Assembly of the cnidarian camera-type eye from vertebrate-like components


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Animal eyes are morphologically diverse. Their assembly, however, always relies on the same basic principle, i.e., photoreceptors located in the vicinity of dark shielding pigment. Cnidaria as the likely sister group to the Bilateria are the earliest branching phylum with a well developed visual system. Here, we show that camera-type eyes of the cubozoan jellyfish, Tripedalia cystophora, use genetic building blocks typical of vertebrate eyes, namely, a ciliary phototransduction cascade and melanogenic pathway. Our findings indicate parallelism provide an insight into eye evolution. Combined, the available data favor the possibility that vertebrate and cubozoan eyes arose by independent recruitment of orthologous genes during evolution.

The assembly of diverse animal eyes requires two fundamental building blocks, photoreceptors and dark shielding pigment. The function of photoreceptors is to convert light (stream of photons) into intracellular signaling. The photoreceptor cells (PRCs) are classified into two distinct types: rhabdomeric, characteristic of vision in invertebrate eyes; and ciliary, characteristic of vision in vertebrate eyes (1). In both ciliary and rhabdomeric PRCs, the seven-transmembrane receptor (opsin) associates with retinal to constitute a functional photosensitive pigment. Each photoreceptor type uses a separate phototransduction cascade. Rhabdomeric photoreceptors employ r-opsins and a phospholipase C cascade, whereas ciliary photoreceptors use c-opsins and a phosphodiesterase (PDE) cascade (2, 3). In general, the dark pigment reduces photon scatter and orients the direction optimally sensitive to light. The biochemical nature of the dark pigment appears more diverse than the phototransduction cascades used by the PRCs. Vertebrate eyes use melanin as their exclusive dark pigment. However, among invertebrates, pterins and ommochromes are accumulated in eyes of Drosophila (5), and melanin is found rarely such as in the inverse cup-like eyes of the planarian, Dugesia (6).

Cnidaria, the likely sister group to the Bilateria, constitute the earliest branching phylum containing a well developed visual system. For example, Cuboza (known as “box jellyfish”) have camera-type eyes with cornea, lens, and retina; unexpectedly, the cubozoan retina has ciliated PRCs that are typical for vertebrate eyes (7–9). Cubomedusae are active swimmers that are able to differentiate optical characteristics of vision in vertebrate eyes (1). In both ciliary and rhabdomeric PRCs, the seven-transmembrane receptor (opsin) associates with retinal to constitute a functional photosensitive pigment. Each photoreceptor type uses a separate phototransduction cascade. Rhabdomeric photoreceptors employ r-opsins and a phospholipase C cascade, whereas ciliary photoreceptors use c-opsins and a phosphodiesterase (PDE) cascade (2, 3). In general, the dark pigment reduces photon scatter and orients the direction optimally sensitive to light. The biochemical nature of the dark pigment appears more diverse than the phototransduction cascades used by the PRCs. Vertebrate eyes use melanin as their exclusive dark pigment. However, among invertebrates, pterins and ommochromes are accumulated in eyes of Drosophila (5), and melanin is found rarely such as in the inverse cup-like eyes of the planarian, Dugesia (6).

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In the present work, we characterize genes required for the assembly of camera-type eyes in Tripedalia. We show that the genetic building blocks typical of vertebrate eyes, namely ciliary opsin and the melanogenic pathway, are used by the cubozoan eyes. Although our findings of unsuspected parallelism are consistent with either an independent origin or common ancestry of cubozoan and vertebrate eyes, we believe the present data favor the former alternative.

Results

Ciliary OOpin Is Expressed in Camera-Type Eyes of Tripedalia. We screened an expressed sequence tag (EST) library derived from rhopalia of Tripedalia to identify the jellyfish genes that are involved in vision; orthologues of other invertebrates and vertebrates were identified by phylogenetic analysis. Of the four opsin types present at the base of the bilaterians [rhabdomeric (r-opsin), ciliary (c-opsin), G1-opsins, and peropsin/RGR (12–14)], the Tripedalia opsin EST clustered with the c-opsins, an orthology consistent with the conservation of the characteristic stretch of deduced amino acids between the transmembrane domain VII and cytoplasmic tail [supporting information (SI) Fig. S1]. This region includes the c-opsin fingerprint tripeptide NR/KQ (NRS in Tripedalia) that is critical for coupling to the downstream phototransduction cascade through interaction with a GTP-binding protein subunit GRα in the vertebrate rods and cones (15). An antibody generated against Tripedalia c-opsin recognized a single electrophoretic band in protein extracts prepared from rhopalia and COS-7 cells transfected with c-opsin cDNA (Fig. 1C). Camera-type eyes of adult jellyfish (Fig. 1D) were immunostained with an anti-c-opsin antibody. The c-opsin localized in the retinal ciliated PRCs of both complex eyes (Fig. 1E and F) in a pattern resembling that by staining with anti-acetylated tubulin antibody (Fig. 1G), which specifically labels stabilized microtubules in axons and cilia (13).

