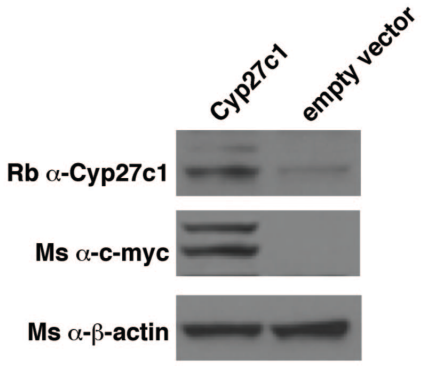
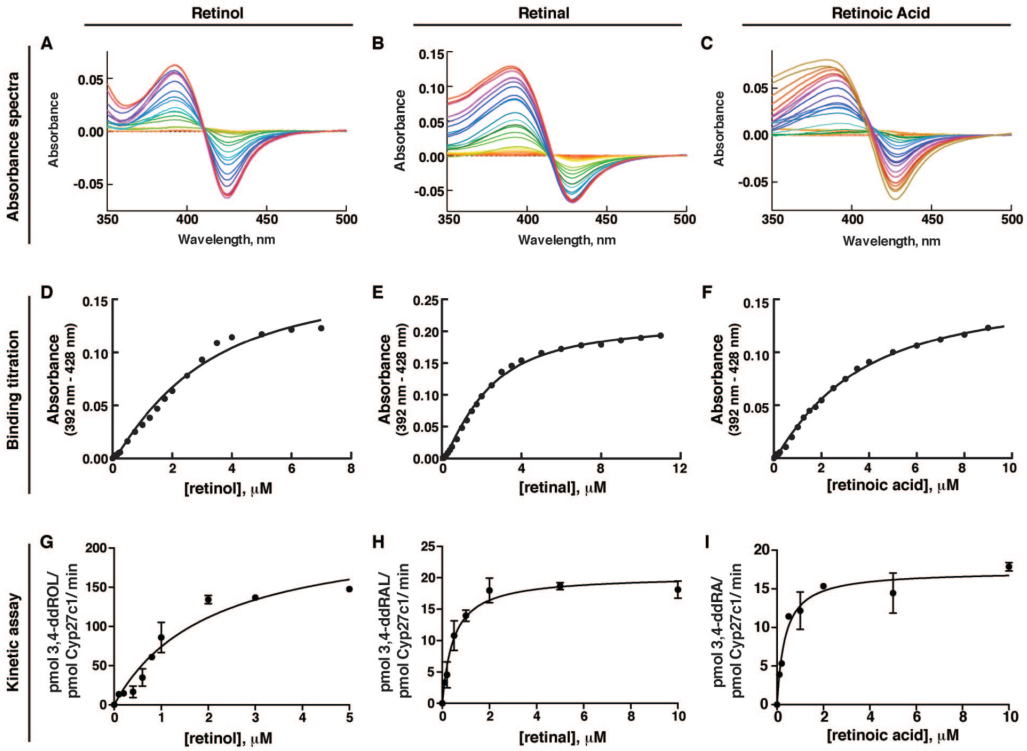


Figure S1. Anti-Cyp27c1 Antibody Reacts With Zebrafish Cyp27c1, Related to Figure 2

Myc-tagged zebrafish Cyp27c1 was expressed in HEK-293 cells, and a cell lysate was collected for Western blot analysis. Antibodies used included a rabbit polyclonal anti-Cyp27c1 antibody, a mouse monoclonal anti-c-myc antibody, and mouse anti-β-actin as a loading control. A strong signal was observed with anti-Cyp27c1 that corresponded to the signal observed with anti-c-myc, indicating that this antibody recognizes Cyp27c1. Only a weak signal was observed in a lysate of HEK-293 cells transfected with empty vector. This could reflect low-level endogenous CYP27C1 expression in HEK-293 cells, or minor off-target binding. We observed two bands with both anti-Cyp27c1 and anti-c-myc. CYP27C1 is predicted to have an N-terminal localization signal. Therefore, the higher and lower molecular weight bands may correspond to full-length and cleaved CYP27C1, respectively. Alternatively, these two bands could represent phosphoisomers. The upper band was also observed with Western blotting of tissue samples (Fig. 2e, 4b), but was much weaker than the lower band.



J

Substrate	K_d, μM ± s.e.m.	k_{cat}, s^{-1} ± s.e.m.	K_m, μM ± s.e.m.	k_{cat}/K_m, $M^{-1}s^{-1}$ ± s.e.m.
Retinol	1.8 ± 0.5	3.8 ± 0.5	1.8 ± 0.5	$1.9 \times 10^6 \pm 0.5 \times 10^6$
Retinal	1.2 ± 0.1	0.33 ± 0.02	0.48 ± 0.1	$0.70 \times 10^6 \pm 0.15 \times 10^6$
Retinoic acid	2.3 ± 0.2	0.28 ± 0.02	0.35 ± 0.08	$0.73 \times 10^6 \pm 0.17 \times 10^6$

Figure S2. Cell-free Assays of Cyp27c1 Activity, Related to Figure 3

(A-C) Characteristic spectra are observed when Cyp27c1 is bound to a substrate. These spectra were observed for three substrates (all-*trans* retinol, retinal, and retinoid acid) across a range of substrate concentrations.

(D-F) Binding titration curves were generated by subtracting absorbance at 428 nm from that at 392 nm across a range of substrate concentrations and used to calculate the dissociation constant (K_d). The K_d for retinal was lower than that of retinol and retinoic acid (J).

(G-I) Steady-state kinetics of Cyp27c1 were determined in a reaction mixture containing the enzyme, along with recombinant bovine adrenodoxin, NADPH-adrenodoxin

reductase, and an NADPH-generating system, and a series of concentrations of all-*trans* retinol, retinal, and retinoid acid. Reaction products were quantified using HPLC.

(J) K_m and k_{cat} values were then calculated for the three substrates and used to determine catalytic efficiency (k_{cat}/K_m). The dissociation constant (K_d), determined from A-C is also presented. Cyp27c1 efficiently catalyzed the desaturation of all three substrates, but showed the highest efficiency with retinol as a substrate.

Error bars = s.e.m.

