Vertebrate features revealed in the rudimentary eye of the Pacific hagfish (Eptatretus stoutii)

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Hagfish eyes are markedly basic compared to the eyes of other vertebrates, lacking a pigmented epithelium, a lens and a retinal architecture built of three cell layers: the photoreceptors, interneurons and ganglion cells. Concomitant with hagfish belonging to the earliest-branching vertebrate group (the jawless Agnathans), this lack of derived characters has prompted competing interpretations that hagfish eyes represent either a transitional form in the early evolution of vertebrate vision, or a regression from a previously elaborate organ. Here, we show the hagfish retina is not extensively degenerating during its ontogeny, but instead grows throughout life via a recognizable PAX6+ ciliary marginal zone. The retina has a distinct layer of photoreceptor cells that appear to homogeneously express a single opsin of the RH1 rod opsin class. The epithelium that encompasses these photoreceptors is striking because it lacks the melanin pigment that is universally associated with animal vision; notwithstanding, we suggest this epithelium is a homologue of gnathosome retinal pigment epithelium (RPE) based on its robust expression of RPE65 and its engulfment of photoreceptor outer segments. We infer that the hagfish retina is not entirely rudimentary in its wiring, despite lacking a morphologically distinct layer of interneurons: multiple populations of cells exist in the hagfish inner retina and subsets of these express markers of vertebrate retinal interneurons. Overall, these data clarify Agnathan retinal homologies, reveal characters that now appear to be ubiquitous across the eyes of vertebrates, and refine interpretations of early vertebrate visual system evolution.

1. Introduction

Vertebrate eyes are remarkably conserved and complex, appearing early in the evolution of this group as the familiar single-chambered camera-style organ with advanced optics at the anterior, and photoreceptors and retinal pigmented epithelium (RPE) lining the posterior of the eye's globe. Across all vertebrate taxa, this morphology deviates little despite diverse visual ecologies and is easily recognizable even in early branching vertebrates including the jawless lamprey. Homology of the eye across vertebrates is evident across various levels—anatomy, development, laminated neural architecture, synaptic wiring, visual cell physiology and molecular markers [1,2]. The visual system is key to the success of vertebrates including their detection of prey, predators, mates and migratory cues, yet the evolutionary origins of the vertebrate eye have remained obscured. Vertebrates are unique among chordates in sharing the following features: a lens, extra-ocular musculature and a neural retina supported by an RPE. The neural architecture of the retina comprises three nuclear layers: the photoreceptors, interneurons and ganglion cells. Photoreceptors (rods and cones) transduce light into neural signals, interneurons (horizontal, amacrine and bipolar cells) compare these signals to compute and decant the various properties of the light stimuli, and retinal ganglion cells (RGCs) send the encoded information through their axons (the optic nerve) to the higher visual centres of the brain. A richly melanized RPE lines the back of the eye chamber, enveloping the photoreceptors, that functions to block stray photons and provide critical support for photoreceptor physiology [3].
It is difficult to overstate (or briefly summarize) the impressive degree of conservation across vertebrate retinas at various levels of eye organization. The vertebrate ocular bauplan is evident across vertebrates (including lamprey), and thus has been retained since early vertebrate evolution prior to the advent of jaws or paired fins (limbs). Celebrated exceptions do exist, where features have been reduced or lost (e.g. in cavenous the eyes degenerate during ontogeny) or elaborated and adapted (e.g. as rod-rich deep sea tube eyes [4] and four-eye fish [5]).

Hagfish are among the rare exceptions to this description in vertebrates. Their eyes are small, unpigmented and lack diagnostic jawed vertebrate features such as a duplex retina (with both rods and cones), lens, cornea and extra-ocular musculature [6,7]. The eyecup is masked by semi-transparent or non-transparent overlying epidermis, the transparency of the skin varies across species [8]. In some species, such as the Atlantic hagfish (*Myxine glutinosa*), the eye is also buried under body wall muscle [6,9]. Though a lens and ocular musculature are absent, photoreceptors are present, as are RGCs [6,9,10]. Moreover, the hagfish neural retina lacks lamination into three nuclear layers, which is suggestive of a pineal-like architecture lacking interneurons, and of a simpler photoreceptive organ that is perhaps more suitable for detecting circadian light rhythms than for resolving images or vision.

Hagfish eyes across two genera, are thus remarkable compared to all other vertebrates for the ocular characters they (seem to) lack, apparently coconitment with their basal phylogenetic position. Combining the phylogenetic position and starkly rudimentary eye has historically positioned hagfish as a transitional form in early vertebrate visual system evolution; although an alternative view is that hagfish eyes may be rudimentary owing to loss of characters.