Spectral Sensitivity of Tripedalia c-Opsin. To address the question of whether the identified Tripedalia c-opsin can function as a true visual pigment, we determined the spectral sensitivity of the opsin protein by photoreceptor degeneration (8). The tripedalian c-opsin exhibited a single broad absorption maximum centered at 495 nm in alkaline ethanol extracts of retina sections. This spectral profile is typical of c-opsins and is consistent with a red visual pigment.


The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU310498 (c-opsin), EU310502 (cox), EU310499 (mitf), EU310500 (catalytic pote), EU310501 (inhibitory pote) and EU310503 (guanylate cyclase)).

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visual opsin, we tested its photochemical properties. Tripedalia c-opsin was expressed in COS-1 cells and reconstituted as a functional photosensitive pigment with 11-cis-retinal. The reconstituted c-opsin was most sensitive to the blue–green region of the spectrum with a peak absorbance (Amax) at 465–470 nm (Fig. 1H), in agreement with the spectral sensitivity of the Tripedalia electroretinogram (16). We conclude that Tripedalia c-opsin is a functional, vertebrate-like photopigment expressed in the PRCs of the camera-type cubozoan eye.

**Tripedalia Orthologues of Vertebrate-Like Phototransduction Genes.** In vertebrates, activated heterotrimeric G proteins use cGMP PDE for signal transduction. In accordance with our identification of a vertebrate-like c-opsin in Tripedalia, we found that the catalytic subunit of pde expressed in the Tripedalia rhopalium phylogenetically clusters with the group of GAF domain-containing PDEs including vertebrate rod- and cone-specific $PDE6$ (Fig. S2A). Furthermore, we identified other components of the ciliary-type cascade associated with deactivation or adaptation of phototransduction, such as the inhibitory subunit of phosphodiesterase ($PDE6D$), phosducin and guanylate cyclase (Fig. S2 B–D). Thus, the nature of the genes expressed in the rhopalium (detected by RT-PCR; Fig. S3) suggests that the camera-type eye of Tripedalia uses a ciliary-type phototransduction cascade similar to that of vertebrates.

**Melanin Granules in Tripedalia PRCs.** A conspicuous ring of dark shielding pigment surrounds the area of c-opsin expression (compare Fig. 1D and E). Most if not all PRCs in the Tripedalia retina contain pigment granules (Fig. 2A). These PRCs thus resemble what one might imagine a prototypical ancestral cell combining photoreceptor and pigment functions to look like (17). The biochemical nature of the Tripedalia pigment was suggested by the identification of an orthologue of the vertebrate ocular and cutaneous albinism-2 ($Oca2$) gene in our EST library (Fig. S4A). $Oca2$ (also known as pink-eyed dilution) is an essential gene for melanin biosynthesis. It is the most commonly mutated gene in cases of human albinism (18). In addition, mutations in $Oca2$ are responsible for pigmentation defects in mouse (19), medaka (20), and independently arisen populations of the cave fish, Astyanax (21). In situ hybridization analysis revealed that Tripedalia $oca$ is conspicuously expressed near the pigmented retina layer of PRCs (Fig. 2C). Control staining with an $oca$ sense probe (Fig. 2D) did not yield a signal. The results of a direct chemical assay (Fontana–Masson) were consistent with melanin being the pigment in the Tripedalia retina (Fig. 2E–G). In vertebrates, development of melanin-producing cells and specification of retinal pigment cells require the conserved
microphthalmia-associated transcription factor, Mitf (22–24). Mitf regulates expression of tyrosinase and tyrosinase-related protein-1 and -2, which are necessary for melanin biosynthesis (for review, see ref. 25). Here, we have cloned a mitf orthologue from Tripedalia (Fig. S4A) expressed in a ring-like pattern just outside of the melanin deposits (Fig. 3 A and B); this area contains the PRC nuclei (Figs. 2 A and 3D). Thus, mitf is a conserved transcription factor with shared expression patterns in the complex eyes of Tripedalia and vertebrates.

**Mitf Expression in Lens and Crystallin Expression in Pigmented PRCs of Nonlens Eyes of Tripedalia.** In addition to expression in the pigmented PRCs of camera-type eyes, mitf mRNA was detected in the outermost cells of the Tripedalia lens (Fig. 3C). Consequently, to investigate a possible relationship between the pigmented PRCs and the cellular lens we examined whether J1-crystallin, the major protein of the Tripedalia lens (26), is expressed in PRCs. The J1-crystallin antibody immunostained the slit and pit eyes as well as the cellular lens (Fig. 3 D and E). The presence of J1-crystallin in the slit and pit eyes of Tripedalia was unexpected because these cup-like eyes lack cellular lenses. They do, however, have pigmented PRCs, suggesting a relationship between the PRCs and cellular lens that warrants further study.