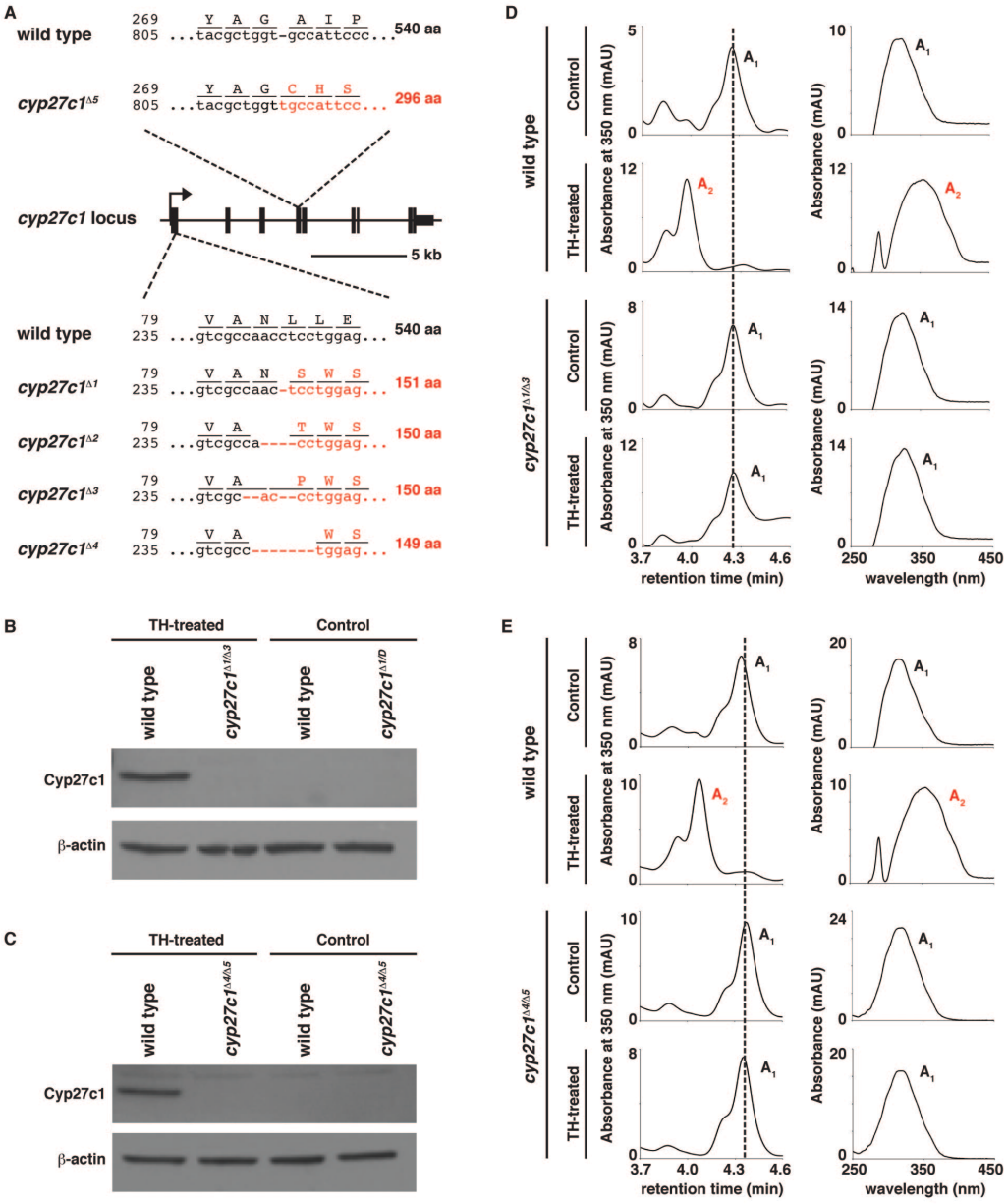


Figure S3. Mutation of *cyp27c1* Eliminates the Capacity to Generate Vitamin A_2, Related to Figure 4

(A) TALENs were used to generate small frameshift mutations in exons 1 and 4 of *cyp27c1.* Four mutations in exon 1 and one mutation in exon 4 were isolated. Each mutation results in severe truncation of the protein.

(B) Heteroallelic *cyp27c1*$^{\Delta 1/\Delta 3}$ mutants were generated and treated with either TH or vehicle control along with their wild-type siblings. Western blotting was used to confirm the loss of Cyp27c1 protein expression. While TH-treatment induces Cyp27c1 protein expression in wild-type fish, Cyp27c1 protein is not detected in *cyp27c1*$^{\Delta 1/\Delta 3}$ tissue.

(C) A similar loss of Cyp27c1 protein was observed in heteroallelic *cyp27c1*$^{\Delta 4/\Delta 5}$ mutants.

(D) HPLC was used to assess retinoid content in wild-type and *cyp27c1*$^{\Delta 1/\Delta 3}$ mutant fish after treatment with TH or vehicle control. Vitamin A_1 and A_2 were distinguished based on differences in retention time and λ_{max}. Wild-type fish switch to vitamin A_2 after TH-treatment, but this response is lost in the *cyp27c1*$^{\Delta 1/\Delta 3}$ mutant fish.

(E) Production of vitamin A_2 is also lost in *cyp27c1*$^{\Delta 4/\Delta 5}$ mutants.

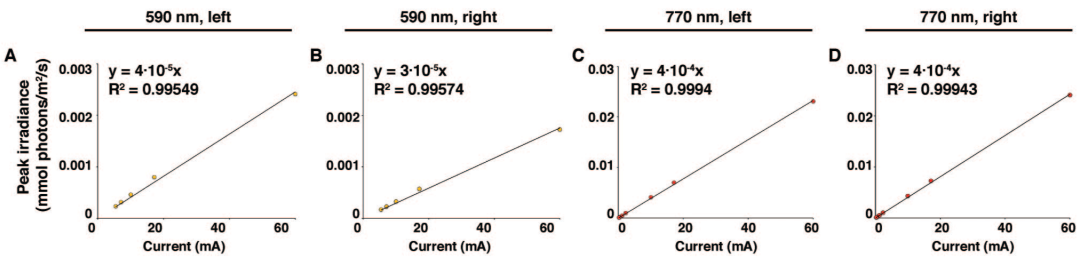


Figure S4. Quantifying Irradiance Levels of the 590 nm and 770 nm LEDs Used in the Behavioral Assay, Related to Figure 5

(A-D) Peak irradiance for each set of LEDs was plotted as a function of current. Linear relationships were observed for all of the LEDs, with R^2 values > 0.999 relative to a linear fit curve passing through the origin. Because the irradiance levels used in the behavioral assay were below the detection limits of the spectrometer, these curves were used to calculate the irradiance levels in the behavioral assay.

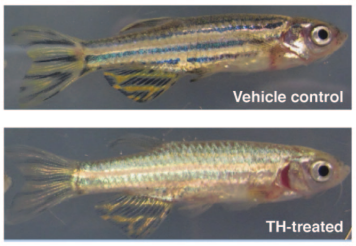


Figure S5. TH-Treatment of Zebrafish Induces a Change in Skin Pigmentation, Related to Experimental Procedures

Wild-type zebrafish are pigmented by alternating rows of melanophores and xanthophores. While this pattern is unchanged with vehicle treatment, TH-treated fish exhibit a reduction in melanophore-based pigment. This change was consistent across strains, and was used to confirm TH-treatment.

Table S1. Differentially Expressed Genes in TH- vs. Vehicle-treated Zebrafish RPE, Related to Figure 2

RNA-seq analysis was conducted on RPE isolated from zebrafish treated with TH or a vehicle control. Significantly differentially expressed genes were identified using a quantile-adjusted conditional maximum likelihood (qCML) test, and filtered to include genes with > 3-fold change, and > 5 average RPKM. FDR < 0.05, n = 3.