Delineating between these two hypotheses is necessary in resolving a substantial gap in understanding how the vertebrate eye and its ‘inimitable contrivances’ evolved. Here, we revisit the rudimentary hagfish retina to better appreciate the earliest steps in the history of vertebrate visual system evolution. Our objective was to explore retinal structure and identify cell types by molecular markers in the hagfish with a focus on Pacific hagfish (*Eptatretus stoutii*) because its visual system seems somewhat less degenerate/rudimentary, compared to other species [6,8]. Because hagfish embryos are prohibitively difficult to procure, we instead consider individuals across a range of sizes, including the smallest individuals attainable, to consider if hypotheses can be informed by characterizing these eyes over their ontogeny. We hypothesized that the hagfish eye possessed a greater number of conserved vertebrate eye characters than previously identified and that some (perhaps not all) features may be rudimentary as a result of secondary loss or arrested development. We reveal hagfish visual system characters that are otherwise conserved across vertebrate taxa, and reason that some aspects of hagfishes’ remarkable eyes have regressed over evolutionary time.

2. Results

(a) The Pacific hagfish retina is simple and unpigmented

Found beneath a layer of translucent skin (figure 1a), the eyes of Pacific hagfish (*E. stoutii*) are small, embedded in the surrounding craniofacial muscle and lack extra-ocular musculature attachments (figure 1b,c). Notably, the eyes completely lack pigmentation (figure 1c,d) and no lens is apparent, though haematoxylin staining indicates the vitreous of the eye is protein-dense (figure 1d). In accordance with an absent lens, no intraocular musculature is identifiable.

In cross section, the typical vertebrate neural retina (exemplified here by zebrafish) is stratified into three distinct cellular layers (outer nuclear layer (ONL), inner nuclear layer, and ganglion cell layer) divided by two synaptic layers (inner and outer plexiform layer) (figure 1e). The retina of *E. stoutii* is not morphologically separated into this same layered organization (figure 1f). Photoreceptors are found at the scleral-most region, with presumptive RGCs basal to this, towards the vitreous (figure 1f). Overall, the *E. stoutii* eye is rudimentary compared to exemplar eyes of other vertebrate taxa (see Introduction), consistent with past reports in various hagfish species [7,8].

(b) Hagfish eyes grow over ontogeny perhaps via PAX6-expressing progenitors at the retinal margins

To characterize if the rudimentary eyes of hagfish are changing over ontogeny, we measured eye size in Pacific hagfish caught off the west coast of Canada. Because the hatching and raising of hagfish embryos is rarely attainable, we collected wild-caught individuals using fish size as a proxy for age. The eye was found to increase in both length and mass during ontogeny over a broad range of fish sizes (figure 2f–g; i.e. greater than fourfold increase in fish mass or length). Thus, the Pacific hagfish eyes appear degenerate (rudimentary and diminutive) but are not overtly degenerating over ontogeny.

Observing larger eyes in larger hagfish was reminiscent of fishes’ eye growth that continues into adulthood, supported by proliferating cells at the outer edges of the mature retina known as the ciliary marginal zone (CMZ) [11–13]. We observed that hagfish eyes possess a comparable region and propose that it contributes to sustained eye growth. In hagfish, the cells at the retinal margins lack photoreceptor outer segments and have irregularly shaped nuclei distinct from cells in the central retina (figure 2h). More elaborated cell morphologies appear gradually towards the central retina, with increasing resemblance to fully differentiated cells (figure 2i–j). The simpler retinal neuroepithelium at the margins is observed to merge with a more distal (scleral) epithelium (the presumptive RPE, described below). This merged retinal margin is positioned at the edge of the eyecup, comparable to the gnathostome CMZ where it would be contiguous with tissue forming the pupil.

A highly conserved orthologue of *PAX6*, expressed in the CMZ of gnathostomes [12], was found to be expressed in the hagfish retina. *PAX6* expression is most concentrated at the tip of the retinal marginal zone (figure 2h–j), suggesting that not only do these cells appear undifferentiated by morphology, but that they also express transcripts related to the maintenance of a multipotent progenitor cell state [14].

(c) Interneurons are found despite the lack of vertebrate-typical retinal layering

Apart from photoreceptors and RGCs, the cell identities within the hagfish retina have remained obscured and a segregated interneuron layer in hagfish is not obvious. This simple retinal composition in hagfish is interpreted by some as a primitive neural architecture reminiscent of the
The distribution of RGCs (previously identified in the hagfish retina [10,17]) were characterized by cell-type specific marker γ-synuclein (SNCG, figure 3g) [18,19] in situ hybridization. RGCs represented a subset of cells identified within the inner retina cell layer, distributed both at the vitreal retinal margin and also in more apical locations. Melanopsin was expressed in at least a subset of RGCs and perhaps other cells (figure 3d), consistent with past reports [17].

In summary, the inner layer of hagfish retina contains subsets of cells expressing markers of RGCs, amacrine cells, bipolar cells and horizontal cells. These cells intermingle with immunoreactivity for synaptic markers that localize in a layer distinct from photoreceptor synapses. Previous characterizations have described hagfish retinas as having only two nuclear layers and speculated that this simplicity is comparable to the vertebrate pineal [7,8,20–22]; the data presented here reveal the hagfish retina has greater complexity, and recognizable neuronal features conserved with retinas of jawed vertebrates.

(d) A homologue of retinal pigment epithelium, the retinal (non)pigment epithelium, interdigitates with photoreceptors lining the hagfish outer retina

In jawed vertebrates, the photoreceptors are sustained by the RPE, a monostratified epithelium with dense melanin. This vital partnership between the neural retina and RPE is unique to vertebrates.

