetic Assays

The reaction mixtures contained recombinant zebrafish Cyp27c1, bovine adrenodoxin (Adx), bovine NADPH-adrenodoxin reductase (ADR), potassium phosphate (50 mM), and various concentrations of a retinoid substrate, which were added to an amber vial and pre-incubated in a water bath at 37°C prior to initiation with an NADPH generating system. Following a 60 second incubation, the reaction was quenched with 1.0 mL of ethyl acetate solution containing 45 µM butylated hydroxytoluene (to prevent radical reactions). The organic solvent was extracted, dried under nitrogen and resuspended in CH<sub>3</sub>CN (100 µL). HPLC-UV was used to separate and detect the products. Briefly, 10 µL of the sample was injected onto a Hypersil GOLD HPLC column (150 mm × 2.1 mm, 3 µm, Thermo Scientific) at 40°C and the products were eluted isocratically (0.5 mL/min) at 30% solvent A (95% H<sub>2</sub>O; 4.9% CH<sub>3</sub>CN; 0.1% formic acid, v/v) and 70% solvent B (95% CH<sub>3</sub>CN; 4.9% H<sub>2</sub>O; 0.1% formic acid, v/v) at a flow rate of 0.5 mL/min. Data analysis and graph generation was conducted using GraphPad Prism v. 5.0d, using a single site hyperbolic equation.

## Single Cell Suction Electrode Recording

Cell preparation was carried out under dim red light. Photoreceptors were dissociated by chopping and triturating the retina in Ringer solution, and transferred to the recording chamber, where they were perfused with Ringer solution. Red cones were identified based on their characteristic morphology and relatively high photosensitivity at 700 nm. Under infrared illumination, the inner segment of a red cone was drawn into a tight-fitting glass pipette for recordings and stimulated with calibrated 20 ms flashes centered at 560, 600, 660, and 700 nm. The light source was calibrated before and after each experiment. The signals were low-pass filtered at 30 Hz (8-pole Bessel), digitized at 1 kHz, and stored on a computer using Clampex 9.2 acquisition software (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed using Clampfit (Molecular Devices, Sunnyvale, CA, USA) and Origin 8.1 (OriginLab, Northampton, MA, USA).

## Phototaxis Assay

The recording setup consisted of a clear plastic rectangular tank (19 cm × 35 cm) with a diffuse light source located across either the right or left end of the tank. The light source was equipped with a switch to toggle between 590 and 770 nm LEDs, and a variable resistor to control illuminance. The fish were recorded with Sony HDR-SR11 video camera using a 940 nm backlight. The behavioral trials were conducted in a dark interior room with no windows. The immediate recording area (tank, light sources, and camera) was draped with a double layer of black fleece and monitored periodically using a small monitor covered in red plastic located outside of the draping.

