Current Biology, Vol. 15, 1–6, October 11, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.08.010

Mix and Match Color Vision: Tuning Spectral Sensitivity by Differential Opsin Gene Expression in Lake Malawi Cichlids

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Summary

Cichlid fish of the East African Rift Lakes are renowned for their diversity and offer a unique opportunity to study adaptive changes in the visual system in rapidly evolving species flocks [1, 2]. Since color plays a significant role in mate choice [3–6], differences in visual sensitivities could greatly influence and even drive speciation of cichlids. Lake Malawi cichlids inhabiting rock and sand habitats have significantly different cone spectral sensitivities [7, 8]. By combining microspectrophotometry (MSP) of isolated cones, sequencing of opsin genes, and spectral analysis of recombinant pigments, we have established the cone complements of four species of Malawi cichlids. MSP demonstrated that each of these species predominately expresses three cone pigments, although these differ between species to give three spectrally different cone complements. In addition, rare populations of spectrally distinct cones were found. In total, seven spectral classes were identified. This was confirmed by opsin gene sequencing, expression, and in vitro reconstitution. The genes represent the four major classes of cone opsin genes that diverged early in vertebrate evolution [9]. All four species possess a long-wave-sensitive (LWS), three spectrally distinct green-sensitive (RH2), a blue-sensitive (SWS2A), a violet-sensitive (SWS2B), and an ultraviolet-sensitive (SWS1) opsin. However, African cichlids determine their spectral sensitivity by differential expression of primarily only three of the seven available cone opsin genes. Phylogenetic analysis suggests that all percomorph fish have similar potential.

Results and Discussion

To examine the spectral sensitivities of the cone visual pigments of the different habitat groups of Malawi cichlids, we have carried out further investigations on *Metriaclima zebra*, a species used previously [7], and have extended the study to include three additional species. Two of the latter are from the Mbuna (rock dwelling) flock (as is *M. zebra*). *Pseudotropheus acei* is found in shallow water on a clear sandy bottom and in bogs. It grazes on algae growing on underwater logs and driftwood. Both males and females are blue with white or yellow tail markings. *Melanochromis vermivorus* lives in shallow water on a rocky shore, free of sediment. It is omnivorous, feeding on plankton and grazing on rock algae. Breeding males are blue or dark purple with black stripes, whereas females are yellow with black stripes. The third species, *Tramitichromis (Lethrinops) intermedius*, is from the sand-dwelling flock and inhabits sheltered water among weeds. It feeds on insect larvae and soft invertebrates extracted from the sediments. Breeding males are silvery with orange and green highlights, whereas females are silver with three large dark spots on their flanks.

Cone Classes

Each of the three species exhibited rods and primarily only three cone pigments, two longer-wave pigments in double cones and the third, short-wave pigment in single cones. In all species, the rods have a wavelength of maximum absorbance (λ_{max}) at 505 nm. Across the three species, seven spectrally distinct cone pigments were identified. In *P. acei* and *M. vermivorus*, the majority of double cones contained a 535 nm pigment paired with a pigment with λ_{max} of 481–485 nm, whereas in *T. intermedius*, the double cones were more long-wave sensitive with a 532 nm pigment paired with a 569 nm pigment (Table 1, Figure 1). The dominant population of single cones differed between species with λ_{max} at 455 nm in *T. intermedius*, 418 nm in *M. vermivorus*, and 378 nm in *P. acei* (Table 1, Figure 1).

This follows the pattern found in previous studies of two other Malawi cichlids, *M. zebra* and *Dimidiochromis compressiceps* [7, 8]. *M. zebra* and *P. acei* have spectrally similar pigments (Table 2), whereas *M. vermivorus* is somewhat different in that although the double cones are spectrally similar to *M. zebra* and *P. acei*, the single cones are violet sensitive with λ_{max} at 418 nm, a pigment not previously reported. *T. intermedius* is similar to *D. compressiceps* with double cone pigments with λ_{max} at 535 and 568 nm and blue-sensitive single cones with λ_{max} at 450 nm.