**Discussion**

The present work reveals surprising similarities in the genetic components used for visual system development in vertebrates and cubozoan jellyfish. If Cubozoa and vertebrates express orthologous c-opsins in their PRCs and make use of the same pigmentation pathway including the key transcription factor Mitf, does this represent a parallel evolution or conservation of an ancestral “eye” program between those evolutionarily distant animal phyla (Fig. 4)? Although our data are formally consistent with both evolutionary scenarios, we believe that they favor the former.

Even though ciliary and rhodobionic photoreceptive systems coexist throughout the animal taxa (1), the present evidence suggests that their evolutionary histories differ. For photode-

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**Fig. 3.** Expression of mitf and J1-crystallin in Tripedalia eyes. (A–C) In situ hybridization (blue) detects mitf expression in the circle around pigment deposits (A and B) and in the lens (C, arrows). (D and E) Immunohistochemistry staining using an antibody to the major cubozoan lens crystallin, J1 (red). Nuclei of cells are visualized by DAPI staining (blue). J1-crystallin expression is localized to lenses of camera-type eyes (D) as well as to the slit and pit eyes (E).

**Fig. 4.** Two scenarios for the use of ciliary phototransduction and melanogenic pathway in eye evolutionary history. A simplified view of the two evolutionary scenarios is compatible with the data in the present work. The use of similar genetic components in vertebrate and cubozoan eyes is either due to common ancestry (A) or independent parallel recruitments in cnidarian and vertebrate lineages (B). The c-opsins and Go/r-opsins arose by duplication and diversification of an ancestral opsin in the early metazoans (27). In the schemes, only the visual (i.e., the eye-specific) PRCs and opsins are considered. Different shading of pigment granules indicates possible distinct chemical composition. CBA: cnidarian–bilaterian ancestor; UBA, ubilaterian ancestor.
after the separation of the cnidarian and bilaterian lineages (Fig. 4) (27).

All eyes have shielding pigment typically found in cells adjacent to the PRCs. Melanin, the dark pigment of Tripedalia eyes, presumably performs the same function in vertebrate eyes as in the simple cup-like eyes of a basal lophotrochozoan, Dugesia (6). Interestingly, Dugesia uses another pigment, an ommochrome, as the body pigment (2, 6). Pterins constitute the dark eye pigment of the polychaete P. dumerilii (4), and pterins and ommochromes are the pigments in eyes of Drosophila (5). Thus, as with the opsins, Tripedalia shares the same dark pigment in the eye with vertebrates.

Unlike in the Dugesia eye, the camera-type Tripedalia eye combines the photoreceptor and pigment functions in the same cell consistent with an ancestral (basal) condition (Fig. 4A). However, the musaeus stage of cubozoans may well be a derived rather than ancestral condition for Cnidaria, complicating discussions about the basal state of the cubozoan visual system (Anthozoans, for example, do not have eyes). Nevertheless, it remains possible that the pigmented PRCs in Tripedalia are descendants of one of the postulated ancient prototypical photosensitive cells diversified by natural selection (17, 28). However, this does not require a common origin for the eyes. It was estimated through computer-based modeling (29) that fewer than a half-million generations would be required under selective pressure to proceed from a cluster of light-sensitive cells to a sophisticated camera-type eye. In theory, this relatively short time interval would allow sophisticated eyes to have originated de novo several times during evolution (polyphyletic eye origin).