We assayed 11 TH-treated *cyp27c1*<sup>1/3</sup> mutants, 12 TH-treated wild-types, 10 vehicle-treated *cyp27c1*<sup>1/3</sup> mutants, and 10 TH-treated wild-type. One TH-treated *cyp27c1*<sup>1/3</sup> mutant was excluded, as it exhibited erratic swimming behavior. Fish were dark adapted overnight and transferred individually to the recording tank. After acclimating in the dark for 30 minutes, each fish was recorded for three trials at 770 nm, and then three trials at 590 nm. Each trial consisted of 3 minutes with the LEDs on and 3 minutes with the LEDs off, with the on/off order randomized. We also alternated the end of the tank illuminated in each trial. We tracked the paths of individual fish using an image difference approach with code written in Matlab (Release 2104a, The MathWorks Inc., Natick, MA, USA). We then calculated the percentage of time spent within 25 mm of the lit wall of the tank. The difference in mean time (as a percentage) spent against the lit end when LED lights were on and off was compared within groups with paired Student's t-tests (SPSS, version 21, IBM, Armonk, NY, USA).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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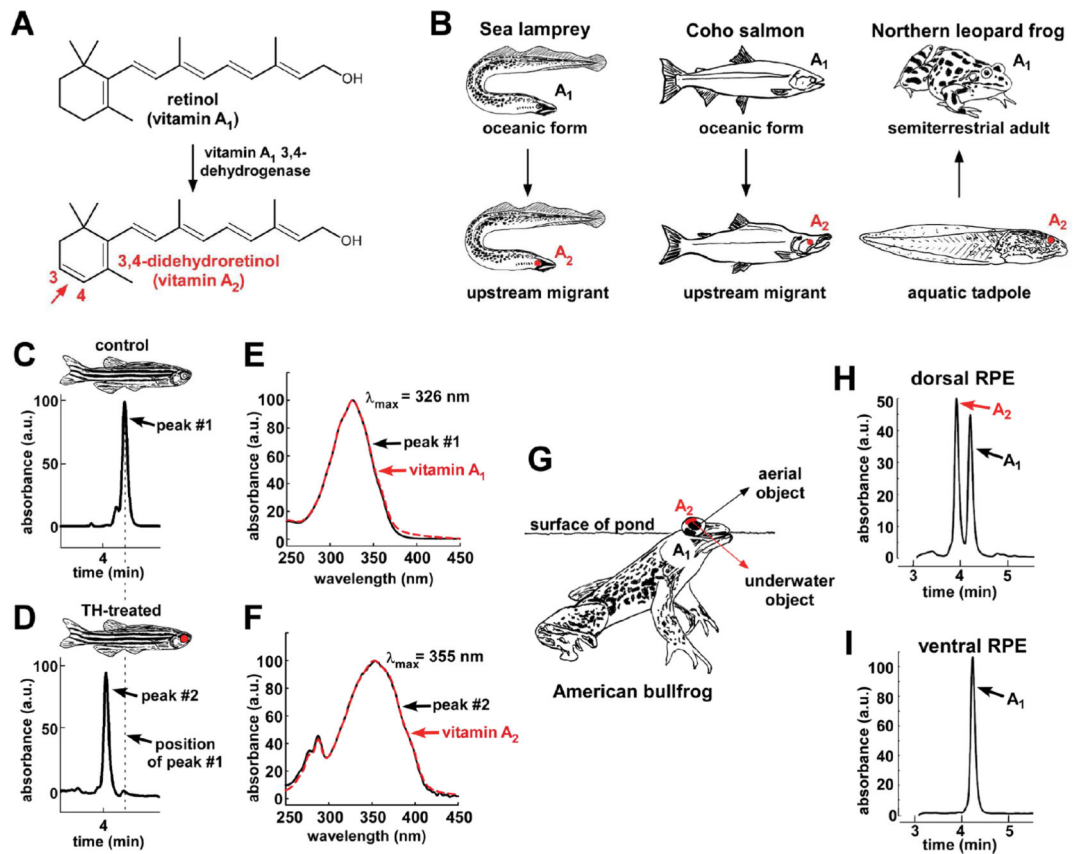
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**Figure 1. Modeling the rhodopsin-porphyrpsin switch**

(A) The conversion of retinol (vitamin A<sub>1</sub>) to 3,4-didehydroretinol (vitamin A<sub>2</sub>) underlies the rhodopsin-to-porphyrpsin switch and is driven by a previously unidentified dehydrogenase that catalyzes the addition of a double bond to the  $\beta$ -ionone ring.

(B) This vitamin A<sub>1</sub> to A<sub>2</sub> switch is widely used by a variety of vertebrates, and can be deployed at key stages of the life cycle: e.g., during upstream migration in sea lamprey (*Petromyzon marinus*) and Coho salmon (*Oncorhynchus kisutch*) and upon metamorphosis in amphibians such as the Northern leopard frog (*Lithobates pipiens*) [13-15].

(C, D) To model this switch, zebrafish were treated with thyroid hormone (300  $\mu$ g/L L-thyroxine [T4]) for three weeks. Retinoids were then isolated from pooled RPE and retina of three individuals, reduced using sodium borohydride and analyzed by HPLC. Retinoids from TH-treated fish have a shorter retention time than those from vehicle-treated fish.

(E) The absorbance spectrum of the predominant retinoid from control fish closely matches the absorbance spectrum of a vitamin A<sub>1</sub> standard, with a  $\lambda_{\max}$  of 326 nm.

(F) The predominant retinoid from TH-treated fish has an absorbance spectrum that matches that of the vitamin A<sub>2</sub> standard, with a  $\lambda_{\max}$  of 355 nm.