In addition to the predominant single and double cones, other cone types were present at substantially lower frequencies. This was most noticeable in *P. acei*, where a second population of double cones with the 535 nm pigment paired with a 505 nm pigment was present (Figure 1). Additionally, in *P. acei*, a further six spectrally distinct single cone types were observed (Table 1; Figure 1H). The majority had λ_{max} at either 505 or 534 nm, but a few had λ_{max} at 476 and 566 nm. In all cases, these pigments are spectrally similar to at least one of the pigments found in the double cones of the three species. Individual examples of short-wave-sensitive single cones were also found with λ_{max} at 415 and 452 nm (Table 1), spectrally similar to the short-wave-sensitive single cones of the other two species.

M. vermivorus also has a small second population of

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Table 1. Visual Pigments of Rods and Cones

	Pseudotropheus acei	*Melanochromis vermivorus*	*Tramitichromis intermedius*
Rods	504.7 ± 1.1 (20)	504.7 ± 1.1 (25)	505.6 ± 0.9 (21)
Single short-wave cones	**378.4 ± 2.5 (19)**		
	415.1 ± 4.7 (1)	**418.1 ± 1.3 (14)**	
	451.5 ± 7.5 (1)		**455.1 ± 1.9 (17)**
Double cones	**481.6 ± 1.0 (25)**	**485.0 ± 1.2 (22)**	
	505.7 ± 2.7 (3)	502.9 ± 3.5 (1)	
	535.1 ± 0.6 (24)	**534.8 ± 0.5 (32)**	**531.6 ± 0.6 (37)**
			568.9 ± 1.1 (27)
Single middle- and long-wave cones	475.9 ± 5.3 (1)		
	505.3 ± 2.3 (5)	505.9 ± 3.9 (1)	
	534.1 ± 2.1 (5)		534.0 ± 1.5 (3)
	565.7 ± 8.0 (1)	555.2 ± 2.2 (3)	

Dominant cone pigments are shown in bold. Numbers of cells analyzed in parentheses. In *P. acei* and *M. vermivorus*, the 505 nm pigment in double cones was paired with the 535 nm pigment. The rare middle- and long-wave single cone pigments are spectrally similar to the double cone pigments.

double cones, spectrally similar to the minority double cones in *P. acei*, and two additional types of single cone, with λ_{max} at 505 and 555 nm (Table 1). The 555 nm pigment is about 10 nm shorter than the LWS pigment found in the other two species, but since only a single LWS gene has been identified in these cichlids, we assume that this pigment is encoded by the LWS gene. In *T. intermedius*, rare single cones were limited to a single population with a 535 nm pigment, spectrally similar to one of the pigments in the double cones.

The presence of minority cone classes raises the important question of whether they are functionally significant. The spectral sensitivities of two species of Malawi cichlid, *Aulonocara jacobfreibergi* and *Metriaclima thapsinogen*, can be constructed from components with peak sensitivities at 369, 488, and 533 nm [10], equivalent to the dominant cone pigment combination found in *P. acei* (this study) and *M. zebra* [7]. However, a small contribution from a 570 nm component was also required, indicating that the minority cone types may well contribute to visual sensitivity.

In summary, in Malawi cichlids, MSP has identified seven spectral classes of cone with λ_{max} around 370, 420, 450 480, 505, 535, and 564 nm.

Opsin Coding Sequences

Genomic DNA was extracted from liver or fin clips preserved in ethanol from the individuals examined by

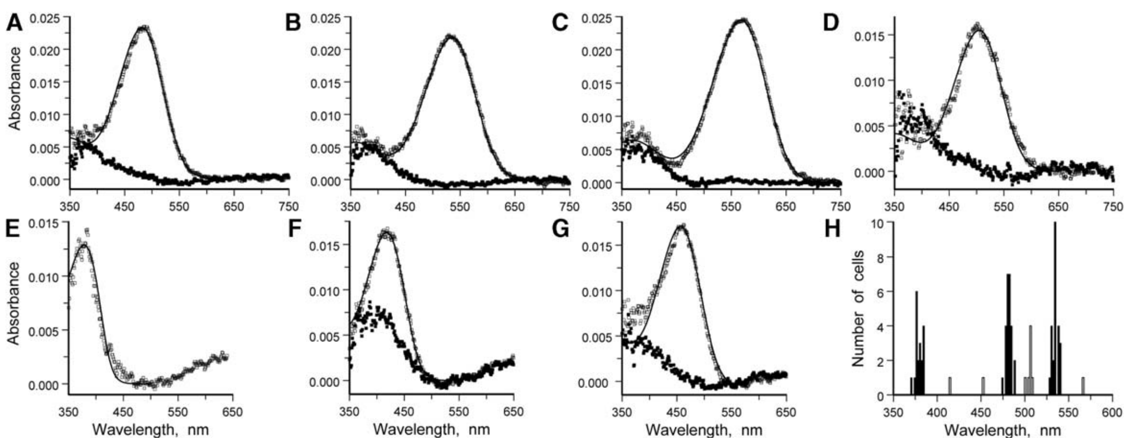

Figure 1. Mean Absorbance Spectra of the Seven Cone Visual Pigments as Determined by MSP