For the common-ancestry model to be true, the cnidarian-bilaterian ancestor (CBA) must have had the same genetic determinants as its descendants. The common-ancestry scenario for cubozoan and vertebrate eyes requires, however, that animals in many bilaterian phyla lost their eyes that were initially assembled by using the same building blocks as in present-day vertebrates and Cuboza (c-opsins, melanin) to explain the exclusive occurrence of rhodomorphic PRCs in invertebrate eyes. There is no obvious explanation for such a specific selection against ciliary PRCs to be used for visual purposes. Eyes in general provide a freely moving animal with a tremendous advantage, and as such there should be a constant selection for eye maintenance, except in, for example, cave or underground animals. Although not definitive, there are at least two additional complications to the common-ancestry model that arise if one invokes the developmental argument that similar transcription factor cascades may direct development of vertebrate and cubozoan eyes. The first is that PaxB, a Pax2/6/7-related transcription factor, is used in Tripedalia (30) instead of Pax6 as in vertebrates (31) as well as flies (32, 33) and other species (34). The second is the apparent evolutionary “promiscuity” of developmental cascades in general; entire regulatory circuits can be co-opted for development of different cell types, tissues, or organs. For example, the Pax–Six–Eya–Dach gene regulatory network has a fundamental role in Drosophila visual system development but is also used for specification of muscle cells or placodes in vertebrates (35). Co-opting orthologous suites of genes for similar functions could be a possible explanation for independent or parallel evolution of cubozoan and vertebrate eyes with ciliary-type PRCs (Fig. 4B) (1, 36, 37). Independent derivation of Tripedalia and vertebrate eyes would also fit conceptually with the early idea that PRCs originated multiple times (38), although it does not address how many times PRCs themselves may have originated. That vertebrates and Cnidaria share many more genes than anticipated (39, 40), including pax, mitf, c-opsin, pde’s, phosducin, guanylate cyclase, and oca2 (ref. 30 and this work), supports the notion that both animal groups use similar sets of genes to generate significantly different body plans. It follows that changes in gene regulation, rather than “new” genes, may drive novelties such as eyes during evolution. Finally, ectopic eye formation by misexpression of Pax6 provides an astounding example of how an eye might arise de novo in a foreign tissue environment (33, 34). The fact that ectopic eyes can be generated experimentally suggests that the same gene, Pax, used by various groups of present-day animals could have been instrumental in creating eyes independently numerous times during evolution.

In addition to sharing the same genetic building blocks in their PRCs (ciliary phototransduction, melanogenic pathway), cubozoans and vertebrates both use a cellular lens to increase visual sensitivity and produce a sharp image in the desired plane of focus. The optical properties of cellular lenses are caused by the high-level expression of proteins collectively called crystallins (ref. 41 and this work). In striking contrast to the conservation of opsins as the visual pigments in the PRCs, the lens crystallins are diverse proteins that are often taxon-specific, i.e., entirely different proteins function as crystallins in different species. Similar transcription factors including those of the Pax gene family have been independently recruited for the regulation of nonhomologous crystallin genes in Tripedalia and vertebrates (30, 42, 43) to achieve a gradient of refractive index within their transparent lenses. The independent recruitment of lens crystallins is consistent with parallel evolution of cubozoan and vertebrate eyes and provides a striking example of the role of convergence in eye evolution.

Finally, the present findings of mitf in the lens and J1-crystallin in the pigmented slit and pit ocelli of Tripedalia support the idea that the cellular cubozoan lens arose from a pigmented cell ancestor. It is known that pigment cells may acquire the capacity to secrete lens-forming material (44). Combined, our data on J1-crystallin and mitf expression suggest that the cellular cubozoan lens with its remarkable ability to refract light without spherical aberration (11) originated from a pigment cell ancestor and that the primitive cup-like eyes located on the cubozoan rhopalia might be evolutionary forerunners of camera-type eyes.

In conclusion, the present study uncovers a surprising molecular parallelism in the eye design of vertebrates and cubozoan jellyfish. Although the current data do not distinguish unambiguously between the common-ancestry and independent-recruitment scenarios, we propose that they lean in the direction of the latter, favoring multiple independent reorganizations of common elements and independent recruitments of similar suites of genes during evolution of the diverse eyes.

Materials and Methods
Jellyfish Collection and Culture. T. cystophora was collected and cultured as described in ref. 43.

Isolation of Rhopalium-Expressed Genes and Phylogenetic Analysis. An EST cDNA library was generated from rhopalia mRNA, and 2,433 individual clones from the library were sequenced by using an ABI capillary sequencer. The accession numbers for the clones are as follows: c-opsin (EU310498), oca (EU310502), mitf (EU310499), catalytic pde (EU310500), inhibitory pde6 (EU310501) and guanylate cyclase (EU310503). Details on phylogenetic analysis including the accession numbers of individual sequences are described in SI Materials and Methods.

RNA in Situ Hybridization. Jellyfish were fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose overnight at 4°C, and embedded and frozen in OCT (Tissue Tek). RNA in situ hybridization was performed as described in ref. 43.

Immunohistochemistry. The cryosections were refixed in 4% PFA for 10 min, washed three times with PBS, permeabilized with PBT (PBS + 0.1% Tween 20) for 15 min, and blocked in 10% BSA in PBT for 30 min. The primary antibodies were diluted in 1% BSA in PBT, incubated overnight at room temperature, washed three times with PBS, and incubated with secondary antibodies in 1% BSA in PBT. The sections were counterstained with DAPI and mounted. Primary antibodies used were: anti-Tripedalia c-opsin, anti-Tripedalia J1-crystallin, and anti-acetylated tubulin (Sigma). The following secondary anti-
bodies were used: Alexa Fluor 488- or 594-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes).