(G) The American bullfrog (*Lithobates catesbeianus*) often sits at the water's surface and possesses exclusively A<sub>1</sub>-based visual pigments in the ventral retina and a mixture of A<sub>1</sub>- and A<sub>2</sub>-based visual pigments in the dorsal retina, possibly facilitating downward vision into the murky, red-shifted water [10, 19].

(H, I) The dorsal third of the RPE from two American bullfrogs was dissected, pooled, and analyzed by HPLC and found to contain a mixture of vitamin A<sub>1</sub> and vitamin A<sub>2</sub>. Only

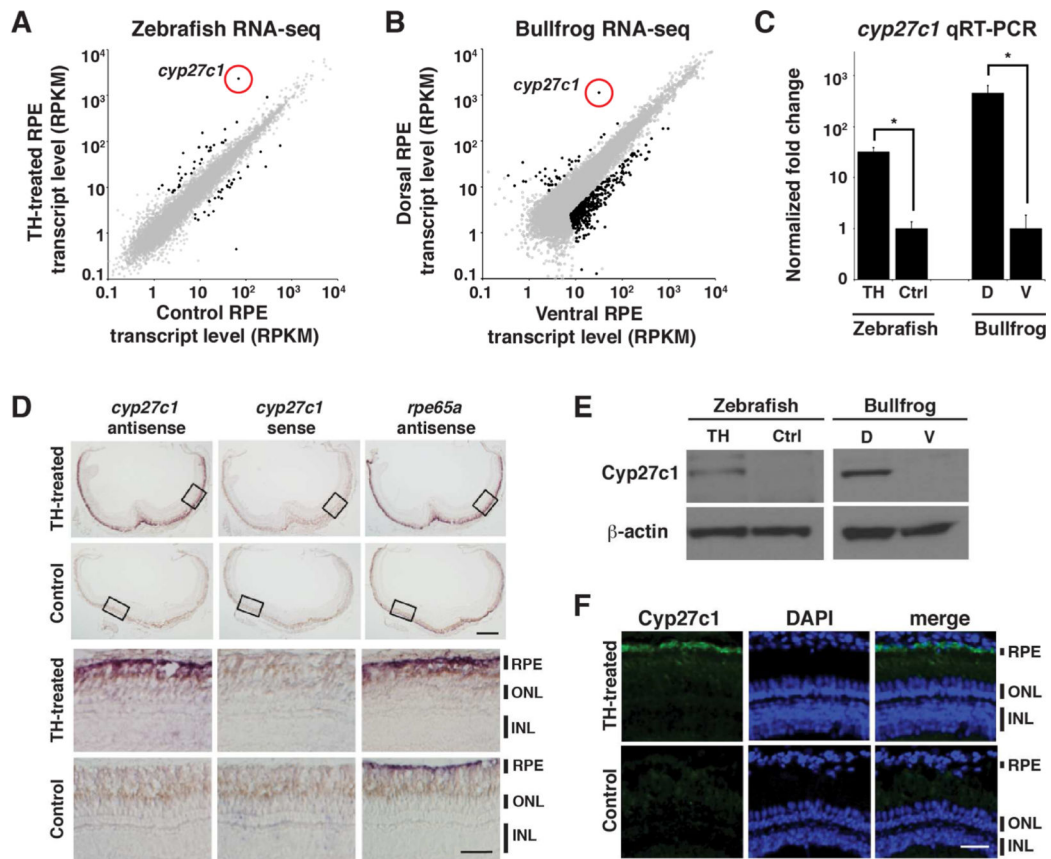
vitamin A<sub>1</sub> was identified in the ventral third of the RPE. All absorbance values are normalized and represented as arbitrary units (a.u.).

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**Figure 2. *Cyp27c1* expression is correlated with the presence of vitamin A<sub>2</sub> in zebrafish and bullfrog RPE**

(A) Zebrafish were treated with TH or a vehicle control for three weeks, after which RPE was isolated and used to construct a cDNA library for transcriptome profiling by RNA-seq. Expression levels (in RPKM, Reads Per Kilobase of transcript per Million mapped reads) of individual transcripts from TH-treated RPE (y-axis) and vehicle-treated RPE (x-axis) are shown as dots, with significantly differentially expressed genes in black (quantile-adjusted conditional maximum likelihood [qCML] test, FDR < 0.05, n = 3).