(A–D) Double cones. (A) P482 from *Pseudotropheus acei*; (B) P535 from *P. acei*; (C) P569 from *Tramitichromis intermedius*; (D) P505 from *P. acei*.

(E–G) Single cones. (E) P378 from *P. acei*; (F) P418 from *M. vermivorus*; (G) P455 from *T. intermedius*.

(H) Distribution of the seven cone pigments from *P. acei*.

Open squares, prebleach; filled squares, postbleach. Spectra were best fitted with a pure rhodopsin template, full lines [26], with no evidence for the presence of porphyropsins. For details of cell numbers and λ_{max}, see Table 1.

Three adult fish from each species were studied. Retinal samples were prepared as previously described [12]. Some samples were lightly fixed in 2% glutaraldehyde for 15–30 s and were used for up to 2 weeks after preparation. Since MSP samples photoreceptors randomly, at least two pieces of retina were analyzed from different regions of each eye, so as to lesson the problem of any regional distribution of different classes of cone. MSP recordings were made in the conventional manner using a Liebman dual-beam microspectrophotometer [27, 28]. Selection criteria were used to discard records that either had low absorbance or were clearly distorted.

Table 2. Comparison of Cone Pigment λ_{max} by Expression and Microspectrophotometry

Species	LWS	RH2Aα	RH2Aβ	RH2B	SWS2A	SWS2B	SWS1
Expressed							
Metriaclima zebra	—	528	519	484	—	423	368
MSP							
Metriaclima zebra[a]		**533**	—	**488**	—	—	**368**
Pseudotropheus acei	566	**534**	505	**482**	452	415	**378**
Melanochromis vermivorus	556	**534**	504	**485**	—	**418**	—
Tramitichromis intermedius	**569**	**532**	—	—	**455**	—	—

Dominant cone pigments are shown in bold.
[a] Ref [7].

MSP. Initially, the same set of five cone opsin genes found previously [7] were identified and sequenced. The primers and sequencing strategy were similar to that used before (Table S1 in the Supplemental Data available with this article online) [7, 11, 12]. The genes were amplified as either one (RH1, SWS1, and RH2A), two (SWS2A, RH2B), or three (SWS2B and LWS) fragments, depending on the size of the introns. The fragments were sequenced and used to assemble the entire gene sequence. This included sequences for all introns except the second intron in RH2B that was over 1500 bp. Previous long PCR experiments have confirmed that this procedure produces contiguous sequences in the genome [7].

The LWS, RH2, and both SWS2 opsin sequences show only minor differences at the amino acid level from the previous sequences [7] (Table S2). However, to account for the additional pigments found in the present study, the four cone opsin classes were subjected to further analysis. Two additional RH2 genes were amplified and sequenced in the three new species and in *M. zebra*. The corresponding gene sequences in a BAC clone of the Nile tilapia, *Oreochromis niloticus*, considered to be representative of the riverine ancestor of the lacustrine flocks [13], were used to design specific primers (Table S1). The new RH2 genes will be referred to as RH2Aα and RH2B (Table 2). RH2Aα shows very strong sequence identity with the extant RH2 sequence (RH2Aβ) (94.9%–96.0% identity, 97.2%–98.3% similarity at the amino acid level), whereas RH2B shows more divergence (80.7%–83.2% identity, 88.1%–89.3% similarity).

These sequences have been deposited with GenBank with accession numbers DQ088627–DQ088652 (Table S1).