We sought to determine if hagfish photoreceptors, which somewhat appear morphologically rod-like (figure 4e), express the rod associated opsin–rhodopsin. Rhodopsin (RH1) is the only visual opsin known to be present in hagfish eyes, though its expression pattern and the presence of other visual opsins in the genome remain to be determined [23]. Through RNA-sequencing, we identified only one visual
ciliary opsin: a highly expressed RH1. Homology searching through the de novo assembled transcriptome using HMMER [24] and BLASTP 2.6.0 [25] did not reveal additional visual opsins. Eptatretus stoutii RH1 shares high sequence similarity with other vertebrates, clustering among other RH1s with high certainty (electronic supplementary material, figure S4).

Eptatretus stoutii RH1 in situ hybridization using RNA probes designed from transcriptome-derived sequences showed high specificity to the photoreceptors (figure 4a–a’). While the central retina has distinct photoreceptor outer segments (arrowheads) and discernable cell layers (a’), the RH1 transcript is highly enriched at the eyecup periphery (purple at both tips of this sectioned retina, *). (c–e) RH1 transcript (magenta) is expressed by the majority of cells in the CMZ (equivalent to box in panel (b), DAPI and TOPRO3 stain nuclei (cyan), scale bars are 100 μm). (f,g) Pacific hagfish eyes grow throughout ontogeny. Each dot represents one eye from one individual, and two different groups of animals are presented in panels (f) versus (g). A nearly isometric increase in eye size is observed over a greater than fourfold increase in animal length or mass. (Online version in colour.)

Figure 2. Hagfish retina has a peripheral tissue expressing PAX6 that presumably contributes to the eye growth that occurs late into ontogeny. This tissue is highly reminiscent of the multipotent and proliferative CMZ that supports eye growth late into ontogeny of early branching gnathostomes. (a) Peripheral hagfish retina, with laminated central retina (at top) merging into a simpler neuroepithelium near the pupil (bottom). More peripherally, the cells are less differentiated (a’), while the central retina has distinct photoreceptor outer segments (arrowheads) and discernable cell layers (a’). (b) PAX6 transcript is highly enriched at the eyecup periphery (purple at both tips of this sectioned retina, *). (c–e) PAX6 transcript (magenta) is expressed by the majority of cells in the CMZ (equivalent to box in panel (b), DAPI and TOPRO3 stain nuclei (cyan), scale bars are 100 μm). (f,g) Pacific hagfish eyes grow throughout ontogeny. Each dot represents one eye from one individual, and two different groups of animals are presented in panels (f) versus (g). A nearly isometric increase in eye size is observed over a greater than fourfold increase in animal length or mass. (Online version in colour.)

Additionally, the photoreceptor outer segments were readily immunolabelled by rod opsin antibodies zebrafish rod opsin (ZPR-3), bovine rhodopsin C-terminus (1D4) and bovine rhodopsin N-terminus (4D2) (Key Resources Table in electronic supplementary material; figure 4b–d). The RH1 protein immunoreactivity was located to the scleral side of the RH1 transcript in the photoreceptor outer segments. This location was immediately apical of an actin-rich strip (electronic supplementary material, figure S3D) akin to the outer limiting membrane in gnathostomes. Overall this polarized cell organization is exactly as is seen in the highly derived and polarized gnathostome photoreceptors.

An epithelium distal to the hagfish photoreceptors is similar to the RPE found in other vertebrates [8,10], but is strikingly disparate (even macroscopically, figure 1; electronic supplementary material, figure S1) owing to its complete lack of melanin pigment. Despite this, RNA-Seq revealed that pigment-related genes are expressed in the eye, including tyrosinase and pre-melanosome protein (PMEL; figure 5a). Thus, we have termed this non-pigmented RPE equivalent the ‘retinal (non)pigment epithelium’ or RnPE.

A major function of the RPE is to maintain the integrity of the photoreceptor outer segments by phagocytosing shed photoreceptor discs. As previously documented [10], we have found ultrastructural evidence of phagosome-like organelles in the hagfish RnPE, which contain what appears to be pieces of photoreceptor outer segments (figure 5c).
The gnathostome RPE also sustains photoreception through retinoid cycling [3,26,27]. RNA-Seq revealed that retinoid cycling genes lecithin-retinol acyltransferase (LRAT), 11-cis-retinol dehydrogenase 5 (RDH5), RPE65, cellular retinaldehyde-binding protein (CRALBP) and cellular retinol binding protein (CRBP) are expressed in the hagfish eye (figure 5a). Interphotoreceptor retinoid-binding protein (IRBP) was not identified. Further, we found that the expression of RPE65 is highly specific and robustly expressed within all cells of the RnPE (figure 5b), akin to its expression in gnathostomes and lamprey [28]. In summary, the presence of phagosomes, the interdigitiation with polarized ciliary photoreceptors, the presence of melanin synthesis machinery and the localized expression of conserved RPE marker RPE65 each support the RnPE being a homologue of gnathostome RPE that was pigmented in the last common ancestor of hagfish and jawed vertebrates.