(B) Dorsal and ventral thirds of bullfrog RPE were isolated and used to construct a cDNA library for transcriptome profiling by RNA-seq. Expression levels of individual transcripts from dorsal RPE (y-axis) and ventral RPE (x-axis) are shown as dots, with significantly differentially expressed genes in black (qCML test, FDR < 0.05, n = 3).

(C) Enrichment of the *cyp27c1* transcript in cDNA samples used for RNA-seq was confirmed by quantitative real-time PCR (qRT-PCR). Expression was normalized to ribosomal protein *rpl13a* for zebrafish and *rpl7a* for bullfrog (two-sided Student's t-test, n = 2-3, \*p < 0.005, error bars = s.e.m.).

(D) *In situ* hybridization of *albino* zebrafish treated with TH or vehicle control for three weeks. Top panels show cross-sections of the whole eye. Bottom panels show high-magnification images of the boxed regions from the top panels. The antisense probe for *cyp27c1* localized exclusively to the RPE in TH-treated fish. No signal was observed with the *cyp27c1* sense probe, but strong signal was observed in the RPE of TH-treated and

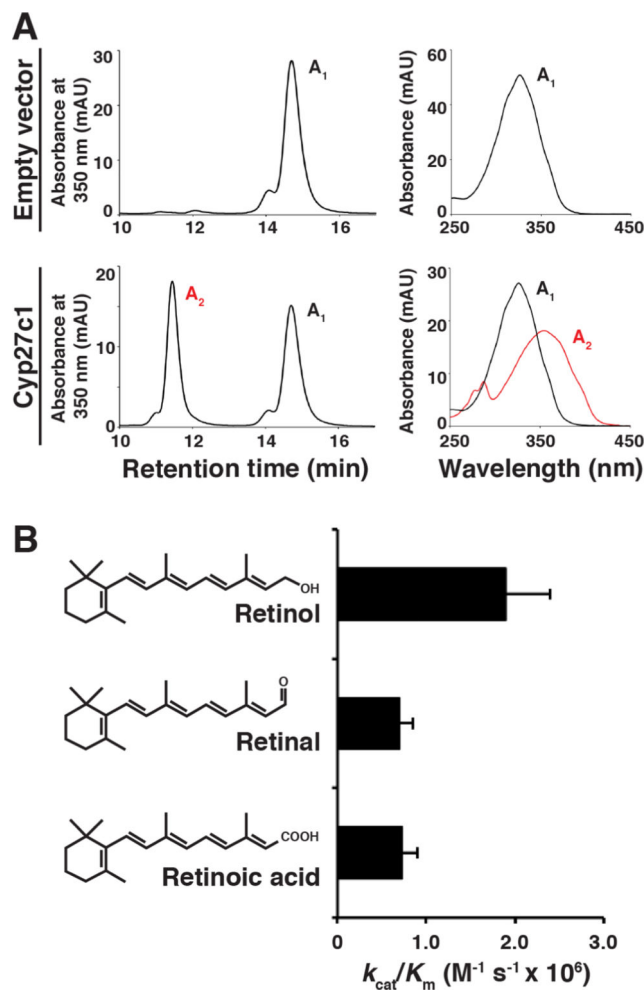


control fish with an antisense probe against *rpe65a*, a gene that is expressed at high levels in RPE. Scale bars = 200  $\mu\text{m}$  low power, 50  $\mu\text{m}$  high power.

(E) Western blot with a rabbit polyclonal anti-Cyp27c1 antibody confirmed enrichment of Cyp27c1 protein in TH-treated zebrafish and dorsal bullfrog RPE.  $\beta$ -actin was used as a loading control.

(F) Immunohistochemistry of *albino* TH- and vehicle-treated zebrafish indicates induction of Cyp27c1 expression in the RPE of TH-treated fish (green), with DAPI used to counter-stain nuclei (blue). Scale bar = 50  $\mu\text{m}$ .

See also Figure S1, Table S1, and Table S2.