Table S2. Differentially Expressed Genes in Dorsal vs. Ventral Bullfrog RPE, Related to Figure 2

A qCML test was used to generate a list of differentially expressed transcripts, which was filtered to include genes with > 3-fold change, and > 5 average RPKM. FDR < 0.05, n = 3. Differentially expressed transcripts were identified using blastx. N/A indicates that no alignment was reported.

Supplemental Experimental Procedures

Animal Husbandry

All procedures were carried out in accordance with animal protocols approved by the Animal Studies Committee of Washington University. Zebrafish were maintained in the Washington University Zebrafish Consortium facility on a 14 hour light, 10 hour dark cycle at ~27°C. Adult fish were fed a blend of commercial quality aquaculture foods such as Lansy NRD 4/6, Salt Creek Progression 4, and Argent Cylop-eze, as well as live *Artemia nauplii*. WT AB* and SJD, *albino, cyp27c1*$^{\Delta 1}$, *cyp27c1*$^{\Delta 2}$, *cyp27c1*$^{\Delta 3}$, *cyp27c1*$^{\Delta 4}$, and *cyp27c1*$^{\Delta 5}$ strains were used in this study. Adult mutant strains were maintained as heterozygous stocks, and crossed to each other to create transheterozygous mutants. Adult zebrafish (>3 months old) were used for all experiments.

Generation of *cyp27c1* Mutant Zebrafish

Left and right TALENs targeting exon 1 (*cyp27c1*$^{\Delta 1}$, *cyp27c1*$^{\Delta 2}$, *cyp27c1*$^{\Delta 3}$, *cyp27c1*$^{\Delta 4}$) and exon 4 (*cyp27c1*$^{\Delta 5}$) of *cyp27c1* were constructed in the MR015 vector by the Genome Engineering and iPSC Center in the Department of Genetics at Washington University. Repeat variable diresidue (RVD) sequences were as follows:

exon 1 left: NH HD HD HD NH NH NI HD HD HD NI NH HD NI HD NG

exon 1 right: HD HD NI NG HD HD HD NH NH NG NI NH NI NI NH NI NI HD NG

exon 4 left: HD HD NG NG NG NI NI NH NI HD HD NI HD HD NI NG NH NG

exon 4 right: NH NI NG NH NH NH NI HD NH HD NI NH HD HD NI NG NG NG NH NH

The mMessage Machine T7 kit (Life Technologies) was used to generate TALEN mRNA. Left and right TALEN mRNAs were mixed in equimolar amounts (12 pg/nL each) in 0.1M KCl, 3% phenol red injection buffer, and 0.5 nL was micro-injected into 1-cell AB* embryos. Germline founders were identified by raising F0 fish to adulthood, crossing to AB* fish and genotyping the offspring at 24 hours post-fertilization (hpf).

Genotyping sequencing primers for exon 1 were TGGTGAGCATTTACGCAGTC and AAGATTGCGTCTGGCTGTTT, which generate a 464 bp product in wild-type fish. The wild-type product is cleaved by *Bst*XI, but the recognition site is disrupted in the mutant. Sequencing primers for exon 4 were TGGTCAGTACATAAATGTTAAGCAA and CCAGCCAAGAGCATTTCTGT, which generate a 509 bp product in wild-type fish. This product contains an *Nla*IV recognition site that is disrupted in mutants. Sanger sequencing of the PCR product was used to identify the precise sequence of the

mutants and confirm the genotype. Four founders were identified with mutations in exon 1 (*cyp27c1*$^{\Delta 1}$, *cyp27c1*$^{\Delta 2}$, *cyp27c1*$^{\Delta 3}$, *cyp27c1*$^{\Delta 4}$) and one founder was identified with a mutation in exon 4 (*cyp27c1*$^{\Delta 5}$).

Thyroid Hormone Treatment

Zebrafish were housed individually without feeding for three weeks in water containing 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). The final pH was within normal limits, and the water was changed every two to three days. We observed an apparent loss of melanin-based skin pigmentation in the TH-treated fish, which we used to confirm successful TH-treatment in all zebrafish experiments (Fig. S5).

Harvesting Tissue

Bullfrog heads were obtained following decapitation. The eyes were marked for dorsal/ventral orientation and were removed, and then the posterior segment was isolated and cut into thirds using a razor blade. The dorsal and ventral thirds of the RPE were then dissected and transferred to various solutions according to the application: Trizol (for transcriptome profiling and qRT-PCR), modified RIPA buffer (65 mM Tris base, 154 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate) with Roche Mini cOmplete protease inhibitor tablet (for Western blot), and PBS (for retinoid analysis by HPLC). RPE from two animals was pooled per replicate. The number of replicates was as follows: RNA-seq and qRT-PCR (n = 3), Western blot (n = 2), HPLC (n = 3). Tissue was immediately frozen on dry ice and stored at -80°C.

Zebrafish were euthanized by rapid cooling. Their eyes were then removed and dissected to isolate either RPE alone (for transcriptome profiling and qRT-PCR) or RPE and retina (for HPLC and Western blot). One eye from each of three fish was pooled per replicate. The number of replicates was as follows: RNA-seq and qRT-PCR (n = 3), HPLC (n = 4), and Western blot (n = 2). Tissue was immediately frozen on dry ice and stored at -80°C. For histology, the cornea was punctured and the lens removed. The tissue was then fixed in 4% paraformaldehyde, equilibrated in a sucrose series and embedded for frozen section as previously described [S1]. One eye from each of six fish was collected for *in situ* and immunohistochemistry. Wild-type SJD fish were used for RNA-seq and qRT-PCR, while AB* wild-types were used for all other experiments.

High Performance Liquid Chromatography (HPLC)

At all steps, tissue was kept light-protected. Conditions were selected to assay retinol and retinal, and tissue was processed as previously described [S2]. Briefly, tissue samples in 500 µL PBS were thawed on ice and sonicated twice for 10 seconds at 20% amplitude (Vibra-Cell, Sonics & Materials Inc., Newtown, CT). The retinoids were then extracted in 3 mL hexanes and dried under nitrogen. Retinal was then reduced to retinol by resuspending the sample in a solution of a few crystals of $NaBH_4$ in 500 µL of ethanol and incubating for one hour in the dark. Samples were again dried under nitrogen and re-suspended in acetonitrile. To assay retinoid content in the cell culture assays, the retinoids were extracted with 5 mL hexanes, dried under nitrogen, and resuspended in mobile phase (acetonitrile:water 80:20 [vol:vol]).

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 resuspended extract and eluted the samples isocratically with a mobile phase of acetonitrile:methanol:dicholoromethane 70:15:15 (vol:vol:vol) at a flow rate of one mL min^{-1} and a column temperature of 18°C. The HEK-293 cell and media samples were eluted isocratically with a mobile phase of acetonitrile:water 80:20 (vol:vol) at a flow rate of one mL min^{-1} and column temperature of 18°C. All samples were monitored at 290, 325, 350, 380, and 400 nm. Retinol (vitamin A_1) and 3,4-didehydroretinol (vitamin A_2) were identified by comparison with authentic standards obtained from Sigma (R7632, St. Louis, MO) and Santa Cruz Biotechnology Inc. (sc-209587, Dallas, TX), respectively.