Figure 3. A subset of cells in the Pacific hagfish retina each express markers of various gnathostome retinal interneurons or retinal ganglion cells. The inner retinal layer has cells exhibiting a variety of morphologies (electronic supplementary material, figure S2) and a subset of express markers of interneuron subtypes (electronic supplementary material, table S1). (a) Markers of retinal interneurons detected in hagfish eyes by RNA-Seq. (b) PAX6 in nuclei of a subset of inner retina cells, suggestive of amacrine cells. (c) Synaptic markers (immunoreactivity for SV2, see also the electronic supplementary material, figure S3) support the existence of interneurons. (d–g) Expression of transcripts encoding melanopsin, PKCα, calbindin or γ-synuclein (SNCG) each in a subset of inner retina cells is consistent with their expression across diverse vertebrates, where they identify bipolar cells, horizontal and/or ganglion cells. DAPI stains nuclei (cyan). Scale bars are 20 µm. (Online version in colour.)

3. Discussion

Although apparently rudimentary in comparison to the elaborate eyes conserved across vertebrate taxa, hagfish eyes appear advanced compared to the photoreceptor clusters of non-vertebrate chordates. This graduating pattern of character complexity is to some degree consistent with hagfish as a transitional form in visual system evolution. However, here we have identified previously undescribed features supporting homology between hagfish and jawed vertebrate eyes, implying these shared features were present in their last common ancestor (summarized in ‘graphical abstract’; electronic supplementary material, figure S7). Thus, we contend hagfish do not represent a transitional form, but that several ocular characters have regressed in hagfish, rendering them difficult to recognize despite decades of interest.

The apparent simplicity of the hagfish retina stands in contrast to the three nuclear cell layers that are nearly ubiquitous across vertebrate retina with an outermost layer comprised of photoreceptors, a middle layer of interneurons (horizontal, bipolar and amacrine cells), and an innermost layer containing RGCs. Obscured lamination in hagfish has likewise obscured the presence of an interneuron cell population [7]. However, the data here reveal that hagfish possess retinal interneurons by identifying the presence of CALBINDIN, PAX6 and PKCα labelling each within a subset of cells in the inner nuclear layer. Moreover, synaptic markers localized into two well-demarcated retinal plexiform layers independently support the presence of retinal interneurons. These parallels with retinal features conserved across jawed vertebrates clearly indicate that hagfish neural retina architecture is more elaborate than previously recognized.

The unique (whorled or shortened) morphology of hagfish photoreceptors has blurred their affinity with vertebrate rods or cones [10]. Our work elevates and expands upon studies characterizing hagfish photoreceptors as rods by their rod-typical spectral sensitivity, and electrophysiological response to low light stimuli. [20,29]. We have shown that the ultrastructurally rod-like photoreceptors of E. stoutii express RH1 transcript, and show robust outer segment immunoreactivity by 1D4, 4D2 (bovine rhodopsin).
and ZPR-3 (zebrafish rod-labelling) antibodies implying that RH1 transcripts are translated and trafficked to the outer membranes, where they are well-positioned to elicit phototransduction. RH1 is the only identified visual opsin in hagfish, and has been proposed to act through a phototransduction cascade similar to that of other vertebrates [23,30].

Our work emphasizes that further studies of hagfish are necessary in appreciating the evolutionary and developmental history of visual photoreceptors of vertebrates: particularly in comparing hagfish photoreceptors to the cones and cone-like rods found in lamprey [31,32]. The shared presence of retinoid cycling components within a separate epithelial tissue in hagfish, lamprey and gnathostomes suggests that this RPE–photoreceptor partnership was present in their last common ancestor and predates the divergence of jawed from jawless vertebrates.

Across hagfish species, a gradient of eye regression is apparent, with Atlantic species (Myxine) exhibiting small eyes buried under muscle and often unobservable when examining intact animals, and Eptatretus species which show far fewer restrictions in receiving environmental light [8]. We found that E. stoutii exhibit macroscopic growth of the eye isometric with body growth and our data suggest...
that this growth is supported by a PAX6+ multipotent cell niche at the retinal margin, the CMZ. Post-natal eye growth has been lost in crown group vertebrates, but ongoing adult retinal neurogenesis is a broadly conserved and prominent feature in fishes [13,34,35].

The absence of key ocular characters in the hagfishes, supported by our data as the result of character loss and eye regression, can now be rationalized against the phylogeny of agnathans. Until recently, it was debated whether hagfish and lamprey were a monophyletic group, or independent branches off the base of the vertebrate tree, contributing to the perplexity regarding the rudimentary hagfish eye. Monophyly of hagfishes and lampreys is now strongly supported [36–43], weakening the hypothesis that hagfish represent an ancient transitional eye, because this interpretation requires that lamprey acquired vertebrate-like ocular characters independently. It is instead more likely that the last common ancestor of jawed vertebrates, lamprey and hagfishes shared these derived characters and the hagfish eye is an extraordinary example of eye regression. Our data reinforce this conclusion, clarifying that vertebrates share key visual system characters (RPE, retinal architecture, eye growth) but that some synapomorphies are concealed by evolutionary regression in the hagfish lineage.