**Generation of Antibodies, COS-7 Cell Transfection, and Western Blotting.** Antibodies directed against Tripedalia c-opsin and J1-crystallin were prepared by immunization of rabbits as follows. The C-terminal region of c-opsin cDNA corresponding to amino acids 274–329 was cloned into the expression vector pET42, expressed in BL21(DE3)RIPL cells (Stratagene), and purified by using His6 tag chromatography. The N-terminal peptide of J1-crystallin AAIVGSLVDAATQPVHVK was attached to KLH via the C-terminal lysine and used for immunization. Monkey kidney COS-7 cells were transfected with CMV-c-opsin (amino acids 1–329) expression vector by using FuGENE6 reagent (Roche). Total extracts were prepared from c-opsin-transfected cells, mock-transfected cells, and cells infected with Western blotting by using anti-c-opsin rabbit serum and chemiluminescent detection kit (Pierce). To avoid formation of multimeric opsin complexes, protein extracts from transfected cells were diluted and heated at low temperature (37°C) before SDS/PAGE.

**Fontana–Masson Method.** The cryosections were hydrated in distilled water and exposed to 0.25% potassium permanganate for 30 min at room temperature. The sections were hydrated in distilled water and exposed to 0.25% potassium permanganate for 30 min, rinsed once in distilled water, placed in 5% sodium thiosulfate at room temperature for 1 min, washed again in water, and mounted.

**Melanin Bleach Procedure.** Bleaching was performed either after Fontana–Masson staining or RNA in situ hybridization. The sections were hydrated in distilled water and exposed to 0.25% potassium permanganate for 30 min at room temperature. The sections were treated with 5% oxalic acid for 5 min, washed with water, and mounted.

**Transmission Electron Microscopy.** Rhopalia excised from juvenile medusae were treated with Karnovsky fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde in cacodylate buffer) for 24 h at 4°C. Fixed tissue was washed 12 h in 0.1% cacodylate buffer at 4°C. Karnovsky-fixed juvenile rhopalia and PFA-fixed adult rhopalia were postfixed in 2% OsO4 for 2 h at 4°C and then washed in water. Samples were dehydrated in series of ethanol solutions, transferred to pure acetone, and embedded in Poly/Bed 812/Araldite 502 resin. Ultrathin sections (600–800 nm) were cut on Ultratome U (Reichert–Jung), placed on copper grids, and treated with 2.5% uranyl acetate for 1 h followed by lead citrate for 15 min. The material was examined by transmission electron microscopy, images were taken with a MEGAview III Soft Imaging System.

**Expression, Reconstitution, and Spectroscopic Analysis of Tripedalia c-opsin.** Tripedalia c-opsin cDNA was expressed in transfected COS-1 cells. Transfected cells were resuspended with 5 µL 11-cis-retinal solubilized with 1% dodecyl maltoside, and the resulting c-opsin photopigment was purified by using immobilized ID (Cell Culture Center, Minneapolis, MN). The UV-visible absorption spectrum was recorded for the c-opsin photopigment from 250 to 650 nm at 0.5-nm intervals by using the Hitachi U3010 dual-beam spectrometer at 20°C. Five replicates were performed in the dark and five more after 3 min of light exposure (with a ~<440-nm cut-off filter). The Amax value was taken from the dark–light difference spectrum.

For additional details, see SI Materials and Methods.
Isolation of Rhopalium-Expressed Genes. The expressed sequence tag (EST) cDNA library was generated from rhopalia mRNA by using pfBluescript II. Individual clones from the library were sequenced using an ABI capillary sequencer, and full-length cDNAs were obtained by SMART RACE (BD Biosciences). A fragment of Tripedalia Mitf cDNA was isolated by reverse transcription-PCR of rhopalia mRNA by using degenerate primers zk665A 5’-AARAARGAYAAYCAAY-3’ and zk665F 5’-TTDATKCRRTDTTRTT-3’. The resulting partial Mitf cDNA was extended by RACE. The accession numbers for the clones are as follows: c-opsin (EU310498), oca (EU310502), mitf (EU310499), catalytic pde (EU310500), inhibitory pde6d (EU310501), and guanylate cyclase (EU310503).

Phylogenetic Analysis. Amino acid alignment created by MUSCLE software (11) with default settings was edited manually, and highly divergent stretches were excluded. The phylogenetic trees were constructed using the Phylib 3.6 package. Bootstrap sample set was generated by SEQBOOT (1,000 replicates), protein distances were estimated using PROTDIST (PAM matrix, 1,000 datasets), and the NJ tree was constructed by NEIGHBOR (1,000 replicates, random input order) and CONSENSE programs. Maximum-likelihood trees were constructed by PROML (JTT matrix, random input order, 500 replicates) and final consensus tree by CONSENSE. The numbers above each node represent the percentage of bootstrap probability based on 1,000 replicates. Tripedalia protein sequences clustered consistently in trees inferred by the maximum-likelihood method. Accession numbers of sequences used in the trees are as follows.

