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RESEARCH ARTICLE

A second visual rhodopsin gene, *rh1-2*, is expressed in zebrafish photoreceptors and found in other ray-finned fishes

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ABSTRACT

Rhodopsin (*rh1*) is the visual pigment expressed in rod photoreceptors of vertebrates that is responsible for initiating the critical first step of dim-light vision. Rhodopsin is usually a single copy gene; however, we previously discovered a novel rhodopsin-like gene expressed in the zebrafish retina, *rh1-2*, which we identified as a functional photosensitive pigment that binds 11-*cis* retinal and activates in response to light. Here, we localized expression of *rh1-2* in the zebrafish retina to a subset of peripheral photoreceptor cells, which indicates a partially overlapping expression pattern with *rh1*. We also expressed, purified and characterized Rh1-2, including investigation of the stability of the biologically active intermediate. Using fluorescence spectroscopy, we found the half-life of the rate of retinal release of Rh1-2 following photoactivation to be more similar to that of the visual pigment rhodopsin than to the non-visual pigment exo-rhodopsin (*exorh*), which releases retinal around 5 times faster. Phylogenetic and molecular evolutionary analyses show that *rh1-2* has ancient origins within teleost fishes, is under similar selective pressure to *rh1*, and likely experienced a burst of positive selection following its duplication and divergence from *rh1*. These findings indicate that *rh1-2* is another functional visual rhodopsin gene, which contradicts the prevailing notion that visual rhodopsin is primarily found as a single copy gene within ray-finned fishes. The reasons for retention of this duplicate gene, as well as possible functional consequences for the visual system, are discussed.

KEY WORDS: Visual pigment, Vision, Gene duplication, GPCR, Teleost

INTRODUCTION

Vertebrate photoreception is mediated by opsins, which are members of the G protein-coupled receptor (GPCR) superfamily of proteins (Terakita, 2005). In the dark, opsins are covalently bound to a light-sensitive chromophore, 11-*cis* retinal, which acts as an inverse agonist to suppress dark state activation (Menon et al., 2001). When activated by light, the chromophore isomerizes to its all-*trans* conformation, which initiates a signaling cascade within the cell (Baylor, 1996). Visual opsins are responsible for initiating the visual transduction cascade, while non-visual opsins are involved in processes such as circadian entrainment (Doyle et al., 2008) and the metabolism of retinal (Bellingham et al., 2003a), with some possibly contributing indirectly to image formation (Cheng et al., 2009). Rhodopsin is the visual opsin expressed in rod photoreceptors responsible for mediating dim-light vision in vertebrates (Nathans, 1992), and was the first GPCR to have its crystal structure resolved at high resolution (Palczewski et al., 2000).

While gene duplications have occurred multiple times in invertebrate opsins (Rivera et al., 2010; Serb et al., 2013) and vertebrate cone opsins (Hunt et al., 1998; Matsumoto et al., 2006), visual rhodopsin is generally considered to be a single copy gene, with only a few exceptions. Several eel species have two rhodopsins, one freshwater (*rh1fwo*) and one marine (*rh1dso*), and expression shifts from the former to the latter following migration during maturation (Beatty, 1975; Hope et al., 1998; Zhang et al., 2000; Zhang et al., 2002). The short-fin pearleye (*Scopelarchus analis*), a deep-sea teleost, also expresses an additional *rh1* gene, *rh1b*, in the accessory retina of adult fish after descending to greater ocean depths (Pointer et al., 2007). Other examples of multiple *rh1* genes are usually the result of species-specific duplication events (Lim et al., 1997). It is also interesting to note that the middle wavelength-sensitive cone opsin groups, which absorb similar wavelengths of light to rhodopsins, have been suggested to make greater contributions to adaptive vision through gene duplication (Gojobori and Innan, 2009).

Despite the scarcity of *rh1* gene duplications, zebrafish (*Danio rerio*) is a logical candidate for opsin gene duplication, considering most of its tissues are directly photoentrainable (Whitmore et al., 2000) and its array of nine visual opsins is a large complement even among teleost fish (Chinen et al., 2003). Many non-visual opsins have been discovered in zebrafish through traditional sequencing studies, including five melanopsins (Bellingham et al., 2002; Davies et al., 2011), teleost multiple tissue (tmt) opsin (Moutsaki et al., 2003), two vertebrate ancient long (VAL) opsins (Kojima et al., 2008) and exo-rhodopsin (Mano et al., 1999). Additionally, a recent functional genomics screen identified 10 novel non-visual opsins (Davies et al., 2015). The non-visual exo-rhodopsin (*exorh*), expressed in the pineal gland of the brain and not in retinal photoreceptors, is not involved in vision, but is also orthologous to *rh1* of non-teleost vertebrates, with *rh1* in teleosts being the product of an ancient retrotransposition event that contains no introns (Fitzgibbon et al., 1995). This duplication is thought to have occurred no later than 284 million years ago, marking the onset of the radiation of ray-finned fish (Hurley et al., 2007), as basal Actinopterygians such as the sturgeon and gar also have intronless *rh1* genes (Bellingham et al., 2003b).

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Journal of Experimental Biology

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We previously identified another rhodopsin-like gene, *rh1-2*, in juvenile and adult zebrafish, but limited functional characterization prevented its classification as either a visual or non-visual opsin (Morrow et al., 2011). This novel gene was found to be expressed in the retina of adult zebrafish, but not in the brain. When regenerated with 11-*cis* retinal, Rh1-2 produced an absorption spectrum with a λ_{max} value of approximately 500 nm (Morrow et al., 2011), similar to both rhodopsin (Chinen et al., 2003) and exo-rhodopsin (Tarttelin et al., 2011). Orthologous sequences were also found in three other cyprinid fish, suggesting that *rh1-2* is not the result of a zebrafish-specific duplication event, and initial phylogenetic analyses hinted at a divergence from *rh1* during the earlier stages of teleost evolution (Morrow et al., 2011).

Here, we further characterized the expression and function of Rh1-2 by comparing it with both rhodopsin and exo-rhodopsin in order to gain a better understanding of its role in photoreception and its potential as a visual opsin. We localized *rh1-2* expression in the retina to a subset of peripheral photoreceptors, a pattern that partially overlaps *rh1* expression but that is distinct from *exorh* expression in the pineal gland of the brain. When monitored using fluorescence spectroscopy following photoactivation, Rh1-2 was shown to release retinal at a rate comparable to rhodopsin and approximately 5 times slower than exo-rhodopsin. Finally, *rh1-2* was identified in three additional species, including one outside of the family Cyprinidae, *Misgurnus anguillicaudatus*, and phylogenetic analyses show the *rh1-2* gene family is likely a sister group to ostariophysian *rh1* genes, having been subjected to purifying selection following duplication and divergence. This study adds new insights into the visual system of zebrafish, and explores the implications of a second visual rhodopsin gene expressed in some teleost fish.