Widespread expression of RH1 in E. stoutii photoreceptors is a key support in the hypothesized regression of hagfish eye features. Typical vertebrate photoreceptors (including some lamprey species [44]) express any one of five visual opsins, with rods expressing rhodopsin (RH1) and cones a number of opsins covering the visual light spectrum (RH2, LWS, SWS1, SWS2) [45]. Phylogenetic analysis supports rhodopsin as the most recently evolved visual opsin, arising from a duplication of RH2 [46]. The lone presence of RH1 expressing photoreceptors in hagfish demonstrates a lost capacity to express other visual opsins. Future work should strive to identify lingering cone networks, if any exist. Using genomic studies will be particularly necessary in interrogating the full extent of opsin gene loss.

The absence of RPE pigment is one of the most striking absences in the hagfish eye. Fossil data for hagfish suggests that the eyes of at least one basal hagfish species possessed RPE pigment implying a pattern of regression in extant species [47]. Though melanin is not detectable in the extant hagfish RPE, it is clear that these hagfish can produce abundant melanin in their skin; thus, genetic drift might not account for loss of melanin production in the RPE. Further, we have shown that the retina expresses pigment-related transcripts including PMEL (required for melanin deposition) and tyrosinase (necessary for melanin synthesis) [48,49]. Melanin loss in the RnPE may have had an additional accelerating role in the degeneration of other eye tissues by leaving them prone to bleaching and oxidative stress which may explain the small and sparse photoreceptors of hagfish [50]. In models of albinism, eye function is seriously impacted in connection with an absence of melanin [51].

As we do not yet have access to embryos of this species, we cannot know if changes to development are responsible for the absence of lens, pigment, and/or cone photoreceptors in adults. It is possible that regression over evolutionary time has eliminated these structures altogether, making studies of other species of hagfish (e.g. the Atlantic hagfish—considered in some ways to have an eye more rudimentary than E. stoutii [8]) invaluable in providing a perspective on character absence across this lineage.

It is difficult to speculate with any authority on the use of the hagfish eye, in part because so little is known of their visual ecology. In one popular view, hagfish are benthic carrion feeders with no opportunity or use for photoreception; this aligns well with Myxine species where the retina is further degenerate and buried under muscle. However, the ecology of hagfish varies beyond this in other species, including recent observations of predatory hunting behaviour at depths under 100 m where photons may be biologically available [6,52]. Some species of the Eptatretus genus live in water less than 10 m depth [21,52–54]. Moreover, we are aware of no reports that detail the ecology of hagfish early in their ontogeny, where behaviour in shallower waters cannot be ruled out. Our data suggest some photoreceptive function for hagfish eyes should not be discounted, including that: (i) a transparent window is held over the eye (whereas the rest of the skin is pigmented and loosely attached, sloughing about the animal with little restriction); (ii) the eye grows substantially (greater than four-fold) throughout life; and (iii) the retina and photoreceptors are more complex than was previously appreciated. It remains to be determined if some photoreceptive function can be ascribed to some hagfish eyes, but in our estimation detection of photons and integrating between the two eyes could be sufficient for entraining circadian rhythms or phototaxis, whereas any ability to resolve images seems unlikely in the species and stages examined to date. It may be argued that loss of pigmentation in the RPE would not favour an ability to infer direction of light sources.

A level of photoreception ability between these (such as detecting objects but not resolving shapes) could occur in some instances, but no data are available at this time to convincingly support or refute any of these interpretations.

The data here reveal several cell- and tissue-level characters in the hagfish retina reminiscent of the eyes of fishes, birds and mammals, underlining the shared evolutionary origins of the vertebrate eye. Thus, the last common ancestor of agnathans and gnathostomes is inferred to have exhibited the following key characters for vertebrate expansion into diverse photic niches: (i) continued retinal growth late into ontogeny supported by a stem cell niche at the retinal margin; (ii) a pigmented RPE supporting a forest of ciliary visual photoreceptors; and (iii) a complex retinal wiring able to compute and decant the photoreceptor outputs into a representation of the visual scene.

We demonstrate how a greater understanding of hagfish eye biology can supplement the findings in lamprey eye biology and better inform evolutionary origins of the eye in the last common ancestor of extant agnathans and jawed vertebrates. Particularly in light of our confirmation of the presence of interneurons and an RnPE, the eyes of hagfish appear less far removed from that of other vertebrates, shifting the view of hagfish eye evolution from rudimentary to that of a more sophisticated vertebrate-style camera eye.