Tissue Processing and Data Analysis for RNA-seq

Both zebrafish and bullfrog experiments were conducted in triplicate. RNA from TH- and vehicle-treated zebrafish RPE was isolated using a Qiagen RNeasy mini kit including a DNase treatment step and assayed for quantity and quality using an Agilent 2100 Bioanalyzer. RNA concentrations ranged from 2.5 to 13 ng/µL, and all RNA integrity number (RIN) scores were >8.0. cDNA libraries were then generated from 5 µL RNA using the Ovation RNA-Seq System V2 kit (NuGen). The quantity and quality of the libraries were assayed using a NanoDrop 2000 UV-Vis spectrometer (Thermo Scientific) and all 260/280 absorbance ratios were > 1.8.

Because a larger yield of tissue was obtained from bullfrog, dorsal and ventral bullfrog RNA was isolated using phenol/chloroform extraction and treated with DNase to remove contaminating genomic DNA. RNA was quantified using a Qubit 2.0 Fluorometer (Life Sciences), with yields ranging from 0.96 µg to 2.3 µg. RNA quality was determined using an Agilent 2100 Bioanalyzer, and the RIN score was >7.2 where reported, although the RIN score was not reported for three samples in which the concentration was below the detection limits of the chip used for this assay. Approximately 1µg of RNA was treated with a Ribo-Zero rRNA removal kit (Epicentre) to enrich for mRNA.

Zebrafish cDNA and bullfrog mRNA were submitted to the Genome Technology Access Center at Washington University for adaptor ligation and high-throughput sequencing on an Illumina HiSeq 2500. Zebrafish reads were aligned to a custom transcriptome curated to remove duplicates using Bowtie v0.12.7 [S3, S4]. This alignment was later repeated with inclusion of all known cyp27 family members (*cyp27a3, a4, a5, a6* and *b1* were added, as only *cyp27a7* and *c1* were present in the original transcriptome). Differential expression analysis was then conducted using edgeR v3.4.2 [S5] The input for edgeR was a read counts file derived from the Bowtie output file using a custom perl script. Transcripts with 0 hits in four or more of the six samples (three replicates, two groups) were removed, and significance was scored at an FDR of 0.05. Significantly differentially expressed genes were then filtered to include only those with enrichment > 3-fold and average expression levels of > 5 reads per kilobase transcript per million aligned reads (RPKM).

We conducted *de novo* transcriptome assembly with the bullfrog reads using Trinity (version r20131110) [S6]. The transcriptome output by Trinity was filtered to include only transcripts containing a predicted open reading frame encoding a minimum of 100 amino acids. This was done using the Transdecoder tool provided with the Trinity package. Reads were then aligned to the filtered *de novo* transcriptome assembly and analyzed for differential expression as described for zebrafish. Raw sequence files, the fasta files used for alignment, and the full output of edgeR for zebrafish and bullfrog are available through the Gene Expression Omnibus (GSE69219). A table of differentially expressed genes is provided in Supplementary Tables 1 and 2.

Quantitative RT-PCR

Bullfrog cDNA was generated by reverse transcription of ~50 µg of RNA using Superscript III reverse transcriptase (Life Technologies), treated with RNase H (Life

Technologies) to remove the RNA template, and isolated using a PureLink PCR purification kit (Life Technologies). Because all of the RNA derived from one of the dorsal replicates was used for sequencing, two dorsal replicates and three ventral replicates were used for qRT-PCR. Zebrafish cDNA generated for RNA-seq was used directly. Primers were designed to flank the last exon/exon junction in the transcript to further select for cDNA. Ribosomal protein transcripts *rpl13a* and *rpl7a* were used as reference genes for zebrafish and bullfrog, respectively. Primer sequences were as follows:

zebrafish *cyp27c1*:	CATGATGATCTGATCGTTGGA	CGGGGGAAGTTCTCCTCAT
zebrafish *rpl13a*:	TCCCAGCTGCTCTCAAGATT	ACTTCCAGCCAACTTCATGG
bullfrog *cyp27c1*:	GAATCCGCAGCTGTATTGGG	AGTCTGGGTCTTGGGTGAAG
bullfrog *rpl7a*:	TGGTGGAAGCAGTAAAGACCA	GCCTTCTCCAGCTTTGCAAT

Five point standard curves were generated with a 1:10 dilution series and performed in triplicate. Primer efficiencies were 99% to 112% over a range including the cycling threshold (C_t) values relevant to the samples being measured. Analysis of the melt curve confirmed that a single product was amplified. Samples were then assayed with 0.5 µL sample/well in triplicate. C_t values for zebrafish and bullfrog *cyp27c1* were normalized to *rpl13a* and *rpl7a*, respectively. A two-tailed Student's t-test was used to calculate statistically significant differences in C_t between treated and control groups.

Antibody Production

To generate a polyclonal antibody against zebrafish Cyp27c1, we created an expression construct encoding a portion of the enzyme encompassing amino acids 293-482, which was predicted to have high immunogenicity. The appropriate DNA fragment was obtained from a full-length cDNA clone of zebrafish Cyp27c1 (detailed below) by PCR with forward and reverse primers containing *Nde*I and *Xho*I sites, respectively.

Cyp27c1 fragment forward primer: GGTACCGAcatatgGACGGCCTCTTTAAATTCAGTCAA

Cyp27c1 fragment reverse primer: GGTACCGActcgagTCCATAGCCGAATGGGAT

This fragment was sub-cloned into the pET-28-a(+) bacterial expression vector, fused with a hexahistidine tag, and transformed into BL21(DE3) *E. coli.* A two liter culture of transformed cells was grown at 37°C under kanamycin selection to an OD_{600} of 0.95, protein expression was induced with the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside, and the culture was incubated overnight at 16°C. Protein expression was confirmed by SDS-PAGE. The Cyp27c1 fragment was purified by nickel-charged affinity chromatography (HisPur Ni-NTA, Cat no. 88221, Thermo Scientific,

Rockford, IL) and dialyzed into PBS. The purified Cyp27c1 fragment was then used by PrimmBiotech (West Roxbury, MA) to immunize two rabbits and three rats. Of these five animals, only one rabbit produced immunoreactive antiserum, which was validated by Western blotting against zebrafish Cyp27c1 expressed in cell culture (described below).