MATERIALS AND METHODS

Opsin sequences

RNA was extracted from adult eyes of various teleost fishes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA libraries were generated using the SMART cDNA Library Construction Kit (BD Biosciences, San Jose, CA, USA). Genomic DNA was extracted from various tissues of teleost fish using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). All specimens were killed prior to tissue extraction using an overdose of tricaine methane sulfonate (MS222, 300 mg l^{-1}; Sigma-Aldrich, St Louis, MO, USA) buffered to neutral pH with sodium bicarbonate prior to fish immersion. Gene fragments of *rh1* and *rh1-2* were amplified from cDNA libraries and genomic DNA, respectively, using either previously designed *rh1-2* (Morrow et al., 2011) or acanthomorph *rh1* primers (Chen et al., 2003), then cloned into the pJET1.2 cloning vector (Fermentas, Waltham, MA, USA). Full-length sequences of *rh1*, *rh1-2* and *exorh* were amplified from zebrafish eye (*rh1*, *rh1-2*) or brain (*exorh*) cDNA libraries. PCR was performed using PfuTurbo (Agilent, Santa Clara, CA, USA); the resulting fragments were cloned into the p1D4-hrGFP II expression vector (Morrow and Chang, 2010). All vectors were sequenced using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

In total, 19 new nucleotide sequences are introduced in this study, including 16 new *rh1* sequences (GenBank accession numbers: KY026025–KY026040) and three new *rh1-2* sequences (GenBank accession numbers: KY026041–KY026043) (Table S1). These sequences were combined with 117 rhodopsin gene sequences (*rh1*, *rh1-2*, *exorh*) obtained from GenBank in order to maintain even sampling across vertebrates within the limits of available data. Among these were four additional *rh1-2* genes not previously studied, three from species of the *Sinocycloheilus* genus that were predicted as rhodopsin-like sequences from a recent cavefish genome project (Yang et al., 2016), and one that was extracted from the carp (*Cyprinus carpio*) genome. A second putative *rh1-2* sequence fragment was also identified within the carp genome, which is not unexpected given a recent tetraploidization (Larhammar and Risinger, 1994), but was not included because of gaps in the first transmembrane domain. Four vertebrate *rh2* sequences were also obtained from GenBank and used as an outgroup. Sequences were aligned using the webPRANK (Löytynoja and Goldman, 2010) implementation of PRANK (Löytynoja and Goldman, 2005). Species and accession numbers for all sequences used in the study are provided in Table S1.

In situ hybridization

Eyes were dissected from 21 and 175 days post-fertilization (dpf) zebrafish, *Danio rerio* (Hamilton 1822), that had been anesthetized with 160 mg l^{-1} tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich); eyes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight, and rinsed in PBS with 0.1% Tween-20 (PBT), then in methanol, before being stored in fresh methanol at −20°C. *In situ* hybridizations were performed on 3 dpf (control) and 5 dpf zebrafish embryos (whole mount), and eyes from 21 and 175 dpf zebrafish, as previously described (Wong et al., 2010). DIG-labeled RNA probes 700 bp in length, as well as unlabeled, full-length blocking RNA for *rh1-2* (control), were amplified from *rh1* and *rh1-2* sequences inserted into the pBluescript cloning vector using T3 RNA Polymerase (Fermentas, Waltham, MA, USA). For our control using unlabeled *rh1-2* blocking RNA, the labeled *rh1* probe was pre-adsorbed with blocking RNA prior to *in situ* hybridization. A probe concentration of 1 ng μl^{-1} was using during the 70°C hybridization. Semi-thin plastic sections were made from 5 dpf embryos, where whole mounts were rinsed with PBT, dehydrated using increasing concentrations of ethanol (from 30% to 90% in PBT), followed by 100% ethanol, and then embedded with increasing concentrations of Spurr's resin in ethanol (3:1, 1:1, 1:3). Samples were then left to polymerize at 65°C in 100% Spurr's resin. Semi-thin coronal sections were cut with a glass knife using an ultramicrotome and dried onto glass slides. Sections were 1.5 µm thick without counterstaining to maximize visualization. Cryosections were performed on 21 and 175 dpf zebrafish eyes, which were washed 3 times in PBS, then put through a sucrose gradient at room temperature, 30 min per step: 5% sucrose in PBS, 2:1 5%:30%, 1:1 5%:30%, 1:2 5%:30%, with a final step in 30% sucrose in PBS at 4°C overnight. Eyes were incubated in 2:1 30% sucrose in PBS:Tissue-Tek OCT compound (VWR, Radnor, PA, USA) for 4 h at room temperature, then 4°C overnight. Cryosections were performed at 20 µm on a Leica CM3050S cryostat, and collected on Superfrost Plus slides (VWR) mounted in 90% glycerol/10% PBS. All images were taken on a Leica DM4500B compound microscope with a Qimaging digital camera and OpenLab 4.0.2 software (Improvision, Coventry, UK).

Protein expression and spectroscopy

The p1D4-hrGFP II expression vector constructs containing full coding sequences of zebrafish *rh1*, *rh1-2* and *exorh* were used to transiently transfect cultured HEK293T cells (ATCC CRL-11268) using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h post-transfection and opsins were regenerated using 11-*cis* retinal, generously provided by Dr Rosalie Crouch (Medical University of

Journal of Experimental Biology (2017) 220, 294-303 doi:10.1242/jeb.145953

South Carolina). Visual pigments were solubilized in 1% *N*-dodecyl-β-D-maltoside (DM) and immunoaffinity purified with 1D4 monoclonal antibody (University of British Columbia no. 95-062, lot no. 1017; Molday and MacKenzie, 1983), as previously described (Morrow and Chang, 2010). Purified visual pigment samples were eluted in sodium phosphate buffer (50 mmol l^{-1}, 0.1% DM, pH 7). The ultraviolet–visible absorption spectra of purified opsin were recorded at 25°C using a Cary4000 double-beam spectrophotometer (Agilent) and quartz absorption cuvettes (Helma, Paris, France). All λ_{max} values were calculated after fitting absorbance spectra to a standard template for A1 visual pigments (Govardovskii et al., 2000).