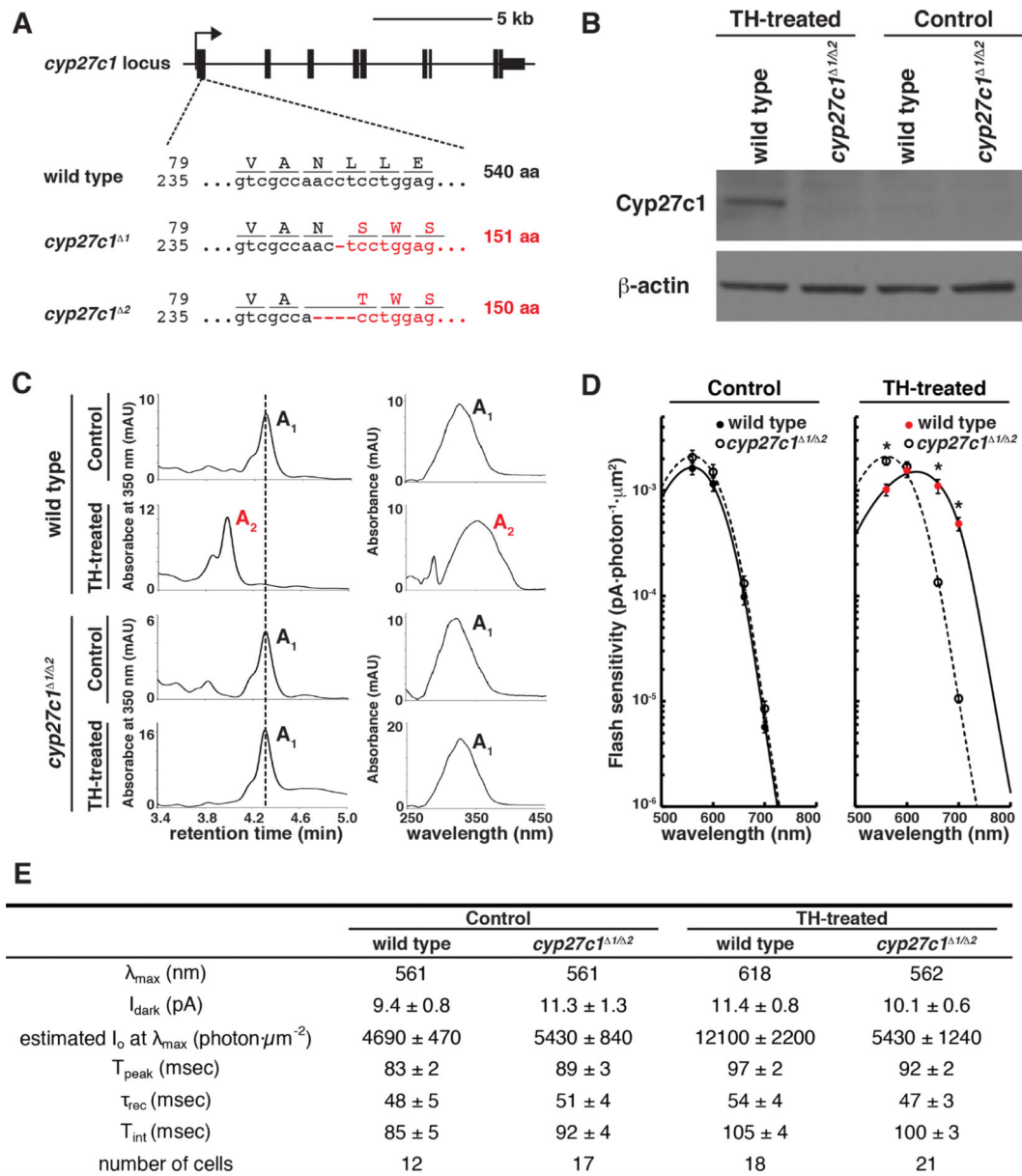


**Figure 3. Cyp27c1 is sufficient to convert vitamin A<sub>1</sub> into vitamin A<sub>2</sub>**

(A) HEK-293 cells were transfected with a *cyp27c1* expression construct or an empty vector and incubated with vitamin A<sub>1</sub> for 24 hours, after which cells and media were harvested for HPLC. Cells transfected with empty vector did not produce vitamin A<sub>2</sub>, but cells transfected with *cyp27c1* converted a substantial fraction of the vitamin A<sub>1</sub> to vitamin A<sub>2</sub>.