4. Methods

(a) Animal ethics and tissue collection

This study was conducted under the approval of the Animal Care and Use Committee: BioSciences (University of Alberta Institutional Animal Care and Use Committee, operating under the Canadian Council on Animal Care), Animal Use Protocol
6. Locket NA, Jørgensen JM. 1998 The eyes of at 4 g l\(^{-1}\) a lethal dose of fish anesthetic MS222 (tricaine methanesulfonate). Eyes were obtained from animals euthanized by immersion in the vessel (MV Alta) and brought to Bamfield Marine Sciences Centre to be housed unfed for 1–3 days in aerated, outdoor holding tanks receiving flow-through seawater at 12°C. Eyes were obtained from animals euthanized by immersion in a lethal dose of fish anesthetic MS222 (tricaine methanesulfonate) at 4 g l\(^{-1}\). Eye tissue was also harvested from fish obtained and used by Dr Greg Goss and laboratory members at the University of Alberta.

5. Borwein B, Hollenberg MJ. 1973 The photoreceptors of the hagfish. In: The biology of hagfishes. In: The biology of hagfishes. Jørgensen, JP Lomholt, RE Weber, H Malte, eds, 405–419. (doi:10.1007/BF00330929)

4. Collin SP, Hoskins RV, Partridge JC. 1997 Tubular eye lineages in teleosts: a comparative study of neurogenesis in the ciliary margin of the frog retina. J. Comp. Neurol. 442. (doi:10.1002/cne.903010308)

3. Strauss O. 2005 The retinal pigment epithelium in visual function. Physiol. Rev. 85, 845–881. (doi:10.1152/physrev.00021.2004)

2. Chow RL, Lang RA. 2001 Early eye development in vertebrates. Annu. Rev. Dev. Biol. 17, 255–296. (doi:10.1146/annurev.cellbio.17.1.255)

1. Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA. 2001 Specification of the vertebrate eye by a network of field transcription factors. Development 128, 5155–5167. (doi:10.1242/dev.00723)

(b) Cryosectioning, riboprobe production, in situ hybridization and immunohistochemistry

**In situ** hybridization and immunohistochemistry were based on described protocols detailed in the electronic supplementary material. Specificity of riboprobes was inferred from: (i) the observation that each riboprobe gave an independent labelling pattern (at the level of tissue distribution and subcellular localization); and (ii) that excluding the riboprobe produced no detectable labelling (electronic supplementary material, figure S6A). Specificity of antibodies was inferred in a similar fashion, and the absence of primary antibody produced no detectable labelling (electronic supplementary material, figure S6B).

(c) RNA-Seq transcript isolation and sequencing

Enucleated eyes were isolated from surrounding muscle in the head, extraneous tissue was trimmed away. Entire eyes (retina, vitreous, lens) were processed through a single homogenate and sent for further tissue processing and sequencing procedures at the Beijing Genomics Institute (BGI) at the Children’s Hospital of Philadelphia (CHOP) Genome Center. Further details concerning methods carried out at the BGI@CHOP Genome Center can be seen in the electronic supplementary material. Sequences of transcripts are reported in Supplemental file ‘eSupp_Transcript_sequence_ID’, and the RNA-Seq data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.79cn5sh7 [55].

*Ethics.* This study was conducted under the approval of the Animal Care and Use Committee: BioSciences (University of Alberta Institutional Animal Care and Use Committee, operating under the Canadian Council on Animal Care). Animal Use Protocol number: AUP00000077. All animals were collected under a permit issued by the Department of Fisheries and Oceans Canada (XR 59 2017), and under animal use protocol approved by the Bamfield Marine Sciences Centre (RS-17-14).

*Data accessibility.* Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.79cn5sh7 [55].

*Authors’ contributions.* E.M.D. carried out the laboratory work, data analysis, participated in the design of the study and drafted the manuscript; W.T.A. participated in data collection, conceived of the study, designed the study, coordinated the study and helped draft the manuscript. Both authors gave final approval for publication and agree to be held accountable for the work performed therein.

*Competing interests.* We declare we have no competing interests.

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References

1. Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA. 2001 Specification of the vertebrate eye by a network of field transcription factors. Development 128, 5155–5167. (doi:10.1242/dev.00723)

2. Chow RL, Lang RA. 2001 Early eye development in vertebrates. Annu. Rev. Dev. Biol. 17, 255–296. (doi:10.1146/annurev.cellbio.17.1.255)

3. Strauss O. 2005 The retinal pigment epithelium in visual function. Physiol. Rev. 85, 845–881. (doi:10.1152/physrev.00021.2004)

4. Collin SP, Hoskins RV, Partridge JC. 1997 Tubular eyes of deep-sea fishes: a comparative study of neurogenesis in the ciliary margin of the frog retina. J. Comp. Neurol. 442. (doi:10.1002/cne.903010308)

5. Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA. 2001 Specification of the vertebrate eye by a network of field transcription factors. Development 128, 5155–5167. (doi:10.1242/dev.00723)

6. Locket NA, Jørgensen JM. 1998 The eyes of hagfishes. In: The biology of hagfishes (eds JM Jørgensen, JP Lomholt, RE Weber, H Malte), pp. 541–556. Dordrecht, The Netherlands: Springer.