Opsin tree. The numbers of Strongylocentrotus purpuratus genes represent the gene ID in public assembly of Sea Urchin Genome Project (hsge.bcm.tmc.edu/projects/seaurchin), Gallus adeno-sine receptor NP._994018.1, Mus serotonin receptor NP._7664001, Branchiostoma belcheri opsin 6 BAC76024.1, Drosophila Rh6 NP._524368.3, Drosophila Rh2 NP._524398.1, Drosophila Rh3 NP._524411.1, Aplys blue rhodopsin NP._001011601.1, Octopus opsin P09241, Sepia rhodopsin AACA26329.1, Mizuho- opeten Gg O15973, Platycerus r-opsin CAC86665.1, B. belcheri Mop O4R1H4, Xenopus melanolopes AACA41235.1, Dario melano- nopsin NP._840074.1, Homo melanolopes NP._1505981, Dario LW NP._571250.1, Homo MW NP._800504.1, Gallus LW NP._909740.1, Homo rhodopsin NP._000530.1, Dario extraocular NP._571287.1, Dario MW4 NP._571329.1, Latimeria Rh2 AAD30520.1, Dario SW opsin NP._571394.1, Xenopus violet P51473, Xenopus green AAO38746.1 Gallus blue NP._990848.1, Salmo VAL opsin O13018, Danio VAL opsin NP._716611, Ciona opsin NP._001027772.1, Xenopus pararhodopsin NP._998830.1, Uta parietopus AAZ79904.1, Xenopus parietopus NP._001039256.1, Mus encephalopsin NP._034228.1, Homo encephalopsin NP._055137.2, Platynereis c-opsin AV63834.1, Takifugu TMT NP._001027778.1, B. belcheri opsin 4 BAC76021.1, B. belcheri opsin 5 BAC76022.1, Aplys pteropsin NP._001035057.1, Aedes opsin EAT43163.1, Anopheles GPRO11 XP._312503.3, Anopheles GPRO12 XP._312502.2, Stronglylocentrotus Sp1 GLEAN3.05569, Mizuhopecuent Gv O15974, B. belcheri opsin 2 BAC76020.1, B. belcheri opsin 1 BAC76019.1, Stronglylocentrotus Sp3.2 GLEAN3.27653, Stronglylocentrotus Sp3.3 GLEAN3.27634, Ratnus Op25 NP._861437.1, Homo Op25 NP._859528.1, Homo peropsin NP._006574.1, Mus peropsin AACS344.1, B. belcheri opsin 3 BAC76023.1, Gallus RGR NP._001026387.1, Mus RGR NP._067315.1, Toddarodes retino-chrome CAA40422.1.


PDE6D alignment. Nemastostella PDE6pred XP._001296547.1, Homo PDE6D NP._002592.1, Mus PDE6D XP._032827.1, Bos PDE6D XP._776845.1, Canis PDE6D NP._00103156.1, Ciona PDE6D NP._001127639.1, PDE6Dalpha XP._394004.2, Strongylcenter PDE6delta XP._001177685.1, Caenorhabditis PDE6delta NP._4985490, Aedes PDE6delta XP._320754.1, Drosophila GA21678-PA XP._001355815.1, Tetrahymena GMPPDE XP._001007755.1.

Oca2 tree. H. sapiens OCA2 NP._0002652.2, Sus scrofa OCA2 NP._999259.2, Oryzias latipes OCA2 NP._001098262.1, M. musculus OCA2 NP._068676, G. gallus OCA2 XP._425579, Nemastomella vectens .oca2 XP._001627452, D. melanogaster RE09889 (P protein) AAN71295.1, Aedes aegypti Ty rantrans (hoepl-like) XP._001658764.1, D. melanogaster Tyra transp (hoep1) NP._6088761hoep1, Clostridium botulinum Ars pump NP._001253041.1, Carboxydermous hydrogenofarmans Ars transp YP._560838.1, Thermococcus kodakarensis Ars pump.


Analysis by RT-PCR. Total RNA was isolated from *Tripedalia* cystophora rhopalia using TRizol reagent (Invitrogen). The RT reaction was performed using random oligonucleotide hexamers in the presence or absence (negative control) of the PowerScript enzyme (Clontech). The specific primers for the phototransduction genes were as follows: guanylyl cyclase, 5’-GGATGTTCTACAGCTATGGCATCAT-3’ (forward) and 5’-CGTTGATCTTTTTCATGATT-3’ (reverse); inhibitory PDE6D, 5’-TCTACGTTAAGCAGTCGAC-3’ (forward) and 5’-GTTCGATCATCACTGTAGTTTC-3’ (reverse); catalytic PDE, 5’-GAAGCTATTCTCCTGATGC-3’ (forward) and 5’-GGTTCTCCCTGACGGATAAGATCC-3’ (reverse); phosducin, 5’-CCAGCAATATCCACAGATC-3’ (forward) and 5’-TCGAAATCGGTTGCTATCC-3’ (reverse).