(B) A cell-free assay system was used to assess the catalytic efficiency of Cyp27c1 for all-*trans* retinol, retinal and retinoic acid. Purified Cyp27c1 was incubated with bovine adrenodoxin (Adx), NADPH-adrenodoxin reductase (ADR), and substrate (with concentrations ranging from 0.1 to 10 μM). The reaction was then initiated using an NADPH-generating system, run for 60 seconds, and quenched. HPLC was used to quantify the products in order to calculate  $k_{cat}$  and  $K_m$ . A high catalytic efficiency ( $k_{cat}/K_m$ ) was observed for all three substrates. Error bars = s.e.m.

See also Figure S2.



**Figure 4. Cyp27c1 is necessary for vitamin A<sub>2</sub> production and red-shifting of photoreceptors *in vivo***

(A) TALENs were used to generate *cyp27c1* mutant zebrafish. Two independent alleles are shown, *cyp27c1*<sup>1</sup> and *cyp27c1*<sup>2</sup>. Both alleles contain frame-shift mutations resulting in premature stop codons at amino acids 151 and 150, respectively.

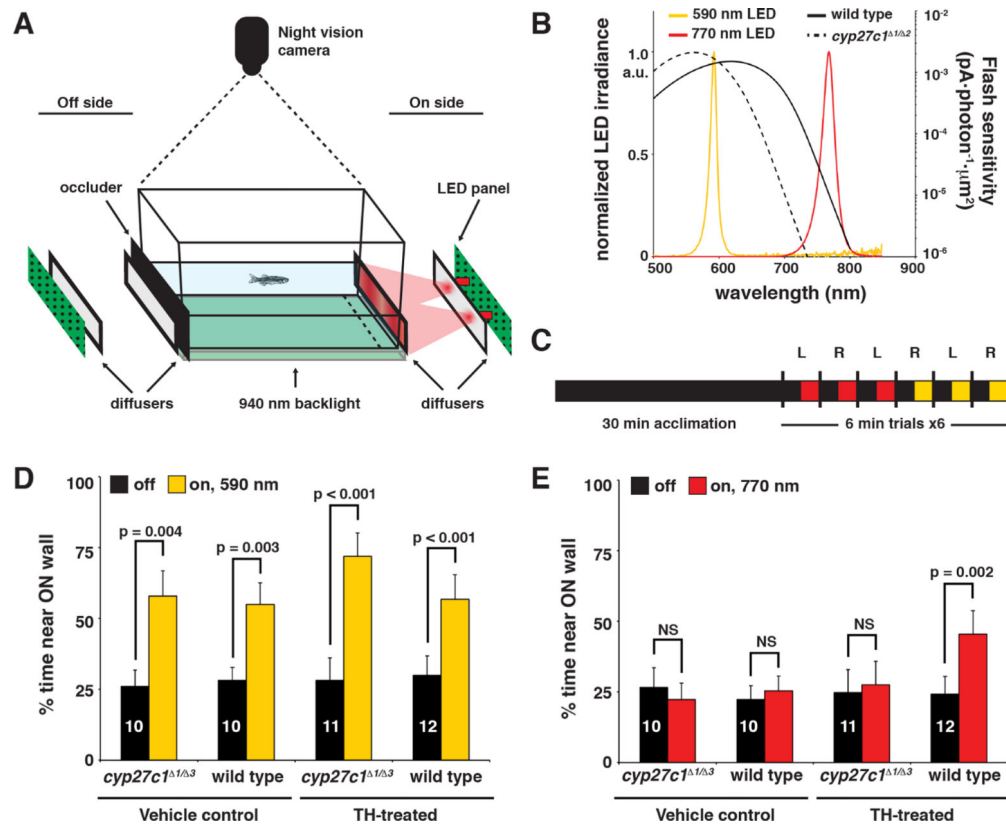
(B) Western blot with rabbit polyclonal anti-Cyp27c1 antibody shows that Cyp27c1 protein is detected in the RPE of wild-type fish but not in the RPE of *cyp27c1*<sup>1/2</sup> fish after three weeks of TH treatment.  $\beta$ -actin was used as a loading control.