7. Lamb TD, Collin SP, Pugh Jr EN. 2007 Evolution of the vertebrate eye: opsins, photoreceptors, retina and eye cup. Nat. Rev. Neurosci. 8, 960–976. (doi:10.1038/nrn2283)

8. Fernholm B, Holmberg K. 1975 The eyes in three genera of hagfish (Eptatretus, Paramyxyne and Myxine): a case of degenerative evolution. Vision Res. 15, 253–259. (doi:10.1016/0042-6989(75)90215-1)

9. Holmberg K. 1970 The hagfish retina: fine structure of retinal cells in Myxine glutinosa, L., with special reference to receptor and epithelial cells. Zeitschrift für Zellforschung und Mikroskopische Anatomie 111, 519–538. (doi:10.1016/BF00309292)

10. Holmberg K. 1971 The hagfish retina: electron microscopic study comparing receptor and epithelial cells in the Pacific hagfish, Polistotrema stouti, with those in the Atlantic hagfish, Myxine glutinosa. Zeitschrift für Zellforschung und Mikroskopische Anatomie 121, 249–269. (doi:10.1016/BF00304676)

11. Wets R, Serbedzija GN, Fraser SE. 1989 Cell lineage analysis reveals multipotent precursors in the ciliary marginal zone of the frog retina. Dev. Biol. 136, 254–263. (doi:10.1016/0012-1606(89)90146-2)

12. Fischer AJ, Bosse JL, El-Hodiri HM. 2014 Reprint of: the ciliary marginal zone (CMZ) in development and regeneration of the vertebrate eye. Exp. Eye Res. 123, 115–120. (doi:10.1016/j.exer.2014.04.019)

13. Kubota R, Hikosaka O, Moshiri A, McGuire C, Reh TA. 2002 A comparative study of neurogenesis in the retinal ciliary marginal zone of homeothermic vertebrates. Dev. Biol. 134, 31–41. (doi:10.1016/S0016-3806(01)00287-3)

14. Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P. 2001 Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105, 43–55. (doi:10.1016/S0092-8674(01)00295-1)

15. Marquardt T, Gruss P. 2002 Generating neuronal diversity in the retina: one for nearly all. Trends Neurosci. 25, 32–38. (doi:10.1016/S0166-2236(00)00228-2)

16. Greferath U, Grünert U, Wäsle H. 1990 Rod bipolar cells in the mammalian retina show protein kinase C-like immunoreactivity. J. Comp. Neurol. 301, 433–442. (doi:10.1002/cne.903010308)

17. Sun L, Kawano-Yamashita E, Nagata T, Tsukamoto H, Furutani Y, Koyanagi M, Terakita A. 2014 Distribution of mammalian-like melanopsin in circostereae retinas exhibiting a different extent of...
visual functions. PLoS ONE 9, 1–8. (doi:10.1371/ journal.pone.0102029)

18. Laboisseiire AA et al. 2019 Molecular signatures of retinal ganglion cells revealed through single cell profiling. Sci. Rep. 9, 15778. (doi:10.1038/s41598-019-52215-4)

19. Soto I et al. 2008 Retinal ganglion cells downregulate gene expression and lose their axons within the optic nerve head in a mouse glaucoma model. J. Neurosci. 28, 546–561. (doi:10.1523/JNEUROSCI.3714-07.2008)

20. Kobayashi H. 1964 On the photo-perceptive function in the eye of the hagfish. Myxine gamarín. J. Natl Fish. Univ. Formely Journal of Shimonoseki Fisheries University 13, 67–83.

21. Femnholm B. 1974 Diurnal variations in the behaviour of the hagfish Eptatretus burgeri. Mar. Biol. 27, 351–356. (doi:10.1007/BF00394371)

22. Collin SP. 2010 Evolution and ecology of retinal photoreceptor development in early vertebrates. Brain Behav. Evol. 75, 174–185. (doi:10.1159/000341904)

23. Lamb TD, Patel H, Chuaí A, Notoli RC, Davies WL, Hart NS, Collin SP, Hunt DM. 2016 Evolution of vertebrate phototransduction: cascade activation. Mol. Biol. Evol. 33, 2064–2087. (doi:10.1093/molbev/msw095)

24. Eddy SR. 2011 Accelerated profile HMM searches. Nucleic Acids Res. 39. (doi:10.1093/nar/gkr520)

25. Blom AM, Rasmussen S, Mikkelsen J, Kaul A, Kryger J, Clausen-Broholm K. 2017 Structural and functional organization of the human genome. Mol. Biol. Evol. 34, 2657–2669. (doi:10.1093/molbev/msx066)

26. Steven DM. 1955 Experiments on the light sense of vertebrates. Biochemistry 2, 2229–2238. (doi:10.1021/bi026911y)

27. Poliakov E, Gubin AN, Stearn O, Li Y, Campos MM, Batten ML. 2011 Molecular phylogeny of early vertebrates: II. Visual cycle proteins are localized in whole brain including photoreceptor cells of a primitive chordate. Vision Res. 43, 3045–3053. (doi:10.1016/j.visionres.2009.07.012)

28. Dickinson DH, Graves DA. 1979 Fine structure of the lamprey photoreceptors and retinal pigment epithelium (Petromyzon marinus L.) Exp. Eye Res. 29, 45–60. (doi:10.1016/0014-4839(79)90165-9)