The protocol used to determine retinal release rates of visual pigments was modified from that of Farrens and Khorana (1995). Briefly, 0.05–0.20 µmol l^{-1} visual pigment samples were incubated in sodium phosphate buffer (50 mmol l^{-1}, 0.1% DM, pH 7) at 20°C using submicro fluorometer cell cuvettes (Agilent) and bleached for 30 s using a Fiber-Lite MI-152 Illuminator external light source (Dolan-Jenner, Boxborough, MA, USA), using a filter to restrict wavelengths of light below 475 nm. Fluorescence measurements were integrated for 2 s at 30 s intervals using a Cary Eclipse fluorescence spectrophotometer, with temperature maintained by a Cary Temperature Controller employing a Peltier Multicell Holder (Agilent) and monitored by a temperature probe. The excitation wavelength was 295 nm (1.5 nm slit width) and the emission wavelength was 330 nm (10 nm slit width); no noticeable pigment bleaching by the excitation beam was detected. Retinal release was demonstrated through a sharp initial rise in intrinsic tryptophan fluorescence, representing a decrease in fluorescence quenching of W265 by the retinal chromophore. Data from the initial rise were fitted to a three-variable, first-order exponential equation $y=y_o+a(1-e^{-bx})$, with half-life values calculated based on the rate constant b ($t_{1/2}=\ln 2/b$). All curve fitting resulted in r^2 values greater than 0.9.

Phylogenetic and molecular evolutionary analyses

A maximum-likelihood rhodopsin gene tree was estimated in PhyML 3.0 (Guindon et al., 2010) under the GTR+I+G model using a BioNJ starting tree, the best of a NNI and SPR tree improvement, and 100 bootstraps. A Bayesian *rh1* gene tree was also constructed in MrBayes 3 (Ronquist and Huelsenbeck, 2003) using reversible jump MCMC with a gamma rate parameter (nst=mixed, rates=gamma), which explores the parameter space for the nucleotide model and the phylogenetic tree simultaneously. The analysis was run for 5 million generations with a 25% burn-in. Convergence was confirmed by checking that the standard deviations of split frequencies approached zero and that there was no obvious trend in the log likelihood plot.

To estimate the strength and form of selection acting on rhodopsin, the alignment, along with the maximum-likelihood gene tree, was analyzed with the codeml package of PAML 4 (Yang, 2007) using random sites models (M0, M1a, M2a, M3, M7, M8a and M8), branch model, branch-site model and clade model C (CmC). Analyses were run on the complete *rh1* alignment and tree as well as on two subsets, one pruned to only include ray-finned fish rhodopsin genes (including *exorh* as the outgroup) and the other pruned to contain only *rh1-2* (no outgroup).

Comparisons between the PAML random sites models were used to test for variation in ω (M3 versus M0) and for the presence of a positively selected class of sites (M2a versus M1a and M8 versus M7 and M8a). All analyses were run starting with the branch lengths estimated by PhyML repeated at least three times with varying initial starting points of κ (transition to transversion ratio) and ω to avoid potential local optima. The model pairs were compared using a likelihood ratio test with a χ^2 distribution.

The branch, branch-site (Zhang et al., 2005) and CmC models (Bielawski and Yang, 2004) were used to test for changes in selective constraint and positive selection on the branch leading to the *rh1-2* clade and between the *rh1-2* clade and other rhodopsins. The branch model estimates a single ω value for each branch and/or clade type specified *a priori*. This model is useful for testing for overall changes in selective constraint between branches/clades. The branch-site and clade models allow ω to vary both among sites and between branches/clades. The branch-site model has four site classes: (0) $0<\omega_0<1$ for all branches; (1) $\omega_1=1$ for all branches, (2a) $\omega_{2a}=\omega_{2b}\geq 1$ in the foreground and $0<\omega_{2a}=\omega_0<1$ in the background, and (2b) $\omega_{2b}=\omega_{2a}\geq 1$ in the foreground and $\omega_{2b}=\omega_1=1$ in the background. This model provides a test for positive selection on specified branches/clades and incorporates a Bayes' Empirical Bayes (BEB) analysis to identify codon sites under positive selection (Yang et al., 2005). CmC assumes that some sites evolve conservatively across the phylogeny (two classes of sites where $0<\omega_0<1$ and $\omega_1=1$), while a class of sites is free to evolve differently among two or more partitions (e.g. $\omega_{D1}>0$ and $\omega_{D1}\neq\omega_{D2}>0$), which can be branches, clades or a mix of the two. Rather than a test for positive selection, this provides a test for divergent selective pressure, although a test for positive selection can be performed if desired (see Chang et al., 2012). These models were applied only to the dataset pruned to contain only ray-finned fish rhodopsins.

RESULTS

rh1-2 is expressed in the outer nuclear layer of peripheral rod photoreceptors

We performed a series of *in situ* hybridizations to investigate the onset of *rh1-2* expression and to localize its expression in the retina. *In situ* hybridizations were performed using 700 bp coding sequence probes for both *rh1* and *rh1-2* in order to localize cellular expression in whole-mount embryos 5 dpf, as well as both juvenile (21 dpf) and adult (175 dpf) eyes. At 5 dpf, expression of *rh1* was strongest in the peripheral retina, although some limited expression was also seen in the central retina (Fig. 1A), while *rh1-2* was only detected in a limited portion of the ventral peripheral retina (Fig. 1B). At 21 dpf, both *rh1* and *rh1-2* expression in the peripheral retina was more prominent relative to expression at 5 dpf (Fig. 1C,D). While the central retina contained more widespread *rh1* expression (Fig. 1E), there was no *rh1-2* expression (Fig. 1F). Expression at 175 dpf was similar to that at 21 dpf, with a strong *rh1* signal throughout the photoreceptor layer (Fig. 1G). However, *rh1-2* was still localized to the ventral peripheral retina, with no staining shown in the central retina (Fig. 1H). Patterns of *rh1* expression were similar to those presented in previous studies (Raymond et al., 1995; Robinson et al., 1995; Takechi and Kawamura, 2005).

Expression of *rh1-2* was consistent with previous RT-PCR results that showed expression in 21 dpf juvenile fish and the adult retina but at significantly lower levels than *rh1* (Morrow et al., 2011). Furthermore, all expression of both *rh1* and *rh1-2* was confined to the outer nuclear layer (ONL), consisting of the cell bodies of rod and cone photoreceptors, suggesting that *rh1-2* protein expression occurs in photoreceptors as opposed to other retinal cells. Another interesting feature of *rh1-2* expression is that it often overlaps *rh1* expression, which suggests the possibility of co-expression of the genes in the same photoreceptor. Because of this overlap and as the nucleotide sequences of *rh1* and *rh1-2* share approximately 75% similarity, a sense-strand probe control experiment was run to exclude the possibility of cross-hybridization. The same 700 bp *rh1* probes were used to stain

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 294-303 doi:10.1242/jeb.145953

3 dpf embryos both with and without the addition of full-length *rh1-2* blocking RNA, present at double the concentration of the *rh1* probe. The presence of the *rh1-2* blocking RNA did not have a significant effect on *rh1* staining, which suggests that there is likely no cross-hybridization between *rh1*/*rh1-2* probes and the opposite transcripts (Fig. S1).