In Vitro Synthesis and Spectral Analysis of Visual Pigments

In order to correlate opsin gene sequences with the different pigments identified by MSP, the SWS1, SWS2B, and the three RH2 opsin sequences were amplified from retinal cDNA, expressed in mammalian cells, and regenerated with 11-*cis*-retinal in vitro. The retinal cDNA came from *M. zebra* [7] in which the principal cone pigments are spectrally very similar to those of *P. acei*. Moreover, a comparison of the opsin gene sequences of *M. zebra* with the three species used in the present study revealed only very minor amino acid differences, of which few had potential significance in spectral tuning (Table S2). A direct correlation of genes with pigments via a similar or identical λ_{max} of the in situ and in vitro pigments can be made for the three cone

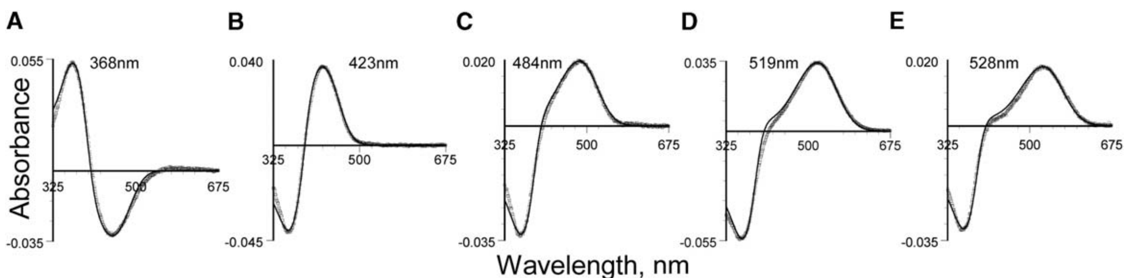

Figure 2. Difference Spectra of Visual Pigments Expressed In Vitro

Absorbance spectra were measured before the pigment was denatured with acid (A) or hydroxylamine treated (B–E). The latter spectra were subtracted from the former and the resulting difference spectra were adjusted to zero by subtraction of a baseline based on points longwave of the absorbance peak, before fitting with visual pigment templates (lines) [26].

(A) *M. zebra* SWS1, 368 nm; (B) *M. zebra* SWS2B, 423 nm; (C) *M. zebra* RH2B, 484 nm; (D) *M. zebra* RH2Aβ, 519 nm; (E) *M. zebra* RH2Aα, 528 nm.

Opsins from *Metriaclima zebra* were amplified from retinal cDNA using Dynazyme EXT (MJ Research), a proofreading polymerase, and primers designed to amplify the entire coding region with added restriction enzyme sites. The result, after restriction enzyme digestion, consisted of *Eco*RI-*Sal*I fragments that could be directionally inserted into the vector pMT3 in the correct reading frame for subsequent expression which was carried out using established methods [17]. Constructs were sequenced through the entire insert including the cloning sites to ensure fidelity of the opsin sequence.