Expression, Reconstitution, and Spectroscopic Analysis of *Tripedalia* c-Opsin. The entire coding region was amplified from the *Tripedalia* c-opsin cDNA clone by primer pairs designed within the 5’ and 3’ edges of the cloning regions with necessary sequences for cloning and translation purposes as in ref. 12: 5’-AAAAAA-GAATTCGACACGGAGATCTAC-3’ (the forward primer with an EcoRI site underlined) and 5’-TTTCTGGTCGCAGCTACA-GAATTTCCAGAG-3’ (the reverse primer with a SalI site underlined). Via the restriction sites set in the primers, the amplified cDNA fragment was cloned into the pMT5 expression vector, which contains the last 15 amino acids of the bovine rod opsin necessary for immunoreactivity purification by 1D4 monoclonal antibody (13). Cultured COS-1 cells (RIKEN Cell Bank) were transfected with the pMT5-cDNA clone, incubated with 5 μM 11-cis-retinal (Storm Eye Institute, Medical University of South Carolina, Charleston, SC), and solubilized with 1% deoxycholate. The c-opsin photopigment was purified using immobilized ID (Cell Culture Center, Minneapolis, MN). The UV-visible absorption spectrum was recorded for the c-opsin photopigment from 250 to 650 nm at 0.5-nm intervals using the Hitachi U3010 dual beam spectrometer at 20°C. Five replicates were performed in the dark and five more after 3 min of light exposure (with a <440 nm cut-off filter) as described in ref. 13. The Savitzky–Gołay least-squares smoothing method was carried out for the absorbance curve, with 100 repetitions to eliminate spurious spikes. The λmax value was taken from the dark–light difference spectrum.

12. Matsumoto Y, Fukamachi S, Mitani H, Kawamura S (2006) Functional characterization of visual and nonvisual phototransduction genes as follows: guanylyl cyclase, 5’-GGATGTTCTACAGCTATGGCATCAT-3’ (forward) and 5’-CGTTGATCTTTTTCATGATT-3’ (reverse); inhibitory PDE6D, 5’-TCTACGTTAACGAAATGACGCAC-3’ (forward) and 5’-GTTCGATCATCACTGTAGTTTC-3’ (reverse); catalytic PDE, 5’-GAAGCTATTCTCCTGATGC-3’ (forward) and 5’-GGTTCTCCCTGACGGATAAGATCC-3’ (reverse); phosducin, 5’-CCAGCAATATCCACAGATC-3’ (forward) and 5’-TCGAAATCGGTTGCTATCC-3’ (reverse).
protein-coupled receptors (GPCRs), is important for G protein interaction (2). G121, P171, and W175 are evolutionary trace residues typical for the opsin family.

Alignment and phylogenetic tree of opsins. The phylogenetic tree was inferred by the neighbor-joining method using murine adenosine and chicken serotonin receptors as outgroup sequences. Nonhomologous stretches were excluded from the analysis. The black lines above the alignment represent the extent of transmembrane helices III, IV, and VII. The color lines on both sides of the alignment demarcate opsin subfamilies. Critical amino acids allowing opsin classification are indicated by the black arrowheads and numbered according to the bovine rhodopsin protein sequence. The lysine residue K296 is critical for covalent binding of retinal via Schiff base linkage, which is stabilized by counterion E113 or E181 (1). The (E/D)RY triade, which is highly conserved among G protein-coupled receptors (GPCRs), is important for G protein interaction (2). G121, P171, and W175 are evolutionary trace residues typical for the opsin family but not for the GPCRs in general (3). The positions of the HPK and the NR/KO motifs conserved among rhabdomeric and ciliary opsins, respectively (4), are boxed in red. The classification of opsin families is given in the colored boxes on the right.

Fig. S51. Alignment and phylogenetic tree of opsins. The phylogenetic tree was inferred by the neighbor-joining method using murine adenosine and chicken serotonin receptors as outgroup sequences. Nonhomologous stretches were excluded from the analysis. The black lines above the alignment represent the extent of transmembrane helices III, IV, and VII. The color lines on both sides of the alignment demarcate opsin subfamilies. Critical amino acids allowing opsin classification are indicated by the black arrowheads and numbered according to the bovine rhodopsin protein sequence. The lysine residue K296 is critical for covalent binding of retinal via Schiff base linkage, which is stabilized by counterion E113 or E181 (1). The (E/D)RY triade, which is highly conserved among G protein-coupled receptors (GPCRs), is important for G protein interaction (2). G121, P171, and W175 are evolutionary trace residues typical for the opsin family but not for the GPCRs in general (3). The positions of the HPK and the NR/KO motifs conserved among rhabdomeric and ciliary opsins, respectively (4), are boxed in red. The classification of opsin families is given in the colored boxes on the right.
modulates phototransduction by interacting with the Strongylocentrotus (74/92%), PDE6D (phosphodiestrase cGMP-specific PDEs used in phototransduction and is phylogenetically close to PDE5 and PDE6. (Kozmik, Fig. S2.