λ_{max} of Rh1-2 is slightly blue-shifted compared with that of rhodopsin

Full-length gene sequences coding for zebrafish rhodopsin, Rh1-2 and exo-rhodopsin were inserted into the p1D4-hrGFP II expression vector (Morrow and Chang, 2010) and transiently transfected into HEK293T cells. All three pigments successfully bound 11-*cis* retinal, producing dark spectra with λ_{max} values of 500.6±0.4, 495.7±0.3 and 496.8±0.5 nm for rhodopsin (*N*=3), Rh1-2 (*N*=3) and exo-rhodopsin (*N*=3), respectively (Fig. 2). These values were consistent over three separate expressions and with previous *in vitro* expression studies (Chinen et al., 2003; Morrow et al., 2011;

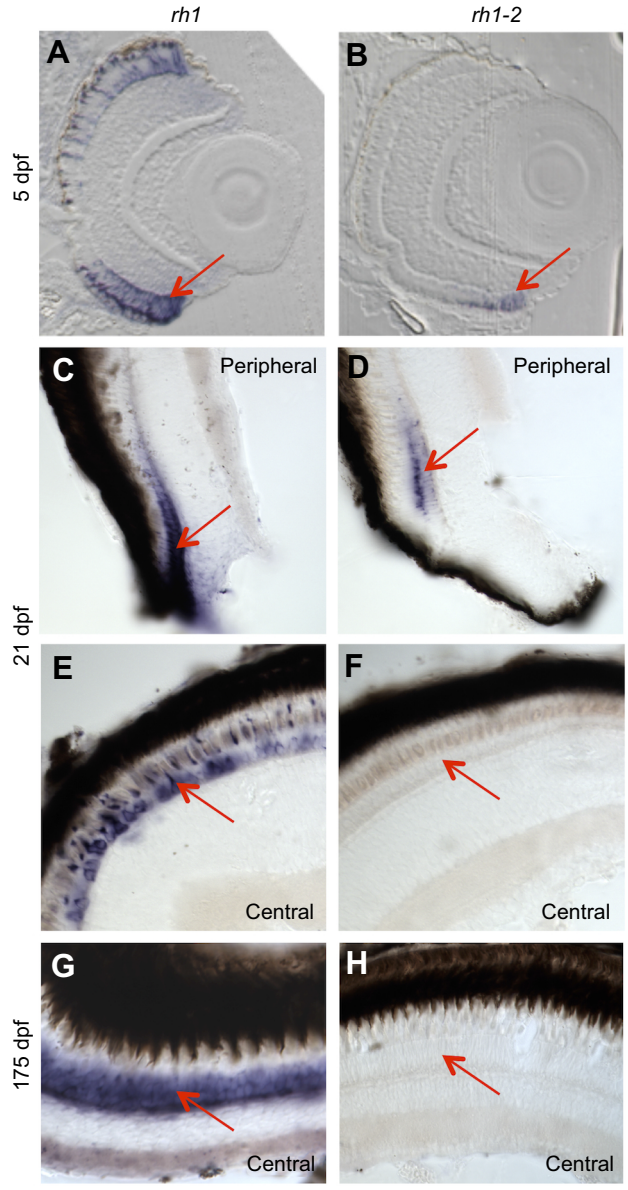

Fig. 1. *In situ* hybridization (ISH) of *rh1-2* in the zebrafish retina at various developmental stages. (A,B) Whole-mount ISH of 5 days post-fertilization (dpf) embryos showing strong expression of *rh1* and weak expression of *rh1-2* in the ventral peripheral retina. (C–F) ISH of juvenile eyes at 21 dpf showing strong expression of *rh1* in both the central and peripheral retina, but only weak expression of *rh1-2* in the ventral peripheral retina. (G,H) ISH of adult eyes at 175 dpf showing strong expression of *rh1* in the central retina, but no signal from *rh1-2*. Both *rh1* and *rh1-2* also showed expression in the peripheral retina at 175 dpf (results not shown). All expression of *rh1* and *rh1-2* was confined to the outer nuclear layer of the retina, consisting of the cell bodies of rod and cone photoreceptors. In all panels, dorsal is to the top and ventral is to the bottom.