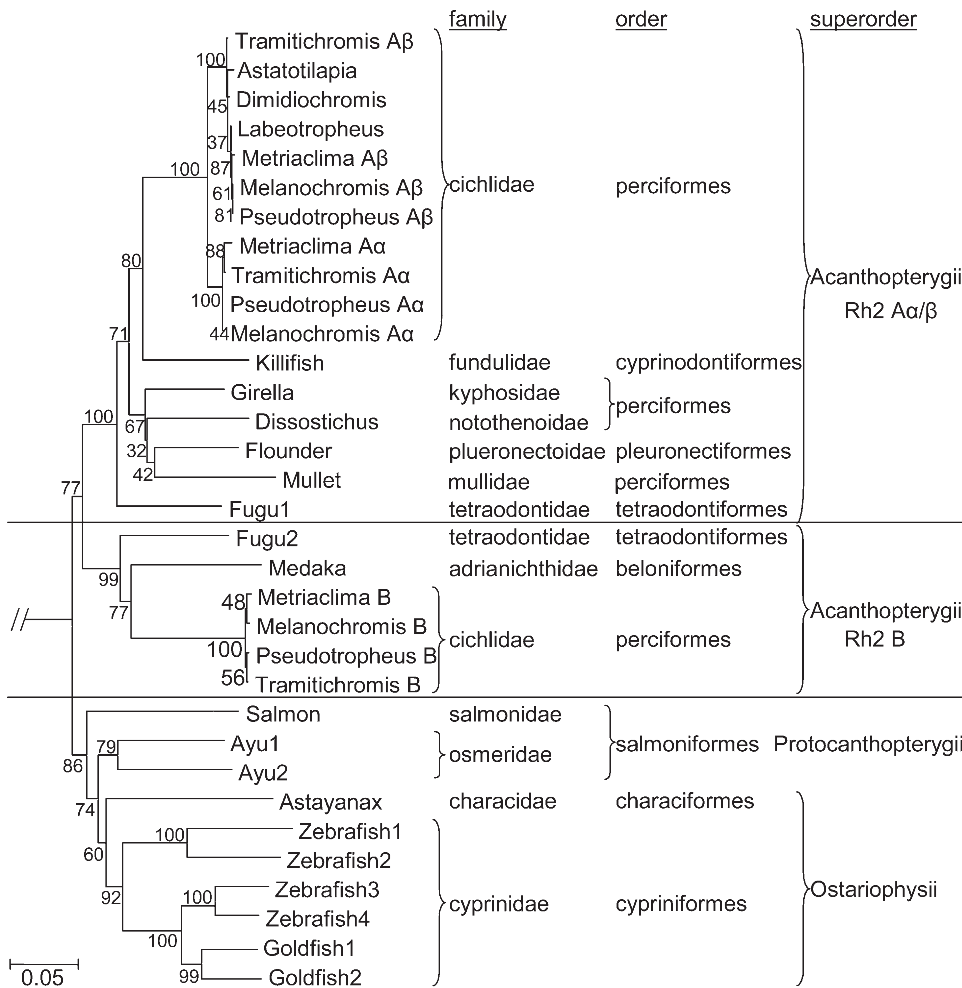

Figure 3. Phylogeny of Teleosts RH2 Cone Opsin Genes

Nucleotide sequences were aligned by Clustal W, and the tree was generated by the neighbor-joining method [29] (p distance, pairwise deletion of missing data) using the MEGA version 2.1 phylogenetic and molecular evolutionary analysis program [30]. The bootstrap confidence values (1000 replicates) are shown for each branch. The goldfish LWS sequence was used as an outgroup. The scale bar is equal to 0.05 substitutions per site. Sequences were obtained from this study and from GenBank (AY660539, AF247130, AF247123, AY296739, AB158259, AY771352, AY631037, Y18680, AF226989, AY599603, AB001603, AY214132, AB098703-4, AH004622, AB087805-8, L11865-6, U12328). The Acanthopterygii represented are all percomorphs. The clade of nonpercomorph sequences contains representatives of the Ostariophysii (cyprinids and characids) and the Protocanthopterygii (salmonids and osmerids).

pigments of *M. zebra*, and the close similarities between the λ_{max} values of the other species in the present study permits a similar correlation for the other two reconstituted pigments (Table 2).

Reconstitution of the SWS1 and SWS2B opsins with 11-*cis*-retinal gave pigments with λ_{max} at 368 and 423 nm, respectively (Figure 2). The SWS1 pigment is UV sensitive and thus responsible for the 378/368 nm pigments in *P. acei*/*M. zebra*. The SWS2B pigment correlates with the 415/418/423 nm pigments in *P. acei, M. vermivorus*, and *M. zebra*, respectively. The 452/455 nm pigments of *P. acei* and *T. intermedius* must therefore be products of the SWS2A gene, which has been confirmed in *O. niloticus* where the gene expresses a 456 nm pigment (T.S., unpublished data).

The presence of a violet-sensitive SWS2B pigment in cichlids, in the bluefin killifish [14], and also in the tetraodontiforms *Takifugu* and *Tetraodon* (GenBank AY598947, AY598948) demonstrates that violet sensitivity is achieved via an SWS2 pigment in fish and may explain the lack of a violet-tuned SWS1 pigment in any teleost species examined to date. This contrasts with the tuning of SWS1 pigments into the violet region of the spectrum in most other vertebrates groups [15–17].

The RH2Aα, RH2Aβ, and RH2B genes gave pigments with λ_{max} at 528 nm, 519 nm, and 484 nm, respectively (Figure 2); two have very similar λ_{max} values to those measured by MSP, though the 519 nm pigment is somewhat shifted from the 505 nm pigment identified by MSP (Table 2). However, the data are from different, but very closely related, species, and it is probable that both λ_{max} values arise from the pigment encoded by the RH2Aβ gene. Further, such spectral differences are not uncommon for expressed visual pigments (e.g., [17,

18]). Nevertheless, we cannot rule out the possibility that another, as yet unidentified, RH2 gene is present or that the 505 nm cones are coexpressing RH2A and RH2B pigments. The spectral difference between the RH2A and RH2B cone pigments is about 30–40 nm, and potential tuning sites are discussed in the Supplemental Data.