Tripedalia the former clustering with the vertebrate retina-specific Phosducin. A subunit of PDE (PDE6D) in the rod outer segment can bind the PDE (inhibitory subunit) groups with NC 2A/B. The percentage of identity/similarity between Tripedalia and other species is as follows: Homo (82/94%), Bos (82/94%), Canis (82/95%), Mus (81/94%), Ciona (80/91%), Strongylocentrotus (74/92%), Caenorhabditis (69/89%), Aedes (62/84%), and Drosophila (59/82%). (C) Phylogenetic tree of the phosducin gene family. Phosducin modulates phototransduction by interacting with the β3 subunits of G protein transducin (6, 7). Phosducin and phosducin-like genes are expressed in Tripedalia, the former clustering with the vertebrate retina-specific phosducins. (D) Phylogenetic tree of the guanylate cyclase (GC) gene family. Tripedalia guanylate cyclase clusters with membrane-associated retina-specific GC2E (also known as retGC-1) and GC2F (also known as retGC-2), both of which are expressed in vertebrate rods and cones (8, 9). These specialized photoreceptor guanylate cyclases in outer segments of rods and cones resynthesize cGMP that is rapidly depleted through activated PDE upon light stimulus.

Fig. S2. Phylogenetic analysis of Tripedalia components of the ciliary phototransduction cascade. (A) Phylogenetic tree of the catalytic subunit of the phosphodiesterase (PDE) gene family. Tripedalia PDE contains two GAF domains and a phosphodiesterase catalytic domain, PDEase I. Tripedalia PDE groups with cGMP-specific PDEs used in phototransduction and is phylogenetically close to PDE5 and PDE6. (B) Alignment of the inhibitory subunit of phosphodiesterase PDE6D. A subunit of PDE (PDE6D) in the rod outer segment can bind the PDE6δβ3 complex and solubilize it, disrupting its normally close association with the disk membrane (5). Among bilaterians, the highest degree of sequence identity/similarity of Tripedalia PDE6D is with PDE6D from various vertebrates. The percentage of identity/similarity between Tripedalia and other species is as follows: Homo (82/94%), Bos (82/94%), Canis (82/95%), Mus (81/94%), Ciona (80/91%), Strongylocentrotus (74/92%), Caenorhabditis (69/89%), Aedes (62/84%), and Drosophila (59/82%). (C) Phylogenetic tree of the phosducin gene family. Phosducin modulates phototransduction by interacting with the β3 subunits of G protein transducin (6, 7). Phosducin and phosducin-like genes are expressed in Tripedalia, the former clustering with the vertebrate retina-specific phosducins. (D) Phylogenetic tree of the guanylate cyclase (GC) gene family. Tripedalia guanylate cyclase clusters with membrane-associated retina-specific GC2E (also known as retGC-1) and GC2F (also known as retGC-2), both of which are expressed in vertebrate rods and cones (8, 9). These specialized photoreceptor guanylate cyclases in outer segments of rods and cones resynthesize cGMP that is rapidly depleted through activated PDE upon light stimulus.

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Fig. S3. Expression analysis of ciliary phototransduction cascade genes. RT-PCR analysis of guanylate cyclase, phosphodiesterases, and phosducin gene expression in rhopalia of T. cystophora. In all cases, no PCR product was obtained in the absence of reverse transcriptase (−RT).
Fig. S4. Phylogenetic analysis of melanogenic pathway-specific genes. (A) Phylogenetic tree of the Oca2 gene family. Oca2 proteins (also known as P protein, P permease, or hoepel) from different organisms and protein sequences of three arsenic pumps (closest homologues to Oca2 family) were aligned, and conserved transmembrane helices were used for phylogenetic analysis. The tree was inferred by the neighbor-joining method using arsenic pumps as outgroup sequences. The numbers above and under the branches indicate bootstrap support (1,000 replicates). (B) Alignment and phylogenetic tree of the Mitf/TFE gene family. The phylogenetic tree was inferred by the neighbor-joining method using Saccharomyces cerevisiae RTG3 as an outgroup sequence. Colored bars above the alignment demarcate the secondary structure motifs (according to ref. 10). Caenorhabditis sequences form an outgroup probably because of the high level of diversification.