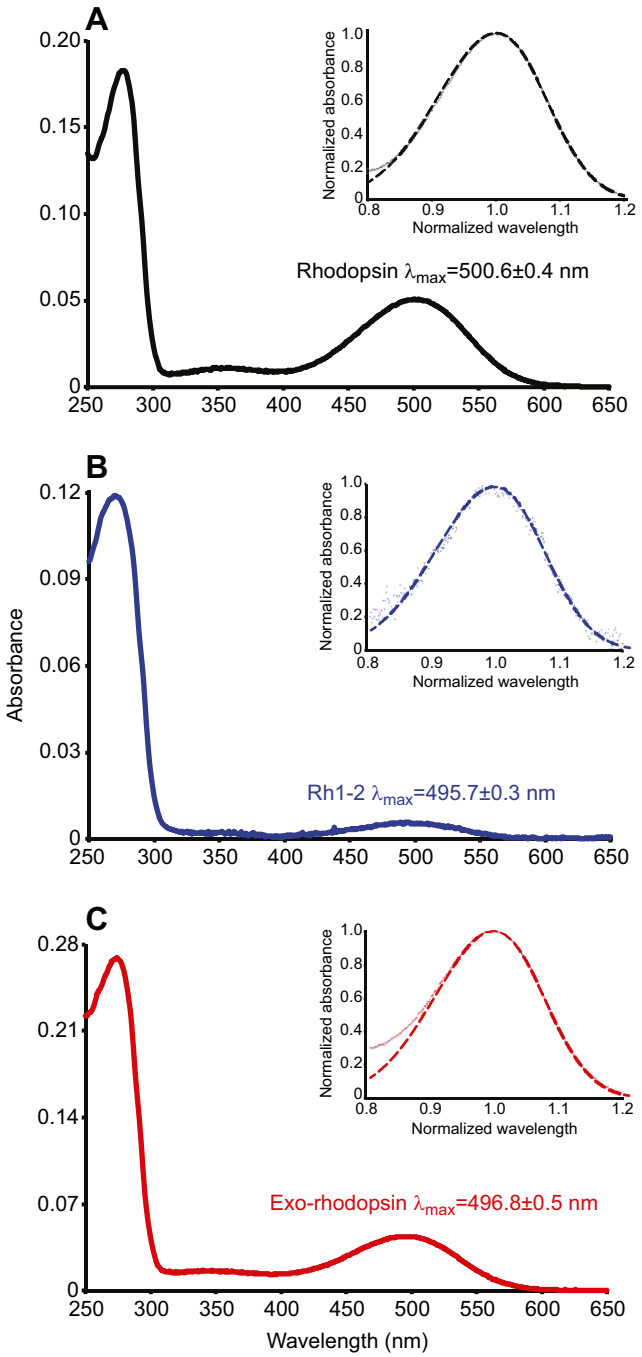

Fig. 2. Absorbance spectra of zebrafish rhodopsins following *in vitro* expression and purification. Dark spectra of (A) zebrafish rhodopsin, (B) zebrafish Rh1-2 and (C) zebrafish exo-rhodopsin, along with respective curve fits to A1 visual pigment templates used to estimate λ_{max} (inset). Both Rh1-2 and exo-rhodopsin are slightly blue-shifted compared with rhodopsin. λ_{max} values represent the mean (±s.d.) of three separate expressions for each pigment (*N*=3). Traces are shown for comparative purposes and are representative of these means.

Journal of Experimental Biology (2017) 220, 294-303 doi:10.1242/jeb.145953

Tarttelin et al., 2011). This shows that the λ_{max} of Rh1-2 is more similar to that of exo-rhodopsin than to rhodopsin, but all three are around 500 nm, characteristic of most rhodopsins and many non-visual opsins (Kojima et al., 2000; Bowmaker, 2008).

Rh1-2 and rhodopsin release retinal at a rate similar

We also measured the rate of release of all-*trans* retinal that occurs after photoactivation, requiring both hydrolysis of the Schiff base linkage between opsin and retinal and dissociation of retinal from opsin. Using fluorescence spectroscopy, we measured the retinal release half-life of zebrafish rhodopsin as 6.5±0.3 min (N=6; Fig. 3), which is comparable to our previous results (Morrow and Chang, 2015). Despite much lower expression levels, Rh1-2 had a very similar retinal release half-life of 7.6±0.8 min (N=3; Fig. 3). Conversely, the non-visual exo-rhodopsin released retinal approximately 5 times faster than rhodopsin, with a half-life of 1.6±0.3 min (N=5; Fig. 3). This is the first time that retinal release has been measured in a non-visual opsin. These results show that the kinetics of photoactivation in Rh1-2 are more similar to those of rhodopsin than to exo-rhodopsin, despite the fact that *in vitro* expression levels suggest that Rh1-2 may be less stable than both rhodopsin and exo-rhodopsin.

rh1-2 gene duplication occurred early in teleost fish evolution

Prior analyses lacked the taxonomic sampling to resolve the origins of *rh1-2*, although there were hints that it might be an ancient gene duplication in teleost fish (Morrow et al., 2011). Here, we amplified additional *rh1* and *rh1-2* sequences in order to better resolve the evolutionary history of *rh1-2*, and its relationship to the *rh1* and *exorh* genes. Both maximum-likelihood and Bayesian phylogenetic analyses recovered with high bootstrap and posterior probability support, respectively, a single clade of *rh1-2* that was most closely related to anchovies and herring (Clupeomorpha) and ostariophysian fishes (Fig. 4; Fig. S2). Interestingly, the *rh1-2* clade was not most closely related to other duplicated ray-finned fish *rh1* genes, such as *exorh*, eel deep-sea rhodopsin (*dso*) and freshwater rhodopsin (*fwo*), or the pearleye *rh1a* and *rh1b*. The resulting topology largely recovered expected species relationships, particularly for major lineages, including the placement of lampreys and ray-finned fish (Hurley et al., 2007; Nakatani et al., 2011; Near et al., 2012). Interestingly, the two clades of eel *rh1* paralogs did not resolve into a monophyletic group, although this may have been due to unusual sequence evolution in nearby clades, which may be resolved with additional sequence data. Together, this suggests that the duplication that led to *rh1-2* occurred in the ancestor of a major group of bony fishes including anchovies, herrings and ostariophysian fishes (Ostarioclupeomorpha). Previous phylogenetic analysis of *rh1-2* found, with weak support, that *rh1-2* was sister to ostariophysians plus acanthomorphs (Morrow et al., 2011). This discrepancy is likely due to the increased taxon sampling in the current study. These results further suggest that additional copies of *rh1-2* have yet to be identified from several groups of ray-finned fish. Additional sequences that become available as emerging genome projects of ostariophysian and clupeomorph fishes are annotated may help to further resolve the placement of this clade.

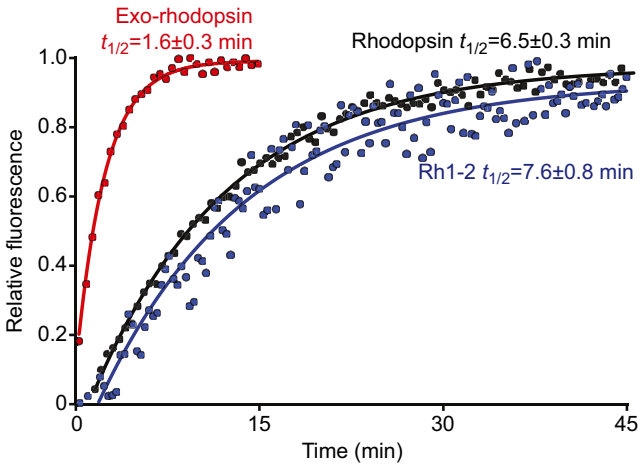

Fig. 3. Retinal release rates of zebrafish rhodopsins at 20°C. The release of all-*trans* retinal is represented by an increase in fluorescence intensity following photoactivation of zebrafish rhodopsin (black), Rh1-2 (blue) and exo-rhodopsin (red). Half-life ($t_{1/2}$) values are the mean (±s.d.) of replicates (rhodopsin: N=6; Rh1-2: N=3; exo-rhodopsin N=5). Traces are shown for comparative purposes and are representative of these means.

