A characterization of adult retinal neurogenesis in the Pacific hagfish (*Eptatretus stoutii*) to elucidate the evolutionary origins of the vertebrate retina

by

Sarah N. Bradshaw

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Department of Biological Sciences

University of Alberta

Abstract

The vertebrate retina is a vital sensory structure that has a murky evolutionary origin. Living vertebrates possess a strikingly complex retina and eye with highly conserved development and physiology. In contrast, the photoreceptive organs of the closest non-vertebrate relatives are comparatively simple clusters of pigment cells and photoreceptors. No extant vertebrates possess characters that are suggestive of a 'transitional state' that could help guide interpretations of how the eye or retina may have initially formed. However, the early-branching jawless vertebrates (Cyclostomata - hagfishes and lampreys) are in an ideal phylogenetic position to shed light upon the origin of the vertebrate eye.

The eyes of cyclostomes are vastly understudied compared to the eyes of jawed vertebrates (Gnathostomata). The available literature has revealed that both hagfish and lamprey eyes contain unique features not present in other vertebrates. Hagfish eyes are particularly notable as the eye (and retina) is small and rudimentary in form. The hagfish eye lacks pigment and a lens. The retinal layers are also more disorganized than in other vertebrates. These observations have led to the interpretation that the hagfish eye could represent the ancestral vertebrate eye condition. Recent morphological and molecular studies, including data in this thesis, now suggest that many of these features in the hagfish eye are due to secondary loss rather than retention of ancestral traits. However, further investigation of the cyclostome retina will be crucial for inferring the state of the ancestral vertebrate (proto-vertebrate) eye.

Although several studies have investigated the morphology of cyclostome eyes, data on development of the eye and retina are extremely limited. This is particularly true for hagfish, whose embryos are notoriously difficult to acquire. Neurodevelopmental data could provide new insights on eye evolution where morphological data from extant organisms or fossil data are

lacking. Here, I provide genomic and RNA sequencing data that suggest many genes critical for gnathostome retinal development are also expressed in the hagfish eye. In addition, recent work suggests adult Pacific hagfish (*Eptatretus stoutii*) have continued retinal growth past embryonic development. Therefore, in this thesis I have begun to characterize the mechanisms driving retinal development in the Pacific hagfish (*E. stoutii*) by taking advantage of a putative proliferative zone in the adult retina.

To achieve this, I applied a brief pulse of EdU to several hagfish to label proliferating cells, if any, in the retina. I also utilized bioinformatics and *in situ* hybridization to assess if homologs of gnathostome retinal genes also drive retinogenesis in this jawless vertebrate. I observed EdU positive cells within the retinal periphery of the hagfish (a region reminiscent of the ciliary marginal zone of gnathostomes) and within the central retina. I also found evidence that hagfish possess homologs of several key genes required for retinal neurogenesis in other vertebrates and that these genes are expressed within the hagfish eye. Finally, through *in situ* hybridization I demonstrated that two of these genes, *OtxA* and *Rx* (retinal homeobox), are expressed in the hagfish retina (including at the proliferative retinal periphery). This work has revealed candidate genes and mechanisms that may be involved in hagfish retinogenesis. This establishes a starting point for future studies to dissect the pathways of retinal neurogenesis in jawless vertebrates. Further efforts comparing retinal development between cyclostomes and gnathostomes are warranted, as this work has also uncovered evidence for deeply conserved mechanisms for retinogenesis within the vertebrate lineage.

Preface

This thesis is an original work by Sarah N. Bradshaw.

This study was conducted under the approval of the Biosciences Animal Care and Use Committee (Animal use protocol number: AUP00000077) which adheres to the Canadian Council for Animal Care guidelines. Animals were collected and held according to a Department of Fisheries and Oceans Canada collection permit (XR 225 2021) and the animal use protocol approved by the Bamfield Marine Sciences Center (BMSC) (RS-21-06).

The majority of thesis chapter 1 was published as part of an invited review article submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Cell and Developmental Biology, Bradshaw SN and Allison WT (2022), "Hagfish to Illuminate the Developmental and Evolutionary Origins of the Vertebrate Retina". This publication was written by SNB and edited by WTA.

Emily Dong generated the *Eptatretus stoutii* RNA sequencing dataset referenced in Chapter 2 and the *E. stoutii* RNA used to prepare the *in-situ* hybridization riboprobes in Chapter 2.

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List of Abbreviations

BCIP - 5-Bromo-4-chloro-3-indolyl phosphate

bHLH - basic helix-loop-helix proteins

BLAST - Basic Local Alignment Search Tool

BMSC - Bamfield Marine Sciences Center

BSA - bovine serum albumin

cDNA - complementary DNA

CMZ - ciliary marginal zone

Crx - cone-rod homeobox gene

CRALBP - cellular retinaldehyde-binding protein

DAPI - 4',6-diamidino-2-phenylindole, dihydrochloride

DIG - digoxigenin

dNTP - deoxynucleoside triphosphates

E. burgeri - *Eptatretus burgeri*

EdU - 5-ethynyl-2'-deoxyuridine

E. stoutii - *Eptatretus stoutii*

EtOH - ethanol

FGF - fibroblast growth factor

FISH - fluorescent *in situ* hybridization

FPKM - fragments per kilobase of transcript per million mapped reads

GCL - ganglion cell layer

IDT - Integrated DNA Technologies

INL - inner nuclear layer

LSM - laser scanning microscopy

MS-222 - tricaine methanesulfonate

NBT- nitro blue tetrazolium

NCBI - National Center for Biotechnology Information

OCT - optimal cutting temperature compound

ONL - outer nuclear layer

Otx - orthodenticle homeobox gene

Pax6 - paired box gene 6

PBS-T - phosphate buffered saline + 0.1% Tween

pH3 - phosphohistone 3

PCNA - proliferating cell nuclear antigen

PCR - polymerase chain reaction

PFA - paraformaldehyde

P. marinus - *Petromyzon marinus*

POD - horseradish peroxidase

RGC - retinal ganglion cell

RNase - ribonucleases

RNAseq - RNA sequencing

RnPE - retinal (non)-pigmented epithelium

RPE - retinal pigmented epithelium

Rx - retinal homeobox gene

Shh - Sonic hedgehog

Six3 - sine oculis homeobox transcription factor gene 3

Six6 - sine oculis homeobox transcription factor gene 6

SNCG - synuclein gamma gene

SSC - saline-sodium citrate buffer

tBLASTn - translated nucleotide BLAST search

TEA - triethanolamine

Chapter 1

Hagfish to Illuminate the Developmental and Evolutionary Origins of the Vertebrate Retina.

Part of this chapter contains work previously published in: Bradshaw SN and Allison WT (2022). Hagfish to Illuminate the Developmental and Evolutionary Origins of the Vertebrate Retina. Front. Cell Dev. Biol. 10:822358. doi: 10.3389/fcell.2022.822358. *Written by SNB. Edited by WTA.*

1.1 Abstract

The vertebrate eye is a vital sensory organ that has long fascinated scientists, but the details of how this organ evolved are still unclear. The vertebrate eye is distinct from the simple photoreceptive organs of other non-vertebrate chordates and there are no clear transitional forms of the eye in the fossil record. To investigate the evolution of the eye we can examine the eyes of a group of early-branching extant vertebrates, the hagfish and lamprey. These jawless vertebrates are in an ideal phylogenetic position to study the origin of the vertebrate eye, but data on eye and retina development in these organisms is limited. New genomic and gene expression data from hagfish and lampreys suggest they have many of the same genes for eye development and retinal neurogenesis as jawed vertebrates. However, functional work to determine if these genes operate in retinogenesis similarly to other vertebrates is missing. In addition, hagfish express a marker of proliferative retinal cells (*Pax6*) near the margin of the retina and adult retinal growth is apparent in some species. Finding evidence of

eye growth late into hagfish ontogeny is unexpected given the degenerate hagfish eye phenotype. Further studies dissecting retinal neurogenesis in jawless vertebrates would allow for comparison of the mechanisms of retinal development between cyclostome and gnathostome eyes and provide insight into the evolutionary origins of the vertebrate eye.

1.2 Introduction

1.2.1 The vertebrate eye is a vital sensory structure with mysterious origins

To survive in adverse environments organisms must be able to perceive and respond to their surroundings. One of the most complex and remarkable sensory systems utilized in the animal kingdom is vision. Through specialized cells (photoreceptors) animals can make use of light information to sense their environment (Arendt, 2003). Across Metazoa, photoreception abilities range from simple light detection to the ability to form images. Organization of photoreceptors into a light-detecting organ (an eye) allows for sophisticated processing of light signals. Representatives of Cnidaria, Mollusca, Annelida, Onychophora, Arthropoda and Chordata are examples of organisms that have developed complex eyes, often associated with image formation (Zuker, 1994). Amazingly, despite these groups being phylogenetically distant, their eye structures appear to have independently evolved to perform similar functions (often through similar mechanisms) reinforcing the importance of visual information across organisms (Fernald, 2000; Nilsson, 2013). With rare exception, vertebrates possess a complex camera-style eye that is remarkably conserved in form and function throughout the group. The retina of the vertebrate eye is specialized to form detailed images, detect motion, enhance contrast and/or perceive color (Yokoyama, 2000; Stenkamp, 2015; Morshedian and Fain, 2017). This allows vertebrates to find food and mates, avoid predation and navigate their surroundings across a variety of habitats.

Despite the eye and retina being of primary importance to vertebrate ecology, a comprehensive appreciation of their evolutionary origins is lacking. Contributing to the struggle to understand vertebrate eye evolution is the complexity of the organ itself. To receive light signals the retina contains highly specialized photoreceptor cells for light detection (these cells require a mechanism for light transduction and supporting cells to maintain their function) (Lamb et al., 2007; Fain et al., 2010). The light signals must then be transmitted to (and subsequently processed within) the brain for an animal to perceive the environment within their visual field. The communication and processing of this information requires an extremely complex neural network. Vertebrates employ multiple neuronal cell types to transmit and initiate processing of light signal information within the visual system (Stenkamp, 2015). In addition, the structure of the camera-style eye is also optimized to direct light onto the retina (to increase visual acuity). Structures such as the pupil and lens focus incoming light onto the retinal photoreceptors, pigment provides directionality for light signals and extraocular muscles allow vertebrates to shift their eye position. Many scientists, including and prior to Darwin, have pondered how these individual components may have arisen and come together through evolution to produce such a multiplex/sophisticated sensory organ (Fernald, 2004; Gehring and Seimiya, 2010).

Another factor confounding a complete understanding of eye evolution is how abruptly the eye appears within the vertebrate phylogeny and the lack of transitional states (Lamb et al., 2007) (Figure 1-1A). Almost all extant vertebrates possess a complete camera-style eye with a clearly laminated retina (Figure 1-1B). In contrast, the closest living relatives of the vertebrates, the cephalochordates (amphioxus) and urochordates (tunicates) do not have visual structures resembling a camera-style eye (Figures 1-1C, D). Instead, these groups possess comparatively

simple clusters of photoreceptive cells associated with pigment cells (Vopalensky et al., 2012; Esposito et al., 2015). This leaves a gap around the chordate/vertebrate transition where eyes may have arisen (Figure 1-1A). Assuming the last common ancestor of vertebrates and other chordates had simple unpaired photoreceptive organs similar to extant non-vertebrate chordates, emergence of the vertebrate eye would have required numerous evolutionary innovations. This includes the development of structures to receive photic stimuli with increased resolution (i.e., a lens), directionality (i.e., the retinal pigmented epithelium) and image formation (i.e., complex photoreceptors) abilities (Nilsson, 2009). Without obvious intermediate stages, we can only hypothesize how these innovations may have taken place in the ancestral vertebrate. Some groups have examined fossil evidence from extinct species to try to find a transitional or early form of the vertebrate eye. However, many types of delicate tissue (such as the eye) do not fossilize well, making fossil data difficult to interpret (Clements et al., 2016; Gabbott et al., 2016). A promising avenue for exploring the origin of vertebrate eyes is to study an obscure but evolutionarily important group, the jawless vertebrates (Agnatha).

The jawless vertebrates consist of two extant groups of anguilliform (elongate, eel-like in body form and swimming style) fishes, the lampreys and hagfish. These organisms are unique among extant vertebrates for lacking jaws and paired fins and possess many distinct features which are considered “primitive” or ancestral compared to jawed vertebrates; whether these features truly represent the ancestral condition for vertebrates or are derived traits that arose in the cyclostome lineage is still a matter of debate (Kuratani and Ota, 2008b; Shimeld and Donoghue, 2012). Lampreys are fishes that begin life as sediment dwelling filter feeders (Osório and Rétaux, 2008). Depending on the species, after metamorphosis lamprey can either become parasitic, feeding on the blood and tissue of aquatic organisms, or non-parasitic (generally do not feed as

adults). Lampreys occur in both fresh and saltwater environments. In contrast, hagfish are marine scavengers that live in the ocean depths and feed mostly on dead or dying animals (although some sources suggest they may be more predatory than traditionally thought) (Martini, 1998). Various molecular and morphological studies agree that these groups comprise their own monophyletic clade, Cyclostomata, forming a sister group to the jawed vertebrates (Gnathostomata) (Mallatt and Sullivan, 1998; Kuraku et al., 1999; Heimberg et al., 2010; Ota et al., 2011; Miyashita et al., 2019). The cyclostomes diverged from other vertebrates around 600 mya, placing the group at a unique phylogenetic position that may be useful for unravelling the origins of the vertebrate eye and retina, especially when contrasted against the gnathostome condition (Figure 1-1A) (Blair and Hedges, 2005).

Despite the importance of these species to appreciating the evolutionary origins of the vertebrate eye, development of the eye in cyclostomes has been poorly studied. The morphology of lamprey and hagfish eyes are important points of comparison for the eyes of other vertebrates. Adult lampreys have well-developed camera-style eyes that are similar to those found in jawed vertebrates (gnathostomes) (Lamb et al., 2007). There are some nuanced differences in retinal organization and retinal cell morphology, but many elements of the visual system of lampreys and gnathostomes appear conserved (Fain, 2020). In contrast, hagfish eyes are strikingly rudimentary. Their small eyes are buried under a layer of skin or muscle depending on the genera (Fernholm and Holmberg, 1975). Hagfish eyes also lack a lens and pigment (features found in the eyes of almost all other living vertebrates). Recently it has been shown that in at least some hagfish the retina contains all the cellular layers seen in other vertebrates (Dong and Allison, 2021), but this had been obscured because the retina is poorly laminated (compared to the four clearly defined retinal layers of gnathostomes) (Lamb, 2013; Dong and Allison, 2021). In

addition, the hagfish eye was shown to grow throughout ontogeny with a proliferative region at the margin of the retina, similar to the ciliary marginal zone of gnathostomes (discussed in **Section 1.4**) (Dong and Allison, 2021). These findings have raised questions about whether the hagfish and lamprey could represent two distinct phases of vertebrate eye evolution, with hagfish showing a more ancestral vertebrate eye form and the lamprey possessing a more derived form. However, others have suggested that hagfish may have once possessed more complex eyes that regressed due to living as scavengers in dim-light environments (Fernholm and Holmberg, 1975; Dong and Allison, 2021). Under this theory, the hagfish eye could have become reduced to its current state (due to lack of use) after the hagfish began living in the deep ocean, similarly to the loss of eyes in other vertebrates living in dim-light environments (i.e., troglobionts such as the cavefish (*Astyanax mexicanus*)) (Sifuentes-Romero et al., 2020). Another related model suggests that the hagfish eye condition is rudimentary because it is neotenic/paedomorphic (retains larval features). This idea is based on the observation that in lampreys, adult animals have complex eyes whereas lamprey larvae possess simpler eye structures (with the larval lamprey eye spot being compared to the hagfish eye) (Lamb et al., 2007; Suzuki and Grillner, 2018). Under the paedomorphosis model, hagfish would have undergone a heterochronic shift resulting in loss of the transition from a larval/juvenile to adult eye condition. Based on the available evidence it is difficult to rule out any of these possibilities.

To gain insight into the evolution of the vertebrate eye, we can take advantage of the available morphological, genetic and developmental information on eye formation in the cyclostomes. However, this type of information, especially that of retinal development, is lacking for both lampreys and hagfish. Both groups are non-model organisms and present unique challenges for study. Several studies have characterized the morphology and cell types within the lamprey eye

(including the transition from larval to adult eye) but few studies have examined ocular gene expression during eye development (Meyer-Rochow and Stewart, 1996; Suzuki and Grillner, 2018; Fain, 2020). Further characterization of retinal and eye developmental pathways within lampreys could be used to strengthen (or refute) the comparisons made between lamprey and gnathostome eyes. There is even more unknown about hagfish eyes. In addition to many species being deep-water, marine animals, historically hagfish embryos have been difficult to attain making development difficult to study in this group (Gorbman, 1997). However, several groups have begun to successfully breed hagfish in a laboratory setting, opening future opportunities to study the embryonic hagfish eye (Holland, 2007; Ota and Kuratani, 2008). Within the current literature, studies have characterized the morphology of adult hagfish eyes and photoreceptors (Holmberg, 1970; Holmberg, 1971; Fernholm and Holmberg, 1975). More recently, hagfish retinal cell types have been studied via gene expression markers (Dong and Allison, 2021). Yet, information on how the hagfish eye develops is still highly lacking. To compare the cyclostome eye condition to the gnathostome condition, more data on cyclostome eye and retina development are necessary.

1.2.2 Organization of the retina is complex and highly coordinated

The vertebrate eye is an organ that is highly conserved (in structure, function, physiology and development) throughout the vertebrates from fishes to mammals (Lamb, 2013). The eyes of cyclostomes and gnathostomes share the same basic organization and neural wiring, although the hagfish eye is lacking some elements seen in the eyes of other vertebrates (i.e., the lens). In the typical vertebrate camera-style eye, the cornea and lens focus light onto the retina at the back of the eye where light detection (and the initial processing/transmission of light information) occurs (Lamb et al., 2007; Koenig and Gross, 2020). The retina consists of four distinct cellular layers

in jawed vertebrates (Figure 1-2A). The outer nuclear layer (ONL) contains photoreceptors (rod cells for dim-light detection and cone cells for bright light photoreception) (Stenkamp, 2015). The inner nuclear layer (INL) contains the cell bodies of various interneurons (bipolar cells, amacrine cells and horizontal cells) involved in the initial processing of light information and transmitting the information from photoreceptors to the ganglion cell layer. The inner nuclear layer also contains Müller glia (cells that provide a supporting role to retinal neurons and a source of stem cells after retinal damage) (Stenkamp, 2015). The retinal ganglion cells (RGC's) of the ganglion cell layer (GCL) transmit light signals to the brain (the axons of the RGC's form the optic nerve). Some RGCs contain melanopsin and are intrinsically photosensitive (ipRGC's) (Ecker et al., 2010; Schmidt et al., 2011). The fourth cellular layer is the retinal pigmented epithelium (RPE) which is adjacent to (and interdigitated with) the photoreceptors. The cells of the RPE provide a supporting role to the photoreceptors by absorbing stray light, phagocytosing dying photoreceptor segments and aiding in the retinoid cycle (Bharti et al., 2006). The retina also contains two synaptic layers with the outer plexiform layer consisting of the synapses between photoreceptors, bipolar cells, and horizontal cells and the inner plexiform layer forming from synapses between the bipolar cells, amacrine cells and retinal ganglion cells (Centanin and Wittbrodt, 2014; Stenkamp, 2015). This is the almost universal retinal organization found in jawed vertebrates, but the retinal layers of cyclostomes differ (see **Section 1.3**).

1.2.3 The eye is formed by a network of developmental pathways

To gain a clearer understanding of the origins of the vertebrate retina, we can study the development of the retina in the jawless vertebrates. Elements of retinogenesis pathways that are missing in the cyclostomes (compared to gnathostomes) may have arisen after the jawless vertebrates diverged from the jawed vertebrates (or were lost from cyclostomes after the lineages

split). Alternatively, if elements of the retinogenesis pathways are similar in both cyclostomes and gnathostomes, this would support that the retinal organization seen in gnathostomes arose before the split between the two groups. Despite their critical phylogenetic position, eye and retina development in the cyclostomes has not been heavily studied. There are several papers that have examined eye/retinal development within the lamprey and very few studies on hagfish. Comparatively, there have been numerous studies investigating retinal development within the gnathostomes (summarized below) which have allowed us to begin to appreciate the impressive complexity of vertebrate retinal development. Increasing our understanding of cyclostome retinogenesis would allow us to develop a more comprehensive evolutionary perspective on how this complex neural structure (and the developmental mechanisms that produce it) arose.

In gnathostomes, the retina arises as an extension of the forebrain (diencephalon). During neurulation the anterior portion of the neural tube is specified as the presumptive eye field via the expression of several transcription factors (*Pax6*, *Otx2*, *Six3*, *ET*) (Zuber et al., 2003). Hedgehog signalling from the ventral midline specifies *Pax6* to be expressed laterally, allowing for the development of two separate eyes (Yang, 2004). Once the eye field forms, the tissue that will develop as the eye forms from two outpocketings of the ventral forebrain known as the optic vesicles (Cardozo et al., 2019). As the vesicles grow outwards and contact the overlying epidermis, signals from this tissue and the surrounding mesenchyme induce the vesicles to invaginate and form the optic cup (Steinfeld et al., 2013). The optic cup is a bilayered structure with the inner layer becoming the neural retina and the outer layer becoming the non-neural retinal pigmented epithelium (RPE). The patterning of the optic cup into distinct territories (i.e., eye stalk, RPE, neural retina) is mediated by extrinsic signals such as hedgehog, Wnt and FGF's (fibroblast growth factor) (Yang, 2004; Steinfeld et al., 2013; Cardozo et al., 2019).

The early neural retina consists of a homogenous population of undifferentiated progenitor cells (Centanin and Wittbrodt, 2014). To form into a mature retina with the seven retinal cell types organized into distinct cellular layers, these cells must proliferate and differentiate in a manner that is tightly regulated spatially and temporally. External signals such as FGFs and Shh (sonic hedgehog) help to control whether cells remain in a multipotent proliferative state or begin to differentiate into a particular fate (Yang, 2004). FGFs (FGF3 and FGF8) from the optic stalk region initiate differentiation of RGC's in the retina by activating expression of the transcription factor *Atoh7* (Martinez-Morales et al., 2005). Hedgehog signalling promotes the expansion of cellular differentiation from the center to the periphery of the retina (neurogenic wave) (Neumann and Nuesslein-Volhard, 2000). Newly differentiated retinal ganglion cells begin to express hedgehog as well, propagating hedgehog signaling within the developing eye (Wang et al., 2005). Notch signaling is critical for maintaining proliferative progenitor cells during retinogenesis (Ahmad et al., 1995; Jadhav et al., 2006; Riesenberg and Brown, 2016). As retinal cells mature, Notch signalling is downregulated, and the effect of intrinsic transcription factor programs (see below) allows the cells to take on new fates (Perron and Harris, 2000). The activity of Gdf6A and retinoic acid also contributes to a proper balance of proliferating and committed cells within the early retina, ensuring the developing retina generates the correct number of cells (Valdivia et al., 2016). Transcription factor networks establish the differentiation pathways that retinal cells will follow to take on their final fates. Retinal ganglion cells are the first cell type to form in the retina by activation of *Atoh7* (Martinez-Morales et al., 2005). *Atoh7* subsequently promotes the expression of additional transcription factors, such as *Pou4f2* and *Isl1*, to specify the retinal ganglion cell fate (Mu et al., 2005). Activation of other cell specification programs is initiated after RGCs begin to form. For most retinal cell types, a major

(or several major) transcription factors initiate the pathway for a particular fate and subsequently activate downstream transcription factor networks to complete specification/differentiation. For example, the production of photoreceptors is initiated by a network of transcription factors including the homeobox genes *rx* (retinal homeobox gene) and *crx* (cone-rod homeobox gene) and the bHLH genes *NeuroD* and *Maf*/*Nrl* (specifically for rod cells) (Chen et al., 1997; Yan and Wang, 1998; Mears et al., 2001; Nelson et al., 2009b; Oel et al., 2020). *Chx10* drives bipolar cell specification (activates *Mash1* and *Math3*) (Hatakeyama et al., 2001). *Ptfla*, *Prox1* and *Foxn4* promote horizontal cell fate, whereas a combination of *Ptfla*, *Foxn4*, *Math3* and *NeuroD* expression produces amacrine cells (Inoue et al., 2002; Fujitani et al., 2006; Ohsawa and Kageyama, 2008). Finally, Müller glia fate is dependent upon the expression of *Rax* (*Rx*), *Hes1*, *Hes5*, *Hesr2* and *Lhx2* (Furukawa et al., 2000; Satow et al., 2001; Melo et al., 2016). These seven cell types initially appear during embryonic retinal development, but in several gnathostome groups (and possibly cyclostomes—**see Section 1.4.2**) production of new retinal cells continues throughout ontogeny at the periphery of the retina (the ciliary marginal zone).

1.3 Cyclostome eyes provide important perspective for understanding vertebrate eye evolution

1.3.1 Hagfish exhibit a rudimentary eye and retinal phenotype that is unique among vertebrates

Compared to gnathostome eyes, the hagfish eye must be considered very rudimentary. Their diminutive eyes are covered by a layer of semi-transparent skin (Genus *Eptatretus*) or a layer of muscle (Genus *Myxine*) (Fernholm and Holmberg, 1975). Most studies have concluded the hagfish eye does not contain a lens, although there is a report of a lens from one species, *Myxine*

garmani (Kobayashi, 1964); this report has not subsequently been supported by other studies. The hagfish retina is more disorganized (lamination between the layers is less clear) than in gnathostomes, but it does appear to contain all the constituents of cellular layers seen in the retinae of other vertebrates (Dong and Allison, 2021) (Figure 1-2D). Hagfish of the genus *Eptatretus* have photoreceptors, but they are difficult to distinguish as rods or cones morphologically (Fernholm and Holmberg, 1975). Hagfish species from the genus *Myxine* have photoreceptors that are even more different from those seen in gnathostomes (the cells have a whorled morphology) (Holmberg, 1970; Fernholm and Holmberg, 1975; Lamb, 2013). The photoreceptor cells of *Eptatretus stoutii* express *rhodopsin*, supporting their similarity to gnathostome photoreceptors (Dong and Allison, 2021). Interestingly, physiological studies have demonstrated that hagfish eyes do respond to light, but if hagfish eyes are removed a response to light signals still occurs (Kobayashi, 1964). This indicates hagfish may also utilize extra-ocular photoreceptors (i.e., photoreceptors in the skin) for light detection (Kobayashi, 1964; Patzner, 1978). *In situ* hybridization data suggests the hagfish retina contains bipolar, horizontal and amacrine cells (Dong and Allison, 2021). *PKC-α* (a marker of rod bipolar cells) and *CALBINDIN* (a marker for horizontal cells) is expressed within the interneuron layer of the hagfish retina. The hagfish retina also has expression of *Pax6* (a marker for retinal ganglion cells and amacrine cells) and *melanopsin* (a marker of intrinsically photosensitive retinal ganglion cells) (Dong and Allison, 2021). In contrast, Müller glia have not been identified (as of yet) in the hagfish retina. The RPE layer of hagfish (retinal non-pigmented epithelium or RnPE for hagfish from here on) is distinct from that of other vertebrates as it does not contain any pigment (pigment allows for the detection of light direction and pigment cells are usually associated with photoreceptors throughout Animalia) (Bharti et al., 2006). The RnPE might participate in other traditional

functions of the RPE such as removal of cellular debris (evidence of phagocytosis of an outer segment) and retinoid cycling (the RnPE expresses genes for several enzymes that drive retinoid cycling) (Dong and Allison, 2021). The impact of having a non-pigmented retinal epithelium on hagfish vision needs to be investigated further. Whether the unique characteristics of the hagfish eye represent an ancestral state of vertebrate eyes or is due to loss has been debated, and possible evolutionary scenarios will be outlined in **Section 1.5**. Despite its rudimentary characters, the hagfish retina appears to share more similarities with the gnathostome eye than traditionally thought, but more work needs to be done to characterize the cell types within the hagfish retina and to determine if they develop using the same mechanisms as in other vertebrates. Comparing the retinogenesis pathways in hagfish, lampreys and gnathostomes may elucidate how the vertebrate retina evolved.

Very little is known about retinal neurogenesis in hagfish, partially due to the difficulty of acquiring hagfish embryos. Examining genomic data for *Eptatretus burgeri* on Ensembl, hagfish appear to have homologs for critical eye transcription factors such as *Pax6*, *Otx2*, *Six3/6* (Table 1-1) and retinal neurogenesis genes such as *Ascl1*, *NeuroD* and *Neurogenin* (Table 1-2). They also appear to have homologs of lamprey *OtxB* (*OtxB* is supported as homologous to gnathostome *Crx* by Yamamoto et al., (2020)) and *rx*/*rax* (Kon and Furukawa, 2020). This suggests that hagfish may have some of the basic genetic machinery utilized by other vertebrates for eye/photoreceptor development. Without data on where these genes are being expressed and how they interact with other potential components of the eye/retina developmental pathways, it is difficult to firmly conclude whether retinogenesis in hagfish proceeds similarly to retinal development in gnathostomes. These types of studies would ideally be performed on embryonic hagfish (a group in Japan has had success acquiring hagfish embryos, creating exciting

opportunities for future research (Ota and Kuratani, (2008))). Another way to study hagfish retinal development in the absence of embryos would be to focus on the ciliary margin (a source of post-embryonic retinogenesis in most other vertebrates, especially teleost fishes and amphibians—see **Section 1.4**). Several pieces of evidence suggest the hagfish retina contains a proliferative ciliary marginal zone (CMZ). Photoreceptor cells located near the ciliary margin have an immature morphology (missing outer segments) whereas cells located closer to the center of the retina appear fully differentiated (Dong and Allison, 2021). Additionally, *Pax6* (a gene known to be expressed in the CMZ of other vertebrates) is expressed in the peripheral hagfish retina. This data, along with the fact that hagfish eyes grow larger over ontogeny, suggests the hagfish retina may still be proliferative throughout ontogeny despite the reduced/degenerate appearance of the eyes and retina (Dong and Allison, 2021). Further characterization of hagfish retinal development would be very insightful for studying the conservation of the retinogenesis pathway in vertebrates. Elements of the retinogenesis pathway missing in cyclostomes could point to elements that did not arise until later (after the jawed-jawless vertebrate split) in the vertebrate lineage.

1.3.2 The structure of the lamprey eye shares many similarities with the gnathostome eye

The eyes of adult lampreys share many morphological and physiological similarities with gnathostome eyes. In lampreys, the adult retina forms the same four cellular layers seen in the gnathostome retina (Figure 1-2B). All six retinal neuron types characterized in gnathostomes have also been identified in lamprey retinae, as well as Müller glia (Villar-Cheda et al., 2008; Suzuki and Grillner, 2018; Govardovskii et al., 2020). Adult lampreys have bipolar, amacrine and horizontal cells, based on immunocytochemical data (Villar-Cerviño et al., 2006; Abalo et al., 2008). All lamprey species studied to date also have at least two distinct morphological

classes of photoreceptors, “long” and “short” (some species have multiple subtypes of long photoreceptors) (Dickson and Graves, 1979). Morphologically both long and short photoreceptors appear cone-like (based on features used to classify gnathostome rods and cones) (Fain, 2020). However, the short photoreceptors are physiologically similar to gnathostome rod cells and are sensitive to single photons of light (Morshedian and Fain, 2015). The long photoreceptors behave like gnathostome cone cells and function best at higher intensities of light. The lamprey retina contains a layer of retinal ganglion cells, but these cells are organized differently than in jawed vertebrates. In gnathostomes the axons of RGC’s travel next to the inner limiting membrane (adjacent to the GCL) whereas in lampreys the RGC axons travel between the IPL and the INL (Fain, 2020). The cell bodies of the retinal ganglion cells lie either in the inner nuclear layer or the inner plexiform layer (the ganglion cell bodies do not lie in a distinct layer). It is currently unknown why the wiring of RGC’s is different in lampreys and how this could affect visual function. The RPE layer appears to be functional in adult lampreys and contributes to debris removal (via phagocytosis) and retinoid cycling as in gnathostomes (Meyer-Rochow and Stewart, 1996).

One important factor to consider when discussing the lamprey retina is that the lamprey eye undergoes dramatic changes throughout ontogeny. In early lamprey larvae, the eye is buried under a layer of skin and only a small central region of the retina differentiates: the central retina produces one type of photoreceptor, bipolar cells and retinal ganglion cells (Dickson and Collard, 1979; Suzuki and Grillner, 2018) (Figure 1-2C). As the lamprey matures, more of the retina undergoes differentiation. In late larvae an additional region peripheral to the central retina differentiates (producing retinal ganglion cells) (Cornide-Petronio et al., 2015). Finally, during metamorphosis retinal differentiation is completed across the most peripheral parts of the retina

and amacrine cells and horizontal cells appear (Rubinson and Cain, 1989; Meyer-Rochow and Stewart, 1996; Abalo et al., 2008). The RPE does not appear to be functional in the larval lamprey (Meyer-Rochow and Stewart, 1996). Outside of the retina, other features of the eye also change throughout lamprey metamorphosis (i.e., the lens completes development during metamorphosis and is only fully functional in adult lampreys) (Meyer-Rochow and Stewart, 1996).

In cyclostomes the mechanisms for retinogenesis are still mostly unknown. In lampreys the eye undergoes a dramatic transformation during metamorphosis. Prior to metamorphosis the lamprey retina contains two distinct regions: a smaller central retina (adjacent to the optic nerve) with cells that are differentiated, and an expansive undifferentiated peripheral region (remains proliferative) (Villar-Cheda et al., 2008). During metamorphosis the central differentiated region expands at the expense of the peripheral region and additional retinal cell types (i.e., amacrine and horizontal cells) appear (Rubinson and Cain, 1989; Villar-Cerviño et al., 2006; Abalo et al., 2008). Interestingly, in adult lampreys the adult eye does not appear to have a proliferative peripheral zone (Villar-Cheda et al., 2008). Compared to gnathostomes, there have been relatively few studies to investigate gene expression in the developing lamprey eye. For example, the signals that initiate retina formation in larval lampreys and the switch from the larval eye state to the adult eye state are still unknown (do FGFs, Wnt and hedgehog signaling drive retinal proliferation and differentiation in lampreys as they do in gnathostomes?). Expression of FGF receptors have been identified in the eyes of early lamprey larvae but further work is required to determine what (if any) role these receptors play in eye development (Guérin et al., 2009).

There have been a few studies exploring the role of transcription factors in lamprey eye development. Three *Pax6* paralogs have been identified in the lamprey genome and are

expressed in the eye (Ravi et al., 2019) (Table 1-1). Yamamoto et al., (2020) identified *OtxA* (homolog to gnathostome *Otx2*—critical for photoreceptor and bipolar cell development), *OtxB* (homolog to gnathostome *Crx*—specifies photoreceptors) and *Chx10* (expressed in gnathostome bipolar cells) expression in the adult lamprey retina. Compared to mice, the lamprey *Crx* and *Chx10* homologs appear to have similar expression profiles to that of gnathostomes (expressed in the photoreceptors of the ONL and interneurons of the INL respectively). However, the majority of *OtxA* expression occurred in lamprey photoreceptors whereas gnathostomes also have strong *Otx2* expression in bipolar neurons. The authors suggest *Otx2* function may have shifted between agnathans and gnathostomes, with *Otx2* gaining a role in bipolar cell specification later in gnathostome eye evolution. Investigation of other retinal development genes could allow us to dissect which elements of the vertebrate retinal system may be ancestral for all vertebrates and which arose later in specific lineages. Examining genomic data available in Ensembl and NCBI (National Center for Biotechnology Information), the lamprey genome possesses homologs for other homeobox genes critical for (gnathostome) eye development such as *Six3/6* and *Rx/Rax* (Table 1-1). Lampreys also have genes necessary for retinal neurogenesis in other vertebrates such as neurodifferentiation factor (*NeuroD1*), *neurogenin*, *Ascl1* and *Atoh7* (Häming et al., 2011; Lara-Ramirez, 2013 (unpublished); Lara-Ramírez et al., 2015; Higuchi et al., 2019) (Table 1-2). *Ascl1* expression has not been explored closely in the lamprey retina but has been identified in the lens of larval lampreys (Häming et al., 2011). If and how these genes are expressed in the retina is unknown. More detailed expression studies (including experiments manipulating gene expression) are needed to establish if the above genes coordinate lamprey retinogenesis similarly to gnathostome retinal development. If retinogenesis pathways are conserved between lampreys

and gnathostomes, this would support that the basic mechanism(s) for vertebrate retinal development evolved in the ancestor of cyclostomes and gnathostomes.

1.3.3 Chordate photoreceptive organs: a window to the state of the ancestral vertebrate eye?

To explore the origins of the vertebrate eye and retinal neurogenesis, one must contextualize the data against visual sensory organs of their closest invertebrate relatives: the nonvertebrate chordates. This group is composed of the lancelet or amphioxus (cephalochordates) and the tunicates (urochordates) (Figure 1-1A). Both groups have photoreceptive cells, but neither possesses the complex, paired eye structures of the vertebrate visual system (Lamb, 2013). Whether the visual/light sensitive cells of the non-vertebrate chordates are related to vertebrate eyes has been debated (see below). In addition to work examining the morphology and function of these organs, several studies have begun to examine the development of amphioxus and tunicate photoreceptive organs, allowing for comparisons to be made to retinal neurogenesis in vertebrates. If amphioxus and tunicate photoreceptors are ancestral/homologous to the vertebrate eye, they could be used to study the early transitional stages leading to the vertebrate eye. The developmental processes driving neurogenesis in non-vertebrate chordates could also provide evolutionary context for retinogenesis in vertebrates.

It has been suggested that the vertebrate ancestor (proto-vertebrate) may have had photosensitive organs resembling those seen in the extant non-vertebrate chordates. The cephalochordates (amphioxus) and urochordates (tunicates) only possess simple photoreceptive cells/organs (not organized into an eye) used for simple light-guided behavior (i.e., phototaxis). Amphioxus possesses four distinct regions that carry photosensitive properties: the frontal eye, lamellar body, Joseph cells and dorsal ocelli (Pergner and Kozmik, 2017). The frontal eye and the lamellar body are of particular interest as they contain ciliary photoreceptors (vertebrate

photosensory organs also contain ciliary photoreceptors). Several studies have proposed homology between the frontal eye of amphioxus and the lateral eyes of vertebrates on the basis that both structures contain similar cell types and gene expression profiles. The frontal eye contains a pigment cell directly adjacent to a row of photoreceptors that express two distinct amphioxus ciliary opsins (op1 and op3) (Vopalensky et al., 2012) (Figure 1-1B). This organization has been compared to the photoreceptors and adjacent RPE layer of the vertebrate retina. In addition, developing amphioxus pigment cells express *Mitf* and *tyrosinase* (as does the developing vertebrate RPE) and amphioxus photoreceptors express *Otx2* and potentially amphioxus-*rx* (similar to the gene expression profile of vertebrate photoreceptors) (Vopalensky et al., 2012). Although the dorsal eye does not possess a layered retinal structure as seen in vertebrates, there are several adjacent groups of neuronal cells that have been hypothesized to be homologous to other retinal cell types seen in vertebrates. One group of neurons within the frontal eye expresses a combination of *pax4/6* and *rx*, which has led to these cells being compared to vertebrate interneurons (Vopalensky et al., 2012). A separate set of frontal eye neurons have been suggested to be retinal ganglion cells or bipolar cells (or at least share a similar function) based on their projection to the amphioxus cerebral vesicle (homologous to vertebrate forebrain/midbrain). Based on this developmental data, the cell types in the amphioxus frontal eye have been proposed to be homologous to cell types in the vertebrate retina, although the photoreceptors themselves are not as complex nor is the frontal eye organized like the vertebrate retina (Vopalensky et al., 2012; Pergner and Kozmik, 2017; Lacalli, 2018). In addition, amphioxus have homologs of genes involved in the vertebrate phototransduction cascade and the retinoid cycle (Albalat, 2012; Lamb and Hunt, 2017).

Ascidian (tunicate) larvae also possess a photosensitive structure, the ocellus (Figure 1-1C). This organ consists of a pigment cell associated with multiple photoreceptor cells and lens cells (the tunicate lens cells are not believed to be homologous with the lens of the vertebrate eye) (Eakin and Kuda, 1971; Kusakabe et al., 2001; Horie et al., 2005; Esposito et al., 2015). Ascidian photoreceptors are ciliary (as are vertebrate photoreceptors) and express a homolog of opsin *Ci-opsin1* (Eakin and Kuda, 1971; Kusakabe et al., 2001). The tunicate homolog of *Rx* helps to form the ocellus and *Onecut* and *Neurogenin* are also expressed in the ocellus (vertebrate homologs of these genes contribute to retinal development) (D'Aniello et al., 2006; Esposito et al., 2015). These features support tunicate photoreceptors may be homologous to vertebrate lateral eye photoreceptors. Tunicate pigment cells have also been compared to the RPE cells of the vertebrates, and as with amphioxus pigment cells they express homologs of *Mitf* and *tyrosinase* (Sato and Yamamoto, 2001; Yajima et al., 2003). Finally, as with amphioxus, the tunicate genome contains homologs of several genes required for phototransduction and retinoid cycling in vertebrates (Kusakabe and Tsuda, 2007; Esposito et al., 2015). The function of these genes within the amphioxus frontal eye and tunicate ocellus would need to be assessed further to support a conserved function between cephalochordates/urochordates and vertebrates, but at the very least certain genetic elements employed to form the vertebrate retina may have existed in the chordate lineage prior to the emergence of the vertebrates.

Overall, characterization of cell types and gene expression profiles within the photosensitive structures of extant chordates suggest the basic cell types that contribute to the vertebrate retina may have already arisen within the last common ancestor of all (vertebrate and non-vertebrate) chordates. The vertebrate eye is presumed to have developed from a simpler structure composed of several photoreceptor cells aggregated together and associated with pigment cells (an ancient

arrangement that occurs in photoreceptive organs throughout Metazoans) (Arendt, 2003; Fain et al., 2010). The vertebrate retina, tunicate ocellus, and the amphioxus frontal eye each consist of pigment cell(s) associated with ciliary photoreceptors (contrasting rhabdomeric photoreceptors that underpin vision in many invertebrates). Therefore, the state of photoreceptive organs in amphioxus and tunicates represents a plausible idea of what the early form of the vertebrate eye may have been. However, the laminated vertebrate retina is highly complex and there must have been many modifications to get from a "chordate-like" proto-vertebrate photoreceptive organ to the vertebrate retina (and eye) plan. The transitional stages that would have led from relatively simple clusters of photoreceptive cells to complex vertebrate camera-style eyes would have involved retinal (or photoreceptor associated) cell types increasing in number and variety and being arranged into distinct layers (Land and Fernald, 1992; Nilsson, 2009).

This process may be related to the emergence of sensory placodes and neural crest in the vertebrate lineage. Sensory placodes are embryonic thickenings of the head ectoderm which contribute to the formation of multiple sensory organs (Schlosser, 2006). Placodes first appeared in vertebrates (i.e., are not found in the non-vertebrate chordates) and are believed to have facilitated an increase in the complexity of vertebrate sensory organs by producing high concentrations of sensory cells clustered into particular areas (Schlosser et al., 2014). This arrangement would set up ideal conditions for the formation of sensory organs in the early vertebrates compared to the scattered sensory cells of other chordates. In vertebrates only the lens placode contributes to eye formation, and it does not directly produce sensory cells (i.e., photoreceptors), but instead forms the non-neural lens (Schlosser, 2006). However, the lens placode does interact with surrounding tissues to induce eye formation by undergoing morphogenetic movement to aid in forming the optic cup and by releasing signals to promote

formation of the neural retina (Cardozo et al., 2019). Perhaps the evolution of the lens placode in the vertebrate ancestor created a region of cells specified for a photoreceptor/interneuron fate via a similar signaling mechanism. Once photoreceptors and visual neurons began to develop in higher concentrations near the lens placode, this may have paved the way for the simpler photoreceptive neurons of chordates to become grouped into an organized retina-like structure (this organization may also have been coordinated by the early vertebrate lens placode). Neural crest cells are another vertebrate innovation. They are a population of migratory cells that contribute to multiple structures (especially within the head) in developing vertebrate embryos and are often associated with formation of sensory organs (Yu, 2010; York et al., 2020). Neural crest is important for proper morphogenesis of the developing vertebrate eye—when neural crest is lost the optic cup does not form properly (Bryan et al., 2020). In early vertebrates, neural crest may have allowed for the shift to a more complex eye structure by providing an additional set of cells to coordinate eye/optic cup formation. Ongoing proliferation late into ontogeny (post-embryonic neurogenesis) is another factor that could have also contributed to evolution of the cup-like eye morphology. The photoreceptors themselves would have also needed to gain further morphological complexity and efficiency for light detection and processing (vertebrate ciliary photoreceptors are more complex structurally compared to those of tunicates or amphioxus) (Lamb et al., 2007; Pergner and Kozmik, 2017). Assuming the early vertebrate had a phototransduction cascade and elements of the retinoid cycling pathway similar to extant non-vertebrate chordates, these pathways would also require further changes to reach the state seen in extant vertebrates (Kusakabe and Tsuda, 2007; Albalat, 2012; Lamb and Hunt, 2017; Pergner and Kozmik, 2017).

Alongside the increase in complexity of visual cells, the vertebrates also have more complex visual pathways in the central nervous system. This had to be coordinated with retinal evolution to allow for the connection between light detection in the eye and visual information processing in the brain to remain linked. There are many factors that need to be considered when postulating about vertebrate eye evolution, but the current evidence suggests certain chordate photoreceptive organs/cell types may be homologous to the vertebrate eye. Therefore, a comparison of the features (and developmental processes producing those features) between the amphioxus frontal eye, tunicate ocellus and the vertebrate retina may provide a more complete image of the state of the ancestral chordate/vertebrate eye and how the photoreceptive organs of these groups arose from it.

1.4 Adult retinal neurogenesis – a source of new retinal cells throughout ontogeny

1.4.1 The ciliary marginal zone and Müller glia: pools of retinal stem cells in gnathostomes

Retinogenesis is most thoroughly characterized during embryonic development but in many vertebrates retinogenesis continues robustly in adult animals. The ciliary marginal zone (CMZ) is a region of stem cells that occurs around the periphery of the neural retina in multiple vertebrate groups (Figure 1-3A). This proliferative cell population generates new retinal neurons throughout ontogeny (Johns, 1977; Fischer et al., 2014) (Figure 1-3B). Cells of the CMZ are multipotent and capable of forming any of the retinal cell classes (Raymond et al., 1988). This proliferative zone has been identified in teleost fishes, amphibians, and birds (the CMZ is relatively limited in mature birds) but is not apparent in mammals (Johns, 1977; Hitchcock et al., 2004; Fischer et al., 2014) (Figure 1-3C). Despite this it has been noted that some cell populations within the peripheral retina of mammals express markers related to retinal

progenitors and can be induced to take on a progenitor identity in cell culture (Das et al., 2005; Jian et al., 2009). The possibility of regressive evolutionary loss of the CMZ in mammals, and perhaps also in sharks (Hernández Núñez et al., 2021) lends tentative support to the speculation that lampreys have lost the CMZ, as discussed below.

Within gnathostomes, the differentiation of cells from the CMZ appears to recapitulate many of the same mechanisms as embryonic retinal development, but this is an area still under investigation (Link and Darland, 2001; Wehman et al., 2005; Xu et al., 2020). In the most peripheral region of the CMZ cells retain full stem cell characteristics and are multipotent (Raymond et al., 2006). Moving from the periphery towards the center of the retina, CMZ cells become more differentiated (begin to express neurogenic/proneural genes and ultimately become fully specified). Xu et al., (2020) have identified several groups of CMZ cells (based on gene expression markers) that are comparable to groups arising during embryonic retinal development. The most peripheral group of cells express *fabp11a*, a gene seen in early embryonic retinal stem cells. *Her4.2* expression (another gene expressed in early embryonic retinal stem cells) was found slightly more centrally. Even more centrally several genes related to the differentiation of retinal cells (i.e., *atoh7*—RGC's, *vsx*—bipolar cells, *rcn*—photoreceptors, etc.) are expressed. These results suggest that the cells from the CMZ pass through several competence stages across the CMZ before taking on their final differentiated state. A similar sequence of stages was documented from cells developing from the CMZ in *Xenopus* (Perron et al., 1998). It is still unknown how the CMZ functions in jawless vertebrates and whether the retinal margin of hagfish can be considered conserved (in structure and function) with the CMZ of gnathostomes. However, if the CMZ of hagfish operates similarly to gnathostomes (i.e., embryonic and adult retinogenesis occur via shared mechanisms) this would

support the adult hagfish CMZ as a valuable tissue for studying hagfish retinal development in the absence of embryos.

Müller glia are an additional source of multipotent cells for retinal regeneration in vertebrates late into ontogeny (particularly after injury) (Raymond et al., 2006; Lenkowski and Raymond, 2014). Müller glia are the only non-neural cell population within the retina and serve a supporting role for the various retinal neurons. However, Müller glia can also return to a stem-cell fate to then re-differentiate into neurons after injury (Raymond et al., 1988; Bernardos et al., 2007; Fimbel et al., 2007; Goldman, 2014; Lenkowski and Raymond, 2014). The return to a multipotent state is mediated by several genetic networks including activation of *Ascl1* (Gao et al., 2021).

1.4.2 Evidence for adult retinal growth in the cyclostomes

The evolutionary origin of the CMZ is unknown but a putative CMZ has been identified in hagfish (Dong and Allison, 2021). The retinal margin of adult hagfish expresses the CMZ marker *Pax6* and contains immature photoreceptor cells. In addition, hagfish eye size increases with body size (supporting the eye grows throughout ontogeny). These findings contrast earlier assumptions that the hagfish eye is degenerate and degenerating, which would be consistent with a release from selective pressure for processing light/visual information (why maintain an eye with no purpose?). As hagfish possess a proliferative retina past embryonic development, this would suggest the eye still has biological/ecological significance despite its small size and simple organization. Interestingly, larval lampreys also possess a proliferative retinal region, but the adults do not (Villar-Cheda et al., 2008) (Figure 1-3D,E). The cells of the peripheral lamprey retina appear to stop proliferating after metamorphosis. This phenomenon has only been documented in one species (*Petromyzon marinus*), so it is unclear if all lampreys undergo loss of

retinal proliferation during ontogeny or if it is species-specific. Examining the retinae of other lamprey species and life stages would be necessary to elucidate whether any lampreys have a CMZ and post-embryonic retinal growth or if the absence of adult retinogenesis is consistent across this group. The finding that post-embryonic retinogenesis occurs in hagfish but not lampreys suggests that lampreys either have lost the CMZ during evolution, or hagfish and gnathostomes converged on the CMZ condition. Regardless, the existence of the CMZ (or a CMZ-like tissue) in hagfish is surprising. Moreover, this data suggests the hagfish eye is an actively growing tissue and the retina may still be utilized by these organisms despite their dim-light habitat. Research comparing the mechanisms driving adult retinogenesis in cyclostomes and gnathostomes could reveal whether the CMZ of hagfish and gnathostomes arose in a shared ancestor or evolved independently in each lineage.

Müller glia have been studied heavily in teleost fishes and have also been identified in the lamprey retina (Raymond et al., 2006; Fernández-López et al., 2016). However, there is no evidence for the occurrence of Müller glia in the hagfish retina and this is an area where further investigation is warranted.

1.5 Models of vertebrate eye evolution

Given the critical position of hagfish and lampreys in the vertebrate phylogeny, these groups can provide important information for uncovering the history of vertebrate eye evolution. Several scenarios have been proposed to explain how the rudimentary eyes of hagfish fit into the sequence of vertebrate eye evolution and whether the hagfish eye can be considered representative of an "ancestral" state for vertebrates. These models also need to consider that

lampreys possess sophisticated eyes (comparable to gnathostomes), yet also form a monophyletic group with hagfish. There are multiple possibilities for how the eyes of lampreys and hagfish diverged from each other and from gnathostomes (involving gain or loss of features in specific lineages). Given the existing body of evidence we articulate three alternative scenarios for how the hagfish eye may fit into the narrative of vertebrate eye evolution.

1.5.1 Hagfish eyes as the ancestral state

One scenario holds that the hagfish eye represents an ancestral state for the vertebrate eye. This hypothesis interprets the lack of a lens or pigment and the rudimentary features of the hagfish eye/retina as ancestral features related to an early state of eye evolution (Lamb et al., 2007), but no longer has strong support given the monophyly of hagfish and lampreys. Under this model the extant hagfish eye represents a tissue that is akin to a transitional state between the relatively simple photoreceptive cells of (non-vertebrate) chordates and the more complex eyes of other vertebrate groups (Figure 1-4A). In this evolutionary scenario, features such as the lens of the eye and pigment within the retinal epithelium would not have been present in the last common ancestor of hagfish and other vertebrates. The early vertebrate eye would have also had fewer interneurons for processing visual signals compared to later vertebrates (the hagfish condition could be seen as bridging a simpler state comparable to the chordate frontal eye and the complex retina of vertebrates). In this model the hagfish eye (and the hypothetical transitional state) could be compared to the pineal organ of other vertebrates (a photosensory organ with photoreceptors lying directly adjacent to interneurons without additional cellular layers (Lamb et al., 2007; Collin, 2010). This type of visual organ would be sufficient for detecting light but not for forming images.

Assuming the hagfish eye represents a more "primitive" form of the vertebrate eye, the next stage of eye evolution/organization would then be seen in the lamprey. Although still part of the jawless vertebrate clade, lamprey retinae possess four clearly laminated cellular layers (including the pigmented retinal epithelium) and most of the retinal cell types seen in gnathostomes (including several classes of photoreceptors) (Suzuki and Grillner, 2018). The lamprey eye also contains a lens and pigment in the retinal epithelium. This could be seen as a more derived state of the hagfish eye. In comparison, the hagfish eye may represent a more ancestral photoreceptive organ for circadian entrainment rather than image formation (Lamb et al., 2007).

Assuming the hagfish eye is a baseline/ "primitive" state, several ecological drivers could plausibly play a role in the shift towards a more complex eye state in other vertebrates. Changes to the environment and lifestyle of the early vertebrates may have required these animals to develop greater visual acuity (Collin, 2010; Davies et al., 2012). In addition, if the hagfish eye is compared to the vertebrate pineal organ this could also represent a shift from use of photoreception in vertebrates for regulating circadian rhythm to image formation and perception of the environment. The addition of a lens would improve visual acuity and photon capture by focusing light onto the retina (Gustafsson et al., 2008). Pigment within the retinal pigmented epithelium would allow for better perception of incoming light direction (perhaps related to evolving from a dim benthic habitat into environs with more light) (Bharti et al., 2006). Greater retinal lamination and organization would allow for more efficient processing and integration of visual information (Lamb et al., 2007). Finally, evolution of more complex photoreceptors and multiple opsin classes would allow vision over a breadth of light intensities to evolve (Yokoyama, 2000; Lamb et al., 2007). As deep-sea scavengers, hagfish may have not needed sophisticated vision. However, if other vertebrates took advantage of rich photic niches, good

vision would have been critical for avoiding predators and finding prey. This evolutionary pressure would ultimately lead to the formation of a functional and potentially image forming eye.

As hagfish and lampreys constitute a monophyletic clade this scenario is no longer thought to be plausible (Mallatt and Sullivan, 1998; Kuraku et al., 1999; Heimberg et al., 2010; Ota et al., 2011; Miyashita et al., 2019). Lampreys possess relatively sophisticated eyes, and this would imply a large degree of convergence between the lamprey eye state and the gnathostome eye state if the hagfish eye condition were ancestral. Thus, the last common ancestor of hagfish, lampreys and gnathostomes likely had an elaborate eye with many features familiar from extant vertebrates (Figure 1-1A). Additionally, although hagfish and lampreys are the only extant Agnathans, several fossils of early jawless vertebrates have been found, and a few studies have investigated the eyes of these specimens. Gabbott et al., (2016) argue that the fossils of both extinct hagfish and lamprey contain pigment. This is a feature that is more consistent with the eye state of extant lampreys than with hagfish and would support an earlier emergence of the "complex" vertebrate eye (i.e., the hagfish eye condition is likely not ancestral for the vertebrate lineage). One limitation of the fossil data is that ocular structures do not generally preserve well. In addition, there can be multiple interpretations for fossil eye data so ideally other lines of evidence (i.e., developmental, genetic, etc.) should be used to support an early origin of these features. The frontal eye of amphioxus and the ocellus of tunicates (argued to be potentially homologous with the vertebrate eye/retina) also contain pigment cells. If the pigment cells of amphioxus and tunicates are ancestral/homologous to the vertebrate RPE (as supported by Sato and Yamamoto, 2001; Yajima et al., 2003; Vopalensky et al., 2012), this would conflict with the hagfish RnPE being the "ancestral condition" for vertebrates (although it does not exclude the

possibility that other features of the hagfish eye, such as the absence of a lens, may be ancestral). This is an area where an exploration of retinogenesis and/or eye development in cyclostomes may be particularly useful. If the hagfish retina forms in the absence of retinal developmental pathways seen in other vertebrates, this could support the idea that the hagfish eye has ancestral elements (i.e., some features are rudimentary due to hagfish not having evolved genetic/developmental networks employed by other vertebrates).

1.5.2 Hagfish eyes as a product of regressive evolution

An alternative scenario/hypothesis is that the hagfish eye represents a degenerate state due to the hagfish adapting to a lowlight environment (the deep-sea). In this model the ancestral hagfish eye would have been similar to that of the extant lampreys or gnathostomes (containing a lens, photoreceptors and pigment) (Figure 1-4B). Due to lack of use as they adapted to a dim-light marine scavenger existence, hagfish as a group would have had their eye structures reduced resulting in smaller eyes (covered in skin or muscle), loss of the lens, loss of ocular pigment, and reduced organization/complexity of the retina. As previously mentioned, both living amphioxus and tunicates and at least once species of fossil Agnathan appears to have pigmented cells associated with photoreceptors (Yajima et al., 2003; Vopalensky et al., 2012; Gabbott et al., 2016). This would support that the ancestors of extant hagfish likely had a pigmented retinal epithelium. Fossil agnathans (classified as lamprey) possibly had a lens (Gabbott et al., 2016) and there is an (unconvincing) report of a lens in at least one extant hagfish species (Kobayashi, 1964). These findings suggest that more “complex” features may been long present in the cyclostome lineage. Fernholm and Holmberg (1975) demonstrated that even within living hagfish the state of the eye varies among species (especially between those that inhabit different light environments). Hagfish that live at greater depths (and lower light levels) have more

rudimentary eyes (eyes covered by muscle instead of transparent skin, photoreceptor morphology more divergent than in other groups). This phenomenon is not restricted to hagfish as Davies et al., (2009) also showed a light dwelling lamprey species had more complex eyes and a greater variety of photoreceptor subtypes than deeper water species.

There are several genetic and/or developmental mechanisms through which the hagfish eye may have regressed. Other organisms with regressed eyes also tend to live in dim or aphotic environments (such as subterranean and cave-dwelling animals). In these groups (and presumably in hagfish) the eye is no longer useful and therefore the selective pressure to maintain a functional eye is greatly reduced. Genetic drift can then act, resulting in the loss of functional genetic machinery to maintain the eye (Jeffery, 2009; Emerling and Springer, 2014). Additionally, the eye/retina is an energetically expensive organ (Yu et al., 2009). Allowing such a structure to become reduced or lost when it is no longer useful allows resources to be utilized on other structures that may be more beneficial for survival. In the deep-sea environment where light is limited and hagfish can rely on olfaction, a fully functional eye may not be worth maintaining (Yu et al., 2009). Finally, if vision is not vital and other structures/systems are expanded, this can result in pleiotropic effects that lead to eye loss. In cavefish (*A. mexicanus*) the shift from a reliance on vision to other senses (i.e., gustation, mechanoreception) and changes in feeding strategies that accompanied adaptation to an aphotic cave environment are believed to have contributed to the loss of the eyes (Yamamoto et al., 2009; Yoshizawa et al., 2012). These changes altered important signaling pathways, including those required for proper eye development. Hagfish may have undergone similar evolution: selection for improving other senses (e.g., olfaction) in a dim-light environment could have altered expression of developmental genes with "side-effects" that compromised the developmental pathway for

generating an eye/retina. Further work studying the embryonic development of hagfish and potentially manipulating the signalling pathways and genes at play could illuminate whether eye loss is intertwined with the developmental expansion of other sensory systems.

In the wild, hagfish are believed to use vision to a limited extent (if at all). Most hagfish live in deep water, although some species occur in relatively shallow areas where light can reach (Patzner, 1978; Braun, 1996; Martini, 1998). Several studies have suggested that hagfish have limited sensitivity to light. The response of hagfish eyes to light is weak and occurs slowly compared to other vertebrates (Kobayashi, 1964; Patzner, 1978). Interestingly, a behavioural response to light still occurs even if the eyes are removed, suggesting hagfish may have extraocular photoreceptors (Kobayashi, 1964). Light does appear to play a role in circadian rhythm for hagfish and it has been proposed the eye may take on the function of the pineal organ (a pineal has not yet been identified in hagfish) (Ooka-Souda and Kabasawa, 1995; Lamb et al., 2007; Lamb, 2013). This has led to the standing idea that hagfish are more reliant on other senses (i.e., olfaction) to find food due to their deep-sea environment and living primarily as scavengers. However, some studies suggest hagfish can be predatory (perhaps to a greater extent than previously realized) and hagfish eye morphology does vary with the available light environment (Braun, 1996; Martini, 1998; Zintzen et al., 2011). Fernholm and Holmberg (1975) noted that hagfish species living in shallower water had more complex eye features than their deeper water relatives. Species in the genus *Myxine* have very reduced eyes covered by muscle whereas *Eptatretus* and *Paramyxine* species have eyes only covered by a translucent layer of skin (some light can reach the eye) (Fernholm and Holmberg, 1975). The photoreceptors of *Myxine* hagfish are more reduced compared to *Eptatretus* and *Paramyxine* as well. This would suggest some elements of eye function remain intact in light-dwelling species. Furthermore, it has been

reported that hagfish actively avoid (and therefore respond to) light in their environments (Fernholm, 1974). This would support the idea of regression with a transition to dim-light environments. Yet, if regression were the full explanation for why hagfish eyes were reduced it would be odd for hagfish species living in well-lit environments to still have such rudimentary eyes. In *Astyanax mexicanus* (a species with cave-dwelling and surface-dwelling fish) there are populations with both functional vision and varying degrees of eye degeneration (Sifuentes-Romero et al., 2020). However, in hagfish all species surveyed appear to have reduced eyesight. This could be due to the evolutionary timeline of hagfish eye loss (i.e., hagfish may have adapted to this lifestyle over hundreds of millions of years compared to the divergence between surface dwelling and troglobiont cavefish (*A. mexicanus*) which diverged several million years ago). Further work is needed to establish to what extent hagfish utilize visual cues in their environment.

1.5.3 Hagfish eye regression via paedomorphosis

An alternate mechanism for regression of the hagfish eye is paedomorphosis. Several groups have compared the hagfish eye to the eye of larval lampreys (Lamb et al., 2007; Collin et al., 2009). Lamprey larvae have a relatively simple eyespot, only one type of photoreceptor, and simpler retinal organization/lamination compared to adults (as described in **Section 1.3.2**) (Suzuki and Grillner, 2018). The larval eyes are also buried under skin and do not emerge until after metamorphosis. These conditions are comparable to the state of hagfish eyes. This has led to the speculation that hagfish eyes may be paedomorphic. In this scenario ancestrally vertebrate eyes would have undergone a transition to a more complex state during ontogeny/maturation but during hagfish evolution a heterochronic shift(s) in gene expression resulted in the retention of a larval eye state (Figure 1-4C). Extant hagfish have direct development whereas the shift in eye

morphology in lampreys occurs during metamorphosis. Regardless, the ancestor of hagfish and lampreys may have had a shift in eye morphology that extant hagfish have lost. The fact that the lamprey retina undergoes some continual development throughout the larval stage (i.e., late-stage larvae have further retinal differentiation than the early-stage larvae) reduces support for the paedomorphosis model (Cornide-Petronio et al., 2015). If retinal differentiation/maturation is not restricted to a particular developmental stage, some complexity would still be present in the hagfish retina even if a hypothetical metamorphic stage was lost. As developmental data on the retina for embryonic hagfish and lampreys is scarce, it is difficult to support this model using current evidence. Similarly, it is difficult to use fossil data for support (fossils of juvenile cyclostomes are quite rare although some have recently been found (Miyashita et al., 2021)). Future work comparing retinogenesis in hagfish and lampreys could greatly help to explore this idea. If hagfish continue to express genes lost early in lamprey retinal development (or do not express genes that are expressed during lamprey metamorphosis) this could support the theory of heterochrony and a paedomorphic hagfish eye. Experiments manipulating expression of the identified genes to see if eye morphology shifts would provide additional support.

1.5.4 Hagfish are at a vital phylogenetic position to unravel mysteries behind vertebrate eye evolution

A combination of the above scenarios may have contributed to the evolution of the hagfish eye. The majority of current evidence refutes scenario 1 (the hagfish eye as ancestral). Importantly, the hagfish has been placed into a monophyletic clade with the lampreys (a group with a sophisticated eye/retina plan; Figure 1-1) (Mallatt and Sullivan, 1998; Kuraku et al., 1999; Heimberg et al., 2010; Ota et al., 2011; Miyashita et al., 2019). Although it is possible the lamprey eye independently converged onto a complex eye condition with the gnathostomes, this

scenario is not parsimonious. The hagfish eye also clearly has some features that align with the eyes of lampreys and gnathostomes: despite its overall lack of organization the retina still contains the major retinal cell classes, the retinal non pigmented epithelium still maintains certain functions despite having no pigment, and the retina contains a CMZ (Dong and Allison, 2021). These findings suggest that the hagfish eye has more affinity with other vertebrates than previously thought and support the idea that the current state of the hagfish eye may be reduced from a more complex state (scenarios 2 and 3). Complexity also arises, because various hagfish eye features may have regressed via separate mechanisms. For example, paedomorphy could possibly account for lack of well-defined retinal lamination in hagfish, but paedomorphy seems untenable for loss of RPE pigmentation because photoreceptors are associated with pigment across all phyla and developmental stages (Bharti et al., 2006; Vopalensky and Kozmik, 2009).

Several lines of evidence support the regression theory for evolution of the hagfish eye. Fossil data suggests some components of the eye may have arisen via regression (i.e., fossil hagfish appear to have ocular pigment whereas extant hagfish species do not) but not others (i.e., a lens was not identified in the fossil hagfish specimen) (Gabbott et al., 2016). The correlation between the condition of the hagfish eye and the environment (i.e., ambient light levels) in extant species could also support the rudimentary eye features being a result of evolutionary regression. The proposal of the hagfish eye as paedomorphic is interesting (especially in the context of the eye phenotype shift during lamprey ontogeny) but requires more work to determine if heterochrony is at play during hagfish development. Determining whether certain eye features are ancestral, or degenerate would be aided by comparison across hagfish species (particularly species that inhabit photic vs. aphotic environments). Future work comparing the retinogenesis pathways of hagfish to lampreys, gnathostomes, and non vertebrate chordates would be an enlightening

contribution. If complex eye features were indeed lost during evolution there may still be vestiges of associated gene regulatory networks and developmental processes in the hagfish eye (the putative CMZ in the hagfish retina is one possible remnant).

Although the hagfish lineage has been separated from jawed vertebrates for at least 500–600 million years, the hagfish eye has some features that could be considered synapomorphic with gnathostomes and lampreys (i.e., the last common ancestor of gnathostomes and cyclostomes likely had each of the major classes of retinal cell types as these are found in both descendant lineages). Other traits may have been retained from the common ancestor in one lineage and lost in the other or are novel traits that arose in one lineage alone. Work examining gene expression during hagfish eye development and retinal neurogenesis could elucidate which genes were utilized for eye formation in the ancestor of cyclostomes and gnathostomes, versus genes which evolved or were co-opted for different uses in each respective lineage. Overcoming the logistical challenges of studying hagfish retinal development is key. Several research laboratories have successfully bred hagfish embryos, which would be an ideal organism for studying hagfish retinal development (Ota and Kuratani, 2008). However, the discovery of a putative ciliary marginal zone is an additional path for exploring retinal growth in adult specimens. In addition to characterizing cyclostome retinal neurogenesis pathways, manipulative genetic experiments in hagfish and lampreys would be an exciting future avenue for exploring the evolution of retinogenesis in the early vertebrates. Altering cyclostome gene expression to change the timing of certain developmental events (i.e., testing the heterochrony model) or to recapitulate phenotypes (i.e., trying to recover a more “functional” eye in hagfish or knocking down genes in lampreys to recreate the hagfish eye condition) could provide experimental evidence to support the three evolutionary scenarios outlined above.

1.6 Objectives and purpose of study

As members of a lineage that diverged from other vertebrates ~600 mya, hagfish are an ideal species to investigate the origin of the vertebrate eye. The unique, rudimentary morphology of the hagfish eye has previously been interpreted as representing an ancestral or transitional state in vertebrate eye evolution (Lamb, 2013). However, the monophyletic grouping of hagfish with lampreys (which do possess a complex, camera-style eye) makes this scenario unlikely. In addition, the hagfish eye does share similarities with the eyes of other vertebrates despite its comparatively simplistic organization (Dong and Allison, 2021). Another more probable scenario is that the hagfish eye has been reduced over evolutionary time. Existence as deep-sea marine scavengers may have released hagfish from the pressure to maintain a functional eye, resulting in the loss of critical eye structures. Alternatively, expansion of other structures (i.e., the olfactory system) may have led to a reduced eye via pleiotropy. By exploring proliferation and gene expression in the hagfish eye, the goal of this study is to uncover if the mechanisms driving retinogenesis are shared between cyclostomes and gnathostomes (and are therefore ancestral for the whole vertebrate lineage) or if each group has evolved distinct pathways to produce a retina.

This study has two main aims. The first is to confirm and characterize retinal proliferation in the adult hagfish. Previous work suggests hagfish may have a proliferative zone at the margin of the retina (similar to the CMZ of gnathostomes) (Dong and Allison, 2021). If true, this tissue would provide an opportunity to examine retinal development in adult animals where embryos are unavailable. In addition, continued proliferation in the hagfish retina is surprising given the diminutive/degenerate state of the eye. Characterizing the proliferation (i.e., where it occurs in the hagfish retina and what retinal cells are being generated) in adult hagfish may allow us to

determine if the tissue can be considered homologous to the CMZ of other vertebrates. It would also be fascinating to uncover why growth continues in the rudimentary and diminutive hagfish eye. Ciliary marginal zones are well known in teleost fishes and amphibians and occur to a limited extent in birds (Fischer et al., 2014). However, there is no clear evidence of a ciliary marginal zone in lampreys or sharks (Villar-Cheda et al., 2008; Hernández-Núñez et al., 2021). If the neurogenesis at the hagfish retinal periphery occurs via similar mechanisms to the gnathostome CMZ, this would be another example of a commonality between hagfish and gnathostome eyes. The presence of a ciliary marginal zone in hagfish could also mean that this tissue originated early in the vertebrate lineage (and has regressed more than once during retinal evolution).

The second aim is to investigate if the hagfish retina develops via similar cellular and molecular mechanisms to the gnathostome retina. Comparisons of model vertebrate species (i.e., mouse, frog, chicken, zebrafish) has demonstrated that many aspects of eye and retinal development are heavily conserved across the vertebrates. For example, expression of critical transcription factors such as *Otx2*, *Crx*, *Six3*, *Rx,* and *NeuroD* help regulate overall formation of the eye and perform crucial roles during retinogenesis in multiple vertebrate groups (Table 1-1, Table 1-2). Much less work has been done to examine eye/retina formation in the cyclostomes. As a lineage separated from other vertebrates for hundreds of millions of years, a comparison of hagfish retinal development to representative gnathostomes may allow us to draw more conclusions about the state of the ancestral vertebrate eye. If hagfish and gnathostomes possess shared developmental mechanisms for retinogenesis, this would suggest that these pathways are ancestral for vertebrates and evolved before the divergence of the cyclostome and gnathostome lineages. If

different genes or pathways are involved in each group, this could suggest that certain aspects of retinal development independently evolved in the cyclostome and gnathostome lineages.

Overall, the early origin of the vertebrate eye and retina remains unclear. The large timespan involved, and understudied nature of a major vertebrate clade, the Cyclostomata (lampreys and especially hagfish), make studying the evolution of these structures difficult. There is still much to be learned about retinogenesis in agnathans. Unravelling the retinal development pathways of these organisms through genetic/molecular/developmental characterization has the potential to clarify the evolutionary origins of this sophisticated sensory organ. The recent availability of hagfish embryos and the discovery of a proliferative CMZ region in adult hagfish creates exciting new opportunities to investigate the emergence of the vertebrate retina and camera-style eye. This study will utilize the CMZ region in adult hagfish to explore the developmental mechanisms driving hagfish retinogenesis.

Acknowledgements

We appreciate discussions with Emily Dong that contributed to the ideas discussed here.

Figures and Tables

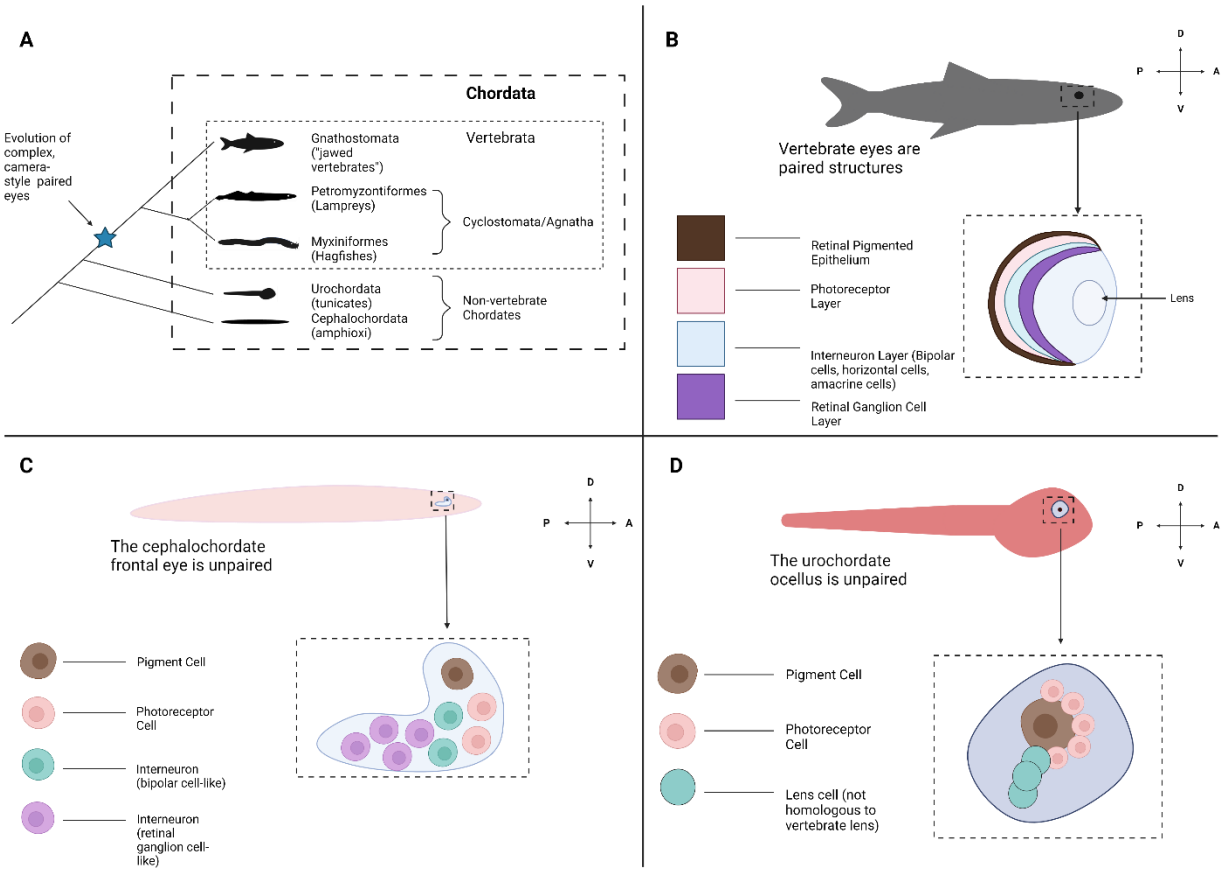


Figure 1-1: Evolutionary origins of the vertebrate eye and the appearance of other photoreceptive organs across Chordata. A) A phylogeny of Chordata. Chordata consists of three subphyla: Cephalochordata (amphioxus), Urochordata (tunicates) and Vertebrata (the Vertebrates). Two clades form Vertebrata: The Hagfish and Lamprey (Myxiniformes and Petromyzontiformes) form a distinct group known as Cyclostomata (extant members of the jawless vertebrates (Agnatha)) and the jawed vertebrates form Gnathostomata. Paired, complex eyes appear within Vertebrata but not in the other Chordate subphyla. B) Most all gnathostomes have the familiar vertebrate eye and retinal structures, conserved from fish through mammals.

The eyes are paired and bilateral. Each eye contains a multi-layered retina for light detection and image formation, a pigmented retinal epithelial layer and a lens to focus incoming light. C) The amphioxus frontal eye is an unpaired photoreceptive organ located at the anterior end of the amphioxus. The frontal eye contains a single pigment cell, several photoreceptor cells, and several interneurons (Vopalensky et al., 2012). D) The tunicate ocellus is an unpaired photoreceptive organ that consists of a large pigment cell and associated photoreceptor cells (Horie et al., 2005). In some species the ocellus also contains lens cells, but these are not believed to be homologous with the lens structure of the vertebrate eye. Graphics created using BioRender.com.

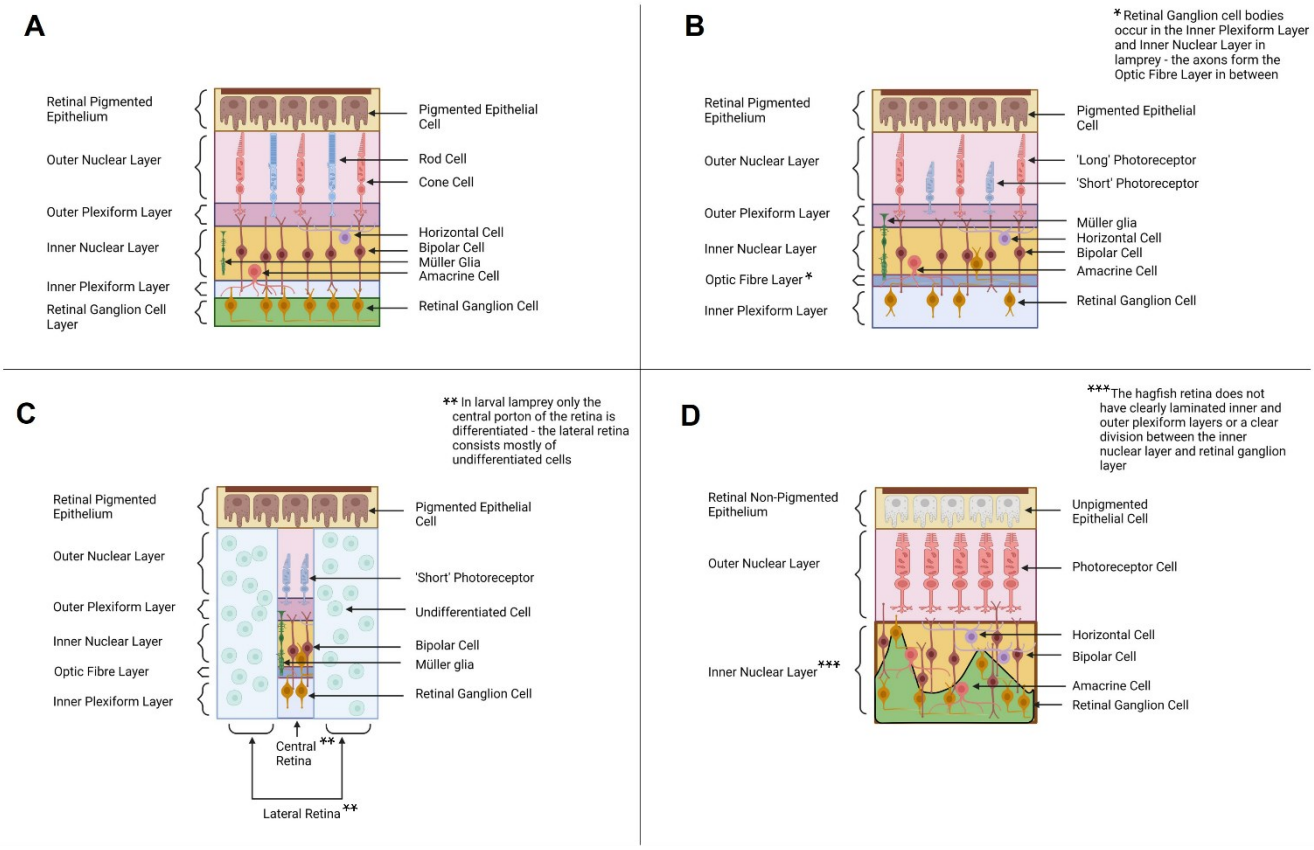


Figure 1-2: Retinal organization across cyclostomes and gnathostomes provides a backdrop for alternative hypotheses of vertebrate eye evolution. A) Across most all gnathostomes, the retina is composed of four distinct cellular layers – the retinal pigmented epithelium, the outer nuclear layer (ONL) containing photoreceptors, the inner nuclear layer (INL) containing bipolar cells and other interneurons and the retinal ganglion cell layer (RGC) containing retinal ganglion cells. B) The adult lamprey retina has a similar organization to the gnathostome retinal plan. The photoreceptors have a distinct morphology and the retinal ganglion cell bodies occur in the inner nuclear layer and the inner plexiform layer rather than forming their own distinct cellular layer. C) The pre-metamorphic larval lamprey retina is distinct from the adult, as it has only a small differentiated central retina (containing one type of photoreceptor, bipolar cells