(C) Wild-type and *cyp27c1*<sup>1/2</sup> siblings were treated with TH or vehicle control for three weeks, and retinas and RPE from three animals were pooled and analyzed for retinoid content using HPLC. A TH-driven switch to vitamin A<sub>2</sub> was observed in wild-type fish, but not in *cyp27c1*<sup>1/2</sup> mutants.

(D) The spectral sensitivity of red cones from TH-treated *cyp27c1*<sup>-1/-2</sup> fish and their wild-type siblings was assessed using single-cell suction electrode recording. The photoreceptors were exposed to a series of dim flashes at 560, 600, 660, and 700 nm, and their response amplitudes were used to calculate flash sensitivity (y-axis) as a function of wavelength (x-axis). No significant difference was observed between vehicle-treated *cyp27c1*<sup>-1/-2</sup> and wild-type red cones. However, TH-treated wild-type red cones were significantly more sensitive to 660 and 700 nm light, and less sensitive to 560 nm light relative to *cyp27c1*<sup>-1/-2</sup> red cones (Student's t-test, \*p < 0.0005, n = 12-21, error bars = s.e.m.). Fitted curves were calculated using a model that incorporates both vitamin A<sub>1</sub>- and A<sub>2</sub>-based pigment. This model predicts 100% vitamin A<sub>1</sub> in vehicle-treated wild-type and *cyp27c1*<sup>-1/-2</sup> fish, 99.7% vitamin A<sub>1</sub> in TH-treated *cyp27c1*<sup>-1/-2</sup> fish, and 95.5% vitamin A<sub>2</sub> in TH-treated wild-type siblings.

(E) Physiological parameters of the red cones are indicated ± s.e.m. These include wavelength of peak sensitivity ( $\lambda_{\max}$ ), dark current ( $I_{\text{dark}}$ ), estimated half-saturation light intensity at  $\lambda_{\max}$  ( $I_0$ ), dim flash response time to peak ( $T_{\text{peak}}$ ), recovery time constant ( $\tau_{\text{rec}}$ ; estimated from a single-exponential fit to the tail of the response), and integration time ( $T_{\text{int}}$ ; estimated as the time integral of the normalized flash response).

See also Figure S3.



**Figure 5. Mutation of Cyp27c1 abolishes the phototactic response to near-infrared light**

(A) The phototaxis assay consisted of 590 or 770 nm LED light sources placed on either the right or left side of a tank (depicted on the right side in the schematic). Light from the LEDs passed through two diffusers before illuminating one end of the tank. On the opposite end of the tank, a black occluder was inserted to prevent reflection off of the ‘off-side’ diffuser. Fish movement was recorded using a night vision camera and a 940 nm infrared backlight located beneath the tank. The percentage of time spent within 25 mm of the lit ‘on’ side (dashed line) was determined. The behavioral assay and subsequent data analysis were conducted in a blinded fashion with respect to the genotype of the fish.

(B) A comparison of red cone sensitivity data (reproduced from Figure 4D) and the measured LED spectra.

(C) Summary of the experimental design. Each dark-adapted fish was acclimated to the testing tank for 30 min in the dark and then recorded during a set of three trials each at 770 and 590 nm. Each trial consisted of 3 min dark, and 3 min lit conditions, with the order of light/dark presentation randomized. The light source was alternated between the left and right sides for each trial.

(D) TH- and vehicle-treated *cyp27c1<sup>Δ1/Δ3</sup>* mutants and wild-type siblings all showed a significant positive phototactic response to 590 nm light (paired Student's t-test,  $p < 0.005$ ,  $n$  as indicated per group, error bars = s.e.m.). All p-values remained significant after Bonferroni correction for multiple testing.

(E) Only TH-treated wild-type fish showed a significant positive phototactic response to 770 nm light. For significantly different groups, p-values are as shown. For vehicle-treated

*cyp27c1*<sup>1/3</sup> mutant and wild-type fish at 770 nm, p-values were 0.449 and 0.177, respectively. For TH-treated treated *cyp27c1*<sup>1/3</sup> mutants at 770 nm, the p-value was 0.490 (paired t-test, n as indicated per group, error bars = s.e.m.). All p-values remained significant after Bonferroni correction for multiple testing. See also Figure S4, Movie S1, and Movie S2.

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