The *rh1-2* clade is under selective pressure similar to other *rh1* genes

Molecular evolutionary analyses were used to determine what changes in selective constraint occurred during and after the duplication that led to *rh1-2*. Random sites models as implemented in PAML revealed that vertebrate rhodopsins as a whole were under strong selective constraint (average ω=0.07, M0) (Table S2) with no evidence of positive selection (M2a versus M1a; M8 versus M8a, P>>0.5 in all cases) (Table S2). Significant among-site rate variation was found, as would be expected for functional protein coding genes (M3 versus M0, P<0.00) (Table S2). This was also true when only ray-finned fish were considered (ω=0.08, M0; M2a versus M1a; M8 versus M8a, P>>0.5 in all cases; M3 versus M0, P<0.00) (Table S2) and when only *rh1-2* was considered (ω=0.09, M0; M2a versus M1a; M8 versus M8a, P>>0.5 in all cases; M3 versus M0, P<0.00) (Table S2).

Accelerated evolution at a subset of sites may have accompanied the divergence of *rh1-2* from *rh1*

To further test for differences in selective constraint between *rh1-2* and other *rh1* genes, we used the branch-site, branch and clade models on the dataset pruned to contain only ray-finned fish rhodopsin sequences. Using the branch-site model, we observed increased positive selection on the branch leading to the *rh1-2* clade (Table S3), which approached significance when compared with the null model (P=0.071), and identified a number of BEB sites with posterior probabilities above 0.8. Conversely, branch-site and CmC analyses with the *rh1-2* clade placed in the foreground found no evidence of either positive or divergent selection, supporting earlier random sites model results, which found that the *rh1-2* clade is under similar selective pressure to other *rh1* genes. Finally, the branch model was used to test for overall changes in selective constraint both on the branch leading to *rh1-2* and on the entire clade. We found that the ω along the branch and for the whole clade did not significantly differ from background ω (P>>0.5) (Tables S3 and S4). This supports the hypothesis that *rh1-2* is a functional gene as it has been maintained under high levels of negative selection, corroborated by the M3 results, which show significant rate variation. Together, these results suggest that *rh1-2* may have experienced a burst of positive selection following duplication and divergence from *rh1*, and was later subject to purifying selection, which highlights an evolutionary path typical of genes that survive duplication and divergence events (Lynch and Conery, 2000).

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 294-303 doi:10.1242/jeb.145953

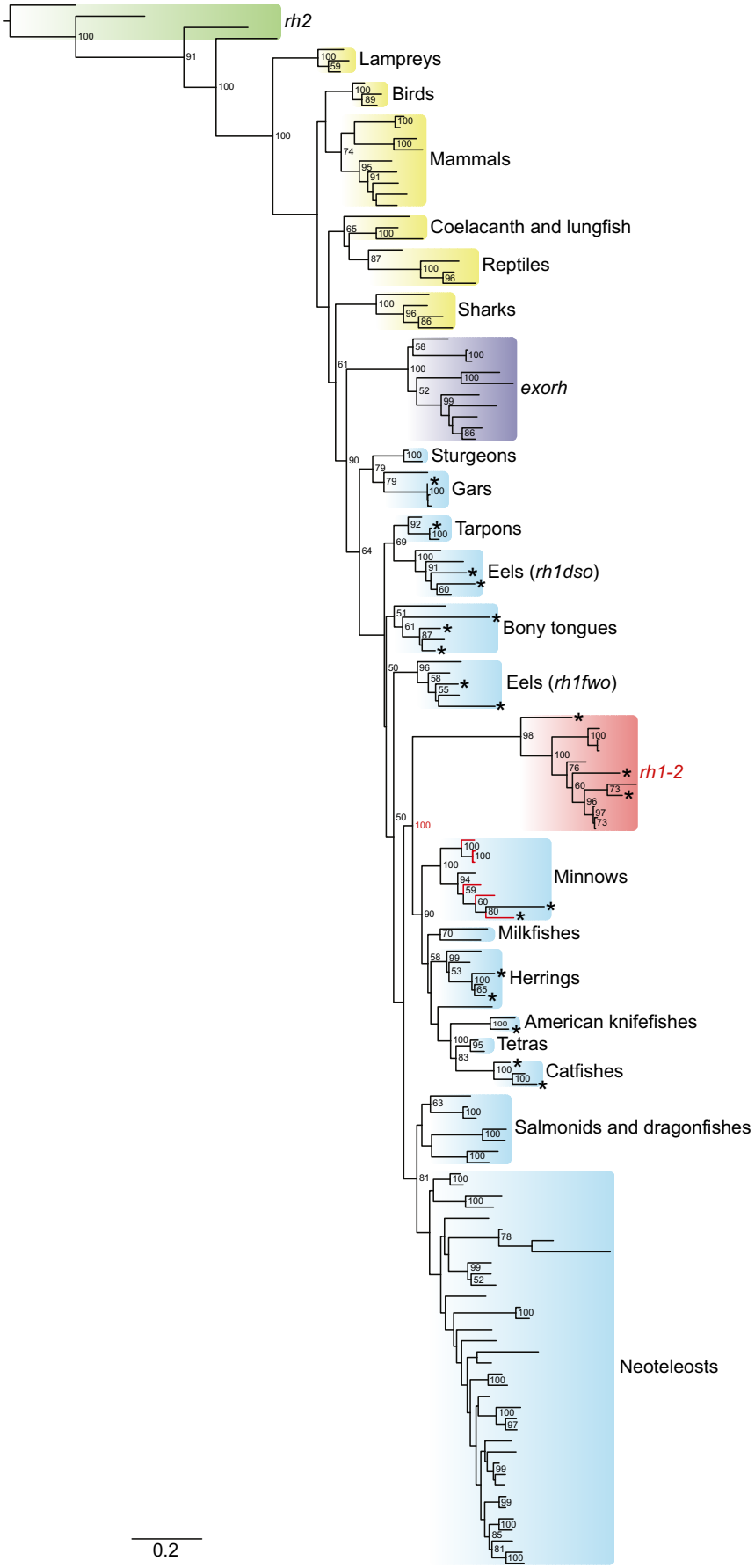

Fig. 4. Maximum-likelihood phylogeny of vertebrate rhodopsin genes, including *rh1*, *rh1-2* and *exorh*. The resulting topology mostly recovered expected relationships, including the positioning of lampreys and ray-finned fishes. Topology supports the placement of the *rh1-2* gene family as the sister group to Ostarioclupeomorpha. Branches with asterisks represent sequences newly introduced in this study. Red branches in the minnow clade represent species that also have an *rh1-2* gene in this phylogeny. Several *rh2* opsin sequences were used as outgroups.