Since the 534, 505, and 484 nm pigments correlate with the RH2Aα, RH2Aβ, and RH2B opsins, the 566 nm pigment must be the product of the LWS gene, again confirmed in *O. niloticus* (T.S., unpublished data). Interestingly, the opsin composition of the double cones varies across species, with the RH2Aα pigment paired with the LWS pigment in *T. intermedius*, whereas in the remaining species, both members of the double cones express spectrally distinct RH2 pigments, with the RH2B/RH2Aα combination predominating.

In summary, it is clear from the four species we have studied that there are seven cone opsin genes that are differentially expressed in Malawi cichlids. Only single copies of the SWS1 UV opsin and the LWS opsin genes are present, whereas the SWS2 class is represented by two copies and the RH2 class by three copies. Three spectrally distinct combinations of expressed genes have been identified (SWS1, RH2B, and RH2Aα; SWS2B, RH2B, and RH2Aα; and SWS2A, RH2Aα, and LWS), but we cannot rule out the possibility in other species of more combinations that include the RH2Aβ gene.

Phylogenetic Analysis

Phylogenetic analysis of the different cichlid RH2 opsin genes (Figure 3) demonstrates the role of gene divergence and duplication in the evolution of this class of opsin. Within teleosts, three sequence clades correspond to the RH2A, RH2B, and remaining (nonpercomorph) RH2 sequences. An ancestral RH2 gene would have diverged significantly between the early Ostariophysii and Protocanthopterygii and the later Acanthopterygii. Within the Acanthopterygii, subsequent gene duplications have led to the three identified RH2 percomorph genes (RH2Aα, RH2Aβ, RH2B), distinct from the RH2 gene of the Ostariophysii and Protocanthopterygii. The RH2A type is found in the Perciformes, Cyprinodontiformes, Pleuronectiformes, and Tetraodontiformes, although only African cichlids have been shown to possess the RH2Aα and RH2Aβ variants, which may represent a recent gene duplication within this group. The RH2B gene is seen in cichlids and in the Tetraodontiformes and Beloniformes [19], and the phylogeny implies that the gene must have been present ancestrally in all species from the percomorph subsection of the Acanthopterygii. In the Ostariophysi and Protocanthopterygii, their distinct RH2 gene shows a totally independent series of duplications [20–22], quite separate from the duplicated RH2 genes in the cichlids.

Selective Forces Driving the Divergence

We have shown that three species of cichlid fish each have diverse visual systems as a result of differential opsin gene expression. The fact that seven opsin genes are expressed in at least three different gene combinations suggests that in cichlids, it is a labile mechanism for varying visual sensitivity, but it is unclear what selective force is driving the divergence. One factor likely to be important is mode of foraging. *T. intermedius* feeds out of the sand and has enhanced sensory pores for finding prey by vibration using lateral lines. This species may not need specialized visual sensitivities for foraging, though its longer-wavelength sensitivity may enhance visual sensitivity in turbid water [23]. *M. vermivorous* and *P. acei* feed on a variety of materials including algae and zooplankton. Their shorter wavelength sensitivity, particularly the ultraviolet sensitivity of *P. acei*, is likely to enhance foraging of zooplankton [24, 25]. However, neither foraging nor habitat can explain the difference in short-wave sensitivity between *P. acei*/*M. zebra* and *M. vermivorous*.

In summary, Malawi cichlids possess at least seven cone opsin genes, but any one species predominantly expresses only three of these. Phylogeny of these opsins suggests that percomorphs, and possibly all Acanthopterygii, possess at least six of these opsin loci and therefore also have the potential to use differential gene expression to tune their visual systems to their photic environment.

Supplemental Data

Supplemental Data include two tables and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/19/■■■/DC1/.

Acknowledgements

This work was supported by a grant from the Leverhulme Trust to J.K.B. and D.M.H. and by a National Science Foundation grant to K.L.C. (IBN-0131285). Thanks to Jan Boyer for help with sequencing the RH2Aα genes. We thank Dr. Rosalie Crouch of the Storm Eye Institute, Medical University of South Carolina, for the 11-*cis*-retinal.

Received: July 14, 2005
Revised: August 3, 2005
Accepted: August 4, 2005
Published: October 11, 2005

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