29. Tsuda M, Kusakabe T, Iwamoto H, Horie T, Nakashima Y, Nakagawa M, Okunou K. 2003 Origin of the vertebrate visual cycle: II. Visual cycle proteins are localized in whole brain including photoreceptor cells of a primitive chordate. Vision Res. 43, 3045–3053. (doi:10.1016/j.visionres.2009.07.012)

30. Fischer AJ, Reh TA. 2000 Identification of a proliferating marginal zone of retinal progenitors in postnatal chickens. Dev. Biol. 220, 197–210. (doi:10.1006/dbio.2000.9640)

31. Hollyfield JG. 1971 Differential growth of the neural crest and evolution of the head/trunk interface in early vertebrates. Proc. Natl Acad. Sci. USA 70, 1937–1941. (doi:10.1073/pnas.70.7.1937)

32. Yu S, Zhang W, Li L, Huang H, Ma F, Li Q. 2008 Photolytic analysis of 48 gene families revealing relationships between hagfishes, lampreys, and gnathostomes and the nature of the ancestral vertebrate. Proc. Natl Acad. Sci. USA 107, 19379–19383. (doi:10.1073/pnas.1013501107)

33. Ichikawa T, Kobayashi H, Nozaki M. 2000 Seasonal migration of the hagfish, Petromyzon marinus (L.). Exp. Eye Res. 70, 222–228. (doi:10.1016/S0014-4839(99)00052-8)

34. Hart NS, Collin SP, Hunt DM. 2016 Evolution of vertebrate phototransduction: cascade activation. Mol. Biol. Evol. 33, 2064–2087. (doi:10.1093/molbev/msw095)

35. Hollyfield JG. 1971 Differential growth of the neural crest and evolution of the head/trunk interface in early vertebrates. Proc. Natl Acad. Sci. USA 70, 1937–1941. (doi:10.1073/pnas.70.7.1937)

36. Julien S, Kociok N, Keppel F, Kopitz J, Kochanek S, Biesemeier A, Blitgen-Heinecke P, Heiduschka P, Schraermeyer U. 2007 Tympanis biosynthesis and trafficking in adult human retinal pigment epithelial cells. Zool. J. Linn. Soc. 144, 277–292. (doi:10.1111/j.1099-0065.2007.00147.x)

37. Ota KG, Fujimoto S, Oisi Y, Kuratani S. 2011 Morphological-molecular conflict in early vertebrate visual system evolution. J. Exp. Biol. 214, 414–426. (doi:10.1242/jeb.072223)

38. Takezaki N, Figueroa F, Zaleska-Rutczynska Z, Klein 37. Yu S, Zhang W, Li L, Huang H, Ma F, Li Q. 2008 Photolytic analysis of 48 gene families revealing relationships between hagfishes, lampreys, and gnathostomes and the nature of the ancestral vertebrate. Proc. Natl Acad. Sci. USA 107, 19379–19383. (doi:10.1073/pnas.1013501107)

39. Ota KG, Fujimoto S, Oisi Y, Kuratani S. 2011 Identification of vertebrate-like elements and their possible differentiation from sclerotomes in the lamprey. Nat. Commun. 2, 372–376. (doi:10.1038/ncomms1355)

40. Ota KG, Kuraku S, Kuratani S. 2007 Hagfish embryology with reference to the evolution of the neural crest. Nature 446, 672–675. (doi:10.1038/nature05633)

41. Delabar C, Gallut C, Barriel V, Janvier P, Gachelin G. 2002 Complete mitochondrial DNA of the hagfish, Eptatretus burgeri: the comparative analysis of mitochondrial DNA sequences strongly supports the cyclostome monophyly. Mol. Phylogenet. Evol. 22, 184–192. (doi:10.1006/mpev.2001.1045)

42. Takezaki N, Figueiroa F, Zaleska-Rutczynska Z, Klein L. 2003 Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by molecular-molecular conflict in early vertebrate visual system evolution. Proc. Natl Acad. Sci. USA 116, 2146–2151. (doi:10.1073/pnas.1817497116)

43. Collin SP, Knight MA, Davies WL, Potter IC, Hunt DM, Trezise AE. 2003 Ancient colour vision: multiple opsins genes in the ancestral vertebrates. Curr. Biol. 13, R864–R865.

44. Bowmaker JK. 2008 Evolution of vertebrate visual pigments. Vision Res. 48, 2022–2041. (doi:10.1016/j.visres.2008.03.025)

45. Okano T, Kojima D, Fukada Y, Shichida Y, Yoshizawa T. 1992 Primary structures of chicken cone visual pigments: vertebrate rhodopsins have evolved out of cone visual pigments. Proc. Natl Acad. Sci. USA 89, 5932–5936. (doi:10.1073/pnas.89.13.5912)

46. Gabbott SE, Donoghue PCJ, Sansom RS, Vintner I, Dolocan A, Purnell MA. 2016 Pigmented anatomy in Carboniferous cyclostomes and the evolution of the vertebrate eye. Proc. R. Soc. B 283, 20161151. (doi:10.1098/rspb.2016.1151)