Journal of Experimental Biology (2017) 220, 294-303 doi:10.1242/jeb.145953

DISCUSSION

Using *in situ* hybridization, along with both absorbance and fluorescence spectroscopy, we have shown that zebrafish *rh1-2* is expressed in peripheral photoreceptors of the retina and codes for a functional opsin protein that releases retinal at a similar rate to rhodopsin following photoactivation. However, *rh1-2* expression only starts around 5 dpf and is weak *in vitro*, both traits that are uncharacteristic of traditional *rh1* genes. Despite low expression levels, Rh1-2 has a similar half-life of retinal release to rhodopsin, almost 5 times greater than that of the non-visual exo-rhodopsin, suggesting the potential for a role in vision as a result of functional similarities to rhodopsin. Phylogenetic analyses place the *rh1-2* clade as a sister group to ostariophysian and clupeomorph *rh1*, suggesting it originated following a fairly ancient duplication event, independent of other *rh1* duplications previously characterized in teleost fish. Below, we discuss potential functional roles for *rh1-2* considering our findings, and the implications of this opsin gene for the zebrafish visual system.

The retinas of teleosts experience persistent neurogenesis in postembryonic fish, with new neurons arising from two different populations of stem cells. The first are multipotent stem cells residing in the ciliary, or circumferential, marginal zone (CMZ), where progenitor cells give rise to Müller glia and all retinal neurons, except for rod photoreceptors, proliferate outward from the peripheral retina (Johns, 1977; Hitchcock et al., 2004; Stenkamp, 2007). The second are Müller glia cells, which give rise to rod precursor cells in the inner nuclear layer; once these rod precursors reach the ONL, they divide and differentiate as rod photoreceptors (Bernardos et al., 2007; Nelson et al., 2008). Our *in situ* results show that expression of *rh1-2* is consistently located in the peripheral ONL of the retina, near the CMZ, but does not persist as cells are repositioned to a more central location in juvenile and adult fish. This suggests that *rh1-2* expression is limited to either rod photoreceptors derived from peripheral Müller glia cells that have recently emerged from the CMZ, or to cone photoreceptors recently derived from retinal progenitors of the CMZ that are still located in the periphery. Either possibility is consistent with the idea that *rh1-2* is more similar to visual opsins and expressed in retinal photoreceptors, as opposed to a non-visual opsin that can be expressed in other retinal cells, including horizontal cells (Cheng et al., 2009) and retinal ganglion cells (Dacey et al., 2005; Panda et al., 2005), as well as in neural tissues outside of the retina, including the pineal gland (Mano et al., 1999), cerebellum (Blackshaw and Snyder, 1999) and spinal cord (Tartellin et al., 2003).

In order to function as a visual rhodopsin, *rh1-2* would likely have to be expressed in rods; however, expression in cones is also possible as there are rare cases where rhodopsins and cone opsins are expressed in the opposing photoreceptor type in reptiles and amphibians (Kojima et al., 1992; McDevitt et al., 1993; Schott et al., 2016). While a ∼496 nm peak corresponding to Rh1-2 was not detected in previous microspectrophotometry studies, which could have helped to localize expression at the cellular level (Nawrocki et al., 1985; Robinson et al., 1993; Cameron, 2002), this is likely the result of either a lack of sensitivity to detect the poorly expressing opsin or confounding signals with other opsins that have similar λ_{max} values, such as rhodopsin, Rh2-3 or Rh2-4 (Chinen et al., 2003). Interestingly, the expression pattern of *rh1-2* most resembles that of *rh2-3* and *rh2-4*, which is confined to peripheral photoreceptors near the CMZ in embryos and juveniles (Takechi and Kawamura, 2005). In adult fish, however, *rh2-3* and *rh2-4* expression expands to additional portions of the peripheral retina (Takechi and Kawamura, 2005), while the pattern of *rh1-2* stays mostly the same. It is possible that even limited *rh1-2* expression in the ventral peripheral retina, which produces a slightly blue-shifted pigment compared with rhodopsin, may be advantageous for detecting the spectrum of down-welling light, a phenomenon that has been noted in cone opsin duplicates (Temple, 2011). Overall, the fact that *rh1-2* has both a different and more restrictive expression pattern than *rh1* in the retina is not unusual, as a diversity of expression patterns seem to be a common feature of duplicated opsin genes in teleost fish (Hofmann and Carleton, 2009).

Along with expression in the ONL, there are several functional features of the Rh1-2 protein that suggest it is likely to be a visual opsin gene. We previously reported that Rh1-2 successfully bound 11-*cis* retinal to form a functional pigment that activates in response to light, with a λ_{max} of approximately 500 nm (Morrow et al., 2011). Here, we present a more precise λ_{max} estimation of 495.7±0.3 nm, calculated via curve fitting to an A1 visual pigment template (Govardovskii et al., 2000). While this is ∼5 nm blue-shifted compared with rhodopsin, and more similar to exo-rhodopsin, all three pigments have λ_{max} values within the typical range of most vertebrate rhodopsins (Bowmaker, 2008). The ∼4 nm blue shift of exo-rhodopsin relative to rhodopsin was suggested to be due to A124 (Tarttelin et al., 2011), as A124G substitutions in some deep-sea fish rhodopsins were associated with red-shifts of up to 3 nm (Hunt et al., 2001). Rh1-2 also has A124, suggesting the potential for a similar spectral tuning mechanism to that of exo-rhodopsin. However, the G124A substitution in zebrafish rhodopsin was recently shown to have no significant effect on spectral tuning (Morrow and Chang, 2015). While identical substitutions in different rhodopsin sequences can lead to variable shifts in spectral sensitivity, it is also possible that the blue shift of Rh1-2 relative to rhodopsin is due to an accumulation of minor substitutions throughout its sequence, as no other differences were identified at sites known to be involved in rhodopsin spectral tuning (Yokoyama, 2000; Hunt et al., 2001).

Following photoactivation, Rh1-2 releases retinal at a rate similar to rhodopsin, suggesting that key structural aspects of rhodopsins, such as Schiff base stability and the hydrogen bond network of the chromophore binding pocket, are likely maintained in Rh1-2 (Janz and Farrens, 2004). This point is reflected in the significantly faster retinal release of cone visual pigments (Chen et al., 2012) and in our measurements of the non-visual exo-rhodopsin, which releases retinal almost 5 times faster following photoactivation. Because retinal release is a step in the retinoid cycle (Kiser et al., 2012), the metabolic cycle responsible for providing new 11-*cis* retinal molecules to regenerate free opsin, the slower retinal release of Rh1-2 also suggests that it may only have access to the retinal pigment epithelium-mediated pathway of retinal regeneration (Lamb and Pugh, 2004), and not the Müller cell-mediated pathway upon which cone opsins rely to help maintain rapid response kinetics (Wang et al., 2009; Wang and Kefalov, 2011). These results support the hypothesis that Rh1-2 is a visual opsin with rhodopsin-like functional properties.