In situ Hybridization, Immunohistochemistry, and Imaging

In situ hybridization probe templates (600-700 bp) were cloned from zebrafish RPE cDNA. Primers included *Eco*RI recognition sites for subcloning into the BlueScript vector pBSK+.

cyp27c1 forward: TCCCCGgaattcCCTGCAGGCAGCTTCTTATC
cyp27c1 reverse: TCCCCGgaattcCCCAGGCGAGTCTCGTATAA
rpe65a forward: TCCCCGgaattcAGCTGAACGAACCTCTTCCA
rpe65a reverse: TCCCCGgaattcGATGGCTTGAACCTCTCAGC

Once subcloned into pBSK+, template sequence and orientation were confirmed by Sanger sequencing. Templates for probe synthesis were generated by PCR with T7 and T3 primers, using the pBSK+ plasmid as a template. DIG-labeled probe synthesis was then performed as previously described [S7]. *In situ* hybridization of 12 µm sections of albino zebrafish eyes was conducted as previously described [S7]. The probes were used at a concentration of 1 µL per 100 µL hybridization buffer [S7].

Immunohistochemistry was performed on 12 µm sections of albino zebrafish eyes. Tissue sections were blocked with 5% normal goat serum, 0.2% Triton-X 100, 0.3 M glycine in PBS for one hour at room temperature and incubated in a 1:100 dilution of rabbit anti-Cyp27c1 in block without glycine overnight at 4°C. Alexa-Fluor goat anti-rabbit 488 (Life Technologies) secondary antibody was applied in block without glycine at a dilution of 1:750 for one hour at room temperature. 4' ,6-Diamidino-2-phenylindole (DAPI) was applied for one minute at a 1:10,000 dilution to counter-stain nuclei.

Images for *in situ* hybridization and immunohistochemistry experiments were taken at 400x using an Olympus BX-51 compound microscope (Olympus, Tokyo, Japan), and processed using Adobe Photoshop CS5 to adjust brightness and contrast.

Cloning *cyp27c1* and Expression in HEK-293 Cells

HEK-293 cells were used to express Cyp27c1 protein for antibody validation by Western blot, and to assay the activity of Cyp27c1. HEK-293 cells were obtained from ATCC and grown in media containing DMEM with 10% FBS, 100 units/mL penicillin, 100 µg/mL

streptomycin and 0.29 mg/mL L-glutamine. Cells were subcultured every 4-7 days and maintained for no longer than 20 passages.

A full-length transcript of zebrafish *cyp27c1* was cloned from zebrafish RPE cDNA by PCR with forward and reverse primers containing *Nde*I and *Bsr*GI sites, respectively. Sanger sequencing was used to confirm that the correct sequence was obtained.

Cyp27c1 forward primer: GGTACCGAcatATGGCTCTTCAAAGTACTATTCTACACA

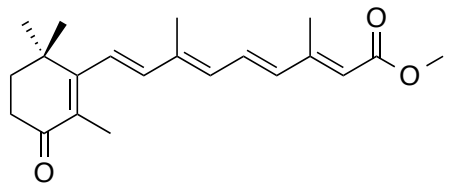
Cyp27c1 reverse primer: GGTACCGAtgtacaTTTTCGGTCTGTAAATCTAAGGTTGA

The subcloned construct consisted of a Tet response element driving the expression of DsRed, separated by an internal ribosomal entry site (IRES) element from *cyp27c1*, which was fused to a C-terminal myc tag. Each construct was transfected along with a vector containing the CMV promoter driving the expression of reverse tetracycline-controlled transactivator (rtTA). An empty expression vector was transfected with the CMV::rtTA vector as a negative control. Transfections were conducted with Mirus TransIT reagent according to the manufacturer's protocol. Expression was induced 24 hours after transfection by treating the cells with 1 µg/mL doxycycline. After another 24 hours, expression was verified by observing DsRed expression under a fluorescent dissecting microscope. For Western blot experiments, the cells were then washed, transferred to modified RIPA buffer (see above), frozen on dry ice, and stored at -80°C. This assay was repeated three times with different cultures. For vitamin A experiments, vitamin A_1 was resuspended in N,N-dimethylformamide at a concentration of 1 µg/µL. Cells were treated with 1.5 µL/mL of vitamin A_1 suspension in media for an additional 24 hours. The cells and media were then collected, frozen on dry ice, and stored at -80°C. This assay was repeated six times.

Retinoid Synthesis for Enzyme Kinetic Assays

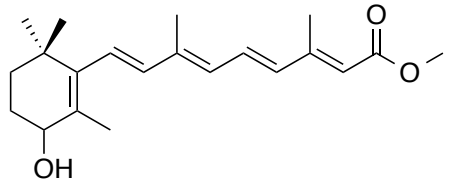
all-*trans* Retinol (catalog no. 95144), retinal (catalog no. R2500), and retinoic acid (catalog no. R2625) were purchased from Sigma-Aldrich (St. Louis, MO). 3,4-Didehydroretinol (called 3-dehydroretinol in catalog) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (catalog no. sc-209587) or prepared by $NaBH_4$ reduction of all-*trans* retinal, synthesized as follows.

4-Oxoretinoic acid methyl ester **(1)** [S8, S9]



Retinoic acid (500 mg, 1.7 mmol) was suspended in anhydrous diethyl ether (10 mL) and the mixture was stirred at room temperature. Diazomethane (in diethyl ether) was added until all retinoic acid was consumed, as judged by thin layer chromatography (TLC). The solvent was removed and the resulting residue was dried *in vacuo*. The yellow solid was dissolved in anhydrous CH_2Cl_2, and MnO_2 (13 g, Fluka, 90%, activated) was added [S9]. The suspension was stirred vigorously under an argon atmosphere. After 24 h the reaction was filtered through a plug of Celite, which was subsequently washed with CH_2Cl_2 (100 mL). The yellow-orange organic solutions were combined and solvent was removed *in vacuo*. The product was purified by column chromatography on silica gel (15% ethyl acetate in hexane, v/v) to give **1** as a yellow residue. The product formed yellow crystals when dried in a vacuum desiccator (213 mg, 39% yield) [S9]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (dd, *J* = 15.1, 11.4 Hz, 1H), 6.36 (d, *J* = 15.1 Hz, 1H), 6.33 (s, 2H), 6.26 (d, *J* = 11.4 Hz, 1H), 5.82 (s, 3H), 3.72 (s, 3H), 2.51 (t, *J* = 6.6 Hz, 1H), 2.36 (s, 3H), 2.03 (s, 3H), 1.89-1.84 (overlapped, 5H), 1.19 (s, 6H); atmospheric pressure chemical ionization (APCI) high resolution mass spectrometry (HRMS) (*m/z*): [M+H$^+$] calculated for $C_{21}H_{29}O_3$ 329.2111; found 329.2107 (D 1.2 ppm).

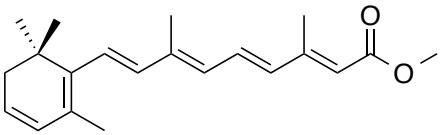
4-Hydroxyretinoic acid methyl ester **(2)** [S8, S9]



Compound **1** (47 mg, 0.14 mmol) was stirred in anhydrous CH_3OH (1 mL), and the mixture was stirred at room temperature. $NaBH_4$ was added and the reaction was stirred at room temperature for 30 min. The reaction was diluted with H_2O (10 mL) and extracted with ethyl acetate. The organic layer was separated, washed sequentially with dilute HCl (aq), saturated $NaHCO_3$ (aq), and brine, and then dried over $MgSO_4$. The product was purified by column chromatography on silica gel (20% ethyl acetate in hexane, v/v) to give an orange solid when dried in a vacuum desiccator (40 mg, 85 %) [9]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.99 (dd, *J* = 15.1, 11.4, 1H), 6.33 (overlapped, 4H),

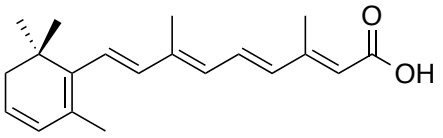
5.79 (s, 1H), 4.01 (bs, 1H) 3.71 (s, 3H), 2.36 (s, 3H), 2.00 (s, 3H), 1.91 (m, 1H), 1.83 (s, 3H), 1.72 (m, 1H), 1.64 (m, 1H), 1.48 (s, 1 H), 1.43 (m, 1H), 1.04 (s, 3H), 1.02 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.7, 141.6, 139.0, 138.3, 135.6, 130.7, 130.2, 130.1, 127.6, 118.4, 70.2, 50.9, 34.7, 34.5, 29.0, 28.4, 27.4, 18.6, 13.8, 12.8; APCI HRMS (*m/z*): [M+H$^+$] calculated for $C_{21}H_{31}O_3$ 331.2268; found 331.2264 (D 1.2 ppm).

3,4-Didehydroretinoic acid methyl ester (**3**)



Compound **2** (40 mg, 0.14 mmol) was dissolved in dry CH_2Cl_2 (2 mL) and triethylamine was added (182 µL). This solution was cooled in an ice bath and methylenesulfonyl chloride (33 µL) was added. The reaction was stirred at room temperature for three hours and quenched by pouring into a separatory funnel containing dilute HCl (aq). The product was extracted with ethyl acetate and the organic layer was separated. The crude product was washed sequentially with dilute HCl (aq), saturated $NaHCO_3$ (aq), and brine and then dried over $MgSO_4$. The product was purified by column chromatography on silica gel (10% ethyl acetate in hexane, v/v) to give an orange oily residue (30 mg, 79%). (See [S10] for ethyl ester.) ^1H NMR (600 MHz, $CDCl_3$) δ 7.00 (dd, *J* = 15, 3.5 Hz, 1H), 6.31 (d, *J* = 15 Hz, 1H), 6.28 (s, 2H), 6.19 (d, *J* = 11.5 Hz, 1H), 5.85 (dt, *J* = 9.5, 1.6 Hz, 1H), 5.78 (s, 1H), 5.76 (dt, *J* = 9.5, 4.5, 1H), 3.71 (s, 3H), 2.36 (s, 3H), 2.09 (d, *J* = 4.4, 1.7 Hz, 2H), 2.01 (s, 3H), 1.87 (s, 3H), 1.04 (s, 6H); APCI HRMS (*m/z*): [M+H$^+$] calculated for $C_{21}H_{29}O_2$ 313.2162; found 313.2162 (D < 0.1 ppm).

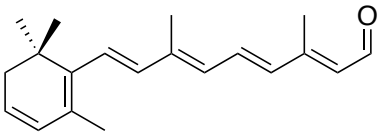
3,4-Didehydroretinoic acid (**4**)



Compound **4** (126 mg, 0.40 mmol) was suspended in a methanolic solution of 10% (w/v) KOH (1.5 mL) and the mixture was heated under reflux for one hour. The reaction was cooled to room temperature and diluted with H_2O (20 mL) and washed with hexane. The aqueous layer was acidified with dilute HCl and cooled to 0°C. The resulting orange precipitate was collected by vacuum filtration and dried to yield crude product. The recovered material was dissolved in CH_3OH and H_2O, followed by concentration *in*

vacuo to afford compound **4** as an orange solid, collected by vacuum filtration (32 mg, 27%) [S11]. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.05 (dd, *J* = 15.1, 11.4 Hz, 1H), 6.33 (d, *J* = 15.1 Hz, 1H), 6.295 (s, 2H), 6.20 (d, *J* = 11.5 Hz, 1H), 5.85 (d, *J* = 9.5 Hz, 1H), 5.80 (s, 1H), 5.75 (dt, *J* = 9.5, 4.4, 1H), 2.36 (s, 3H), 2.08 (d, *J* = 4.4 Hz, 2H), 2.02 (s, *3*H), 1.87 (s, 3H), 1.03 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.4, 155.1, 140.1, 138.3, 136.6, 135.1, 131.7, 129.9, 129.8, 127.8, 127.4, 125.4, 117.5, 39.8, 33.9, 26.7, 20.3, 14.0, 12.8; APCI HRMS (*m/z*): [M+H$^+$] calculated for $C_{20}H_{27}O_2$ 299.2006; found 299.2004 (D 0.7 ppm).

3,4-Didehydroretinal (**5**)



Compound **3** (75 mg, 0.24 mmol) was dissolved in anhydrous CH_2Cl_2 (2.5 mL) and cooled to -78°C. Diisobutyl aluminum hydride in ether (1.0 M, 900 µL) was added dropwise over 15-20 minutes. The mixture was stirred for 2.5 h, and the reaction was allowed to warm to room temperature. Upon completion, the reaction was cooled in an ice bath and CH_3OH (10 drops) was added, followed by H_2O (0.5 mL). The reaction was stirred vigorously at room temperature for 1 hour. The resulting suspension was filtered through Celite, and solvent was removed *in vacuo*. The resulting yellow residue was dissolved in CH_2Cl_2 (3.5 mL) and cooled to 0°C. MnO_2 (375 mg) was added and the mixture stirred for 1.5 h. The reaction was filtered through Celite, which was washed with additional CH_2Cl_2; and then the combined organic solvent mixture was concentrated. The crude product was purified by column chromatography on silica gel (10% ethyl acetate in hexane, v/v) and dried in a desiccator under vacuum to give an oily orange residue (20 mg, 30%) [S12]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.11 (d, *J* = 8.1 Hz, 1H), 7.142 (dd, *J* = 15.0, 11.5 Hz, 1H) 6.38 (d, *J* = 15.1 Hz, 1H), 6.34-6.32 (overlapped, 2H), 6.23 (d, *J* = 11.7 Hz, 1H), 5.98 (d, *J* = 8.1, 1H), 5.86 (d, *J* = 9.5 Hz, 1H), 5.77 (m, 1H), 2.33 (s, 3H), 2.09 (s, *J* = 4.3, 2H), 2.04 (s, 3H), 1.88 (s, 3H), 1.06 (s, 6H); APCI HRMS (*m/z*): [M+H$^+$] calculated for $C_{20}H_{27}O$ 283.2056; found 283.2056 (D <0.1 ppm).

Protein Production for Enzyme Kinetic Assays

Zebrafish Cyp27c1 was expressed in *E. coli* (from an *E. coli* codon-optimized cDNA, GeneWiz) using the vector pCW and purified according to published methods [S13]. Bovine adrenodoxin (Adx) and NADPH-adrenodoxin reductase (ADR) were expressed from cDNAs in *E. coli* and purified according to previously published procedures [S14-S16]. Briefly, a single colony was grown overnight in Luria-Bertani media with 100 µg/mL ampicillin at 37°C with gyratory shaking at 220 rpm; 5 mL of overnight culture was then inoculated into 500 mL of Terrific Broth media containing 0.30 mM ampicillin, 1.3 mM thiamine, and 0.025% (v/v) of a mixture of trace elements [S17]. The cultures were incubated at 37°C with gyratory shaking at 220 rpm for 4 hours, and gene expression was induced with the addition of 1.0 mM IPTG. The incubation continued at 30°C for another 24 hours. All further steps were done at 0-4°C. Cells were harvested by centrifugation at 3000 $\times$ *g* for 10 minutes, and the cell pellet was sonicated in 100 mM potassium phosphate (pH 7.4) containing 0.1 mM EDTA, 20% glycerol (v/v), and protease inhibitor tablets (Roche). Cell debris was then removed by centrifugation at 100,000 $\times$ *g* for 30 minutes.

For purification of ADR, the supernatant was loaded on a nickel-charged affinity chromatography column (Ni-NTA Agarose, Cat no. 30210, Qiagen, Valencia, CA). The column was washed with same buffer containing 20 mM imidazole, and ADR was eluted with the same buffer containing 200 mM imidazole. The eluted ADR was dialyzed against 40 volumes of 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v) and 0.1 mM EDTA three times to remove the imidazole.

For purification of Adx, the supernatant was applied to a 2.5 cm × 20 cm column of DEAE-Sepharose equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and washed with one liter of the equilibration buffer. The column was eluted with the same buffer containing 170 mM NaCl and then 300 mM NaCl Those fractions showing absorbance at 414 nm from the 170 mM pool were highly purified as judged by SDS-PAGE (15% (w/v) gel). The fractions eluted with 300 mM NaCl were dialyzed twice vs. 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and applied to a 2.5 × 10 cm column of DEAE-Sepharose, which was washed with the dialysis buffer and then eluted with the same containing 500 mM KCl. Fractions containing Adx were pooled on the basis of A_{414} measurements. Fractions from the DEAE steps were further purified with gel filtration chromatography on a 2.5 × 100 cm column of Sephadex G-75, using 10 mM potassium phosphate buffer (pH 7.4) containing

0.1 mM EDTA (≤20 mL per run). The total yield was 25,700 nmol Adx, which was stored in aliquots at -70°C.

Spectral Binding Titration Studies

Spectral binding titration studies were performed with zebrafish Cyp27c1 (2 μM) in potassium phosphate buffer (50 mM, pH 7.4) using tandem (Yankeelov) cuvettes. Experiments were conducted at room temperature using an OLIS-Aminco DW2a spectrophotometer (On-Line Instruments, Bogart, GA). After a baseline correction was recorded (350–500 nm), the retinoid (0 – 11 μM) was titrated into cuvettes containing either Cyp27c1 or buffer alone as a reference. Spectra were recorded after each addition. To estimate the dissociation constant K_d, the difference between maximum (390 nm) and minimum (428 nm) absorbance was plotted as a function of the ligand concentration using the quadratic equation below in Prism software (GraphPad, La Jolla, CA), where Y is the absorbance change, E is the Cyp27c1 concentration, and X is the ligand concentration, and A and B are fitted values describing the total absorbance change and the y-intercept, respectively [S18].

$$Y = B + \frac{A((K_d + E + X) - \sqrt{((K_d + E + X)^2 - 4EX)})}{2E}$$

Steady-state Kinetic Experiments

The reaction mixtures contained recombinant zebrafish Cyp27c1 (25 pmol for retinal and retinoic acid, 10 pmol for retinol), Adx (5 μM), ADR (0.2 μM), potassium phosphate (50 mM), and various concentrations of a substrate (0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, or 5 μM retinol or 0.1, 0.2, 0.5, 1, 2, 5, or 10 μM retinal or retinoic acid), 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 [S19]. The substrates had been reconstituted in ethanol and were soluble at all concentrations used in this assay. The final volume was 500 μL and the reaction time was 60 s. The reaction was quenched with 1.0 mL of ethyl acetate solution containing 45 μM butylated hydroxytoluene (to prevent radical reactions). The organic solvent (800 μL aliquot) was extracted and dried under nitrogen. The sample was suspended in CH_3CN (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_2O; 4.9% CH_3CN; 0.1% formic acid, v/v) and 70%

solvent B (95% CH_3CN; 4.9% H_2O; 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.

Western Blotting

Western blotting was conducted on HEK-293 cells, which were collected along with their culture media in the wells, and on RPE and retina isolated from zebrafish and bullfrog as described above. Protein was isolated by sonicating samples twice for 20 seconds at 20% amplitude, spinning the samples down at 14,000 x *g* for 10 minutes at 4°C and collecting the supernatant. Protein concentration was then determined with the Pierce™ BCA™ Protein Assay kit (Thermo Scientific). For each blot, equivalent amounts of protein were loaded in each well of a NuPAGE® Novex® 10% Bis-Tris Protein Gel (Life Technologies), ranging from 5 µg for zebrafish tissue to 15 µg for HEK-293 cell lysates. Gels were run in MOPS buffer, according to the manufacturer's protocol, with Novex® Sharp Pre-Stained Protein Standard. Proteins were then transferred to a nitrocellulose membrane using the XCell II Blot Module from Life Sciences, according to the manufacturer's instructions.

Blocking steps and antibody incubations were performed in 5% non-fat dry milk, 0.05% Tween-20 in PBS. Rabbit anti-Cyp27c1 was used at a 1:500 dilution for tissue samples and at a 1:5,000 dilution for HEK-293 cell lysates. Mouse anti-β actin (Santa Cruz) and mouse anti-c-Myc 9e10 (Santa Cruz) primary antibodies were used at a 1:5,000 dilution, and HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Thermo Scientific) at a 1:4,000 dilution. Blocking was performed for one hour at room temperature, primary antibodies were incubated overnight at 4°C, and secondary antibodies were incubated for one hour at room temperature. Gels were developed using either Immobilon Western Chemiluminescent HRP Substrate (Millipore) or Immun-Star WesternC Chemiluminescent Kit (Bio-Rad) according to the manufacturer's protocol and exposures were taken on Blue Basic Autorad Film (BioExpress). The films were scanned and imported into Adobe Photoshop CS5 for re-orientation and cropping to generate images.

Single Cell Suction Electrode Recording

Adult (4 month old) wild-type and *cyp27c1*$^{\Delta 1/\Delta 2}$ zebrafish were treated with TH or vehicle, dark-adapted overnight and euthanized by rapid cooling. Cell preparation was carried

out under dim red light. Eyes were enucleated and dissected with fine forceps. The retina was peeled off and chopped into small pieces in Ringer solution (111 mM NaCl, 2.5 mM KCl, 1.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.01 mM EDTA, 3.0 mM HEPES and 10 mM glucose; pH adjusted to 7.8 with NaOH). Photoreceptors were dissociated mechanically by trituration of the chopped retina, placed in a recording chamber and perfused with Ringer solution. The recording pipette, lumen diameter of ~2.5–3 μm, was filled with solution containing 110 mM NaCl, 2.5 mM KCl, 1.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 10 mM HEPES; pH adjusted to 7.8 with NaOH.

Single-cell recordings were carried out as previously described for salamander photoreceptors [S20]. Red cones were identified as a member of a double-cone pair, based on their characteristic morphology and their 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. Light wavelength and intensity were regulated with interference filters and calibrated neutral density filters, respectively. 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). Photosensitivities at 560, 600, 660 and 700 nm were calculated from the linear region of the intensity-response curve as the ratio of response amplitude and flash intensity. Spectral sensitivity plots thus obtained were fitted with a mixed A_1 and A_2 template[9]. Intensity-response data were fitted with the Naka–Rushton equation:

$$R/R_{max}=I^n/(I^n+I_o^n)$$

where R is the transient-peak amplitude of the response, R_{max} is the maximal response amplitude, I is flash intensity, I_o is the flash intensity that produces a half-maximal response, and n is the Hill coefficient.

Behavioral Assay Chamber Construction

The recording setup consisted of a clear plastic rectangular tank (19 cm x 35 cm) filled with three liters of TH- or vehicle-treated water. On each end of the tank, half white diffuser sheets (filter #250, LEE Filters, Burbank, CA) were placed 1 cm and 18 cm from the tank. LED panels were placed into holders 20.5 cm from the tank. This arrangement

resulted in a diffuse light source across one end of the tank that could easily be toggled between left and right by inserting and removing LED panels from each side. An occluder constructed from black plastic and matte black fabric was inserted immediately adjacent to the tank on the 'off side' to prevent reflection off of the off-side diffusers due to light transmitted across the tank. The fish were illuminated from below with a 940 nm backlight consisting of a series of LEDs (Lumex Opto/Components Inc, OED-EL-8L) placed on the sides of a light guide panel underneath the tank. A Sony HDR-SR11 video camera with Nightshot (infrared-sensitive) mode activated was mounted above the tank and used to record the fish. The behavioral trials were conducted in a dark interior room with no windows, and the immediate recording area (tank, light sources, and camera) was draped with a double layer of black fleece.

LEDs of two wavelengths (590 nm and 770 nm) were used as light sources for the assay (Martek, MTE5900N-UY and MTE1077N1-R). The LEDs were arranged on two panels to be inserted into the holders on the left and right sides of the tank. Each panel held two LEDs of each wavelength. The panels were wired together, with LEDs of the same wavelength connected in series, and the 590 nm and 770 nm LEDs arranged in parallel. This arrangement allowed for the wavelength to be controlled by a single switch. Furthermore, because only one panel was inserted near the tank at a given time, the second panel could be placed outside of the recording area where it was visible to the researcher, but not the fish. This allowed for verification that lights were on or off at the appropriate time, and that the correct wavelength was selected.

All necessary controls were located outside of the immediate recording area to minimize disturbances to the fish. These included an on/off switch, a switch controlling wavelength, and a decade box for controlling resistance in the circuit (IET LABS, RS-200) to modulate light intensity. In addition, the camera was controlled by remote, and observed periodically with a 7 inch TFT-LCD monitor covered in several layers of red plastic, located outside of the draped area.

Behavioral Assay

Cyp27c1$^{\Delta 1/\Delta 3}$ mutants and wild-type siblings were treated for 2.5 – 3.5 weeks with either TH or a vehicle control and maintained in the same treated water during recording. The experimenter and data analyst were blind to the genotype of the fish. Forty-three fish were assayed: 11 TH-treated *cyp27c1*$^{\Delta 1/\Delta 3}$ mutants (8 male), 12 TH-treated wild-types (7 male), 10 vehicle-treated *cyp27c1*$^{\Delta 1/\Delta 3}$ mutants (5 male), and 10 TH-treated wild-type (6

male). One (of 12) TH-treated *cyp27c1*$^{\Delta/1\Delta3}$ mutant was excluded, as it exhibited erratic swimming behavior. Fish were dark adapted overnight in tanks with the same dimensions as the recording tank, with 1-3 fish per tank. The next afternoon, each fish was transferred individually to the recording tank and allowed to acclimate 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. To eliminate the possibility that fish preference for one side of the tank or the other might skew the results, we alternated the end of the tank illuminated in each trial. This was accomplished by moving the occluder from the 'off' side of the previous trial to the 'off' side of the next trial, removing the LED panel from the 'on' side of the previous trial, and inserting an LED panel in the 'on' side of the next trial. This process took about 1.5 minutes and was performed in darkness.

The intensities of the 590 nm and 770 nm LEDs were determined empirically. We tested a series of irradiance levels over five orders of magnitude at 770 nm in a pilot experiment with four TH-treated wild-type and *cyp27c1*$^{\Delta1/\Delta3}$ mutant fish, and observed a significant response from TH-treated wild-type, but not *cyp27c1*$^{\Delta1/\Delta3}$ mutant fish at 4.8×10^{-9} and 4.8×10^{-8} μmol/m^2/s. For 770 nm LEDs, we selected the dimmest irradiance level at which we observed a response (4.8×10^{-9} μmol/m^2/s). Because we expect the photoreceptors in both groups to be more sensitive to 590 nm light than 770 nm light, we used the 590 nm LEDs at a lower irradiance level (8.4×10^{-11} μmol/m^2/s), for which we observed a robust response from both genotypes in pilot experiments. Irradiance levels were measured using a Black Comet UV-vis spectrometer calibrated across 200-850 nm, fitted with a CR2 cosine corrector (StellarNet Inc, Tampa, FL). Data analysis was conducted with SpectraWiz software provided by the manufacturer.

Fish Tracking and Statistical Analysis

The videos were recorded at 1440 × 1080 pixels and down-sampled to 640 x 360 pixels using Quicktime prior to analysis. 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). The videos were converted to grayscale and noise removed by applying blur (12 pixel diameter). The program generated a background (image without the fish) using commonalities among ten frames chosen throughout the video sequence. By iteratively comparing frames, the program found the largest region of change in pixel grayscale value for every frame (using a pixel difference threshold of >3%) and then

recorded the x-y coordinates of its center of mass. We plotted the path of fish movement for every video and each of these was visually examined to confirm accuracy of the tracking software.

We then calculated the percentage of time spent within 25 mm of the lit wall of the tank. Each trial consisted of 3 min with the LEDs off and 3 min with the LEDs on. The same 'lit end' was used for the paired on and off videos. For each fish, the mean time spent within 25 mm of the lit end was calculated from three trials for each wavelength. These individual fish mean values were then used to calculate group means for all fish in each treatment. 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).

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