An alternative hypothesis concerning the role of *rh1-2* is that it is a gene duplicate experiencing low levels of expression that has no unique functional role in photoreception. Where traditional rhodopsin genes maintain a high level of expression in the retina, the duplication event that gave rise to *rh1-2* failed to transfer the same regulatory elements that drive *rh1* expression (Kennedy et al., 2001), resulting in much lower expression levels. Additionally, *in vitro* expression of Rh1-2 suggests it is considerably less stable than rhodopsin. Despite having some characteristics of a redundant

Journal of Experimental Biology (2017) 220, 294-303 doi:10.1242/jeb.145953

gene duplicate, this classification is usually attributed to genes resulting from relatively recent gene duplication events, with the vast majority of gene duplicates being silenced within a few million years (Lynch et al., 2001). However, unlike a more recent rhodopsin gene duplication, which may generate species-specific duplicates (Lim et al., 1997), our analyses suggest a much more ancient origin for the *rh1-2* gene, within the *rh1* gene family of a major group of bony fishes, which would place the duplication leading to the birth of *rh1-2* somewhere between 153 and 248 million years ago (Nakatani et al., 2011; Chen et al., 2013). PAML analyses also suggest the potential for increased evolutionary rates at a variety of sites along the branch leading to the *rh1-2* clade, followed by strong selective constraint characteristic of *rh1* genes within the *rh1-2* clade. This pattern of evolutionary rates is typical of genes that survive duplication and divergence events (Lynch and Conery, 2000), including retrogenes (Gayral et al., 2007). This pattern will likely become more significant when additional *rh1-2* sequences are discovered and included in future analyses.

Aside from *rh1-2*, only two other *rh1* gene duplications are known to have been retained in Actinopterigian fishes, making *rh1*, along with *sws1*, the least common opsin gene to experience duplication. The first example is from eels, which express the *rh1fwo* gene with the 11-*cis* 3,4-dehydroretinal (A2) chromophore in the early stages of life, where a red-shifted rhodopsin is thought to provide an advantage in the more long wavelength-shifted spectral environment of freshwater (Bridges, 1972; Loew, 1995). During maturation, eels migrate to a marine environment, with a more restricted and blue-shifted light spectrum, coupled with expression of a blue-shifted *rh1dso* gene, regenerated with 11-*cis* retinal (A1) chromophore (Hope et al., 1998; Zhang et al., 2000). This switch of both opsin and chromophore is a clear example of an adjustment of the visual system as a result of a change in photic environment. Another example is the deep-sea pearleye, *S. analis*, which has a more traditional *rh1A* gene, along with *rh1B*, expressed alongside *rh1A* in adult fish living over 900 m below the surface (Pointer et al., 2007). The pearleye has unique cylindrical eye morphology, containing both a main retina, used for image formation, and an accessory retina, likely only capable of gross light perception (Collin et al., 1998), with *rh1B* expression being localized in this accessory retina (Pointer et al., 2007). Zebrafish do not experience an ontogenetic migration, possess only A1 chromophore-based visual pigments (Allison et al., 2004), do not occupy deep-sea habitats and do not have abnormal eye or retinal morphology, suggesting that it is unlikely that *rh1-2* serves a similar function to the duplicated rhodopsin genes in either eels or the pearleye. This is supported by our phylogenetic analyses, which found *rh1fwo*, *rh1dso*, *rh1B* and *rh1-2* all form distinct lineages that resulted from separate duplication and divergence events.

Perhaps the most intriguing result highlighted in this study is the partially overlapping expression patterns of *rh1* and *rh1-2*, suggesting the possibility that the two genes may be co-expressed in a subset of photoreceptors, which could influence both the structure and function of these cells. Previous studies have hypothesized that cone opsin co-expression in humans could provide a developmental advantage (Xiao and Hendrickson, 2000). Alternatively, cone opsin co-expression in the cichlid fish *Metriaclima zebra* is thought to contribute to spectral tuning, although the λ_{max} differences in these opsins is 35–48 nm (Dalton et al., 2014), far exceeding the 5 nm difference between rhodopsin and Rh1-2. However, rhodopsin also serves an important structural role in rod photoreceptors, where it is packed into the outer segments and forms an array of dimers (Fotiadis et al., 2003). This arrangement could help to maximize the capacity of the rod outer segments, but likely also serves a functional purpose, with higher order rhodopsin oligomers being a more active species than monomers (Fotiadis et al., 2006). Considering the relatively low stability of Rh1-2 compared with rhodopsin, the incorporation of the former into a rhodopsin oligomer array could influence the structure of peripheral rod photoreceptors.

Co-expression of multiple *rh1* genes also raises the possibility of the formation of heterodimers, common in some GPCRs where it allows for differential binding between ligand and G protein (Waldhoer et al., 2005; Monnier et al., 2011). In fact, a functional dichotomy where one monomer responds to stimuli and the other binds the G protein was predicted for rhodopsin by molecular dynamics simulations (Neri et al., 2010), while alternative conformations for each monomer following activation could promote distinct functions from otherwise identical subunits (Jastrzebska et al., 2013). The presence of a rhodopsin/Rh1-2 heterodimer would likely influence the properties of a rod photoreceptor; however, further study is required to investigate this possibility. These studies will be challenging because of the low expression levels of *rh1-2* and its significant sequence similarity to the highly expressed *rh1*. Nonetheless, the potential for this interaction as well as the presence of the *rh1-2* gene in other teleost fish should promote additional investigation into the influence of a second visual rhodopsin gene on the vertebrate visual system.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
J.M.M. helped design the study, sequenced rhodopsin genes, expressed and characterized rhodopsins, and drafted the manuscript. S.L. helped design the study and performed *in situ* hybridization. M.D.F. and C.K. performed *in situ* hybridization. R.K.S. and E.A.G. ran the phylogenetic and selection analyses and provided text for the manuscript. F.S. contributed to the sequencing. V.T. helped design the study and provided guidance and edits for the manuscript. B.S.W.C. led study design, helped draft the manuscript and supervised all aspects of the project.

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Data availability
In total, 19 new nucleotide sequences were submitted to NCBI GenBank, and are avialable under the following accession numbers: 16 new *rh1* sequences (KY026025–KY026040) and three new *rh1-2* sequences (KY026041–KY026043).

Supplementary information
Supplementary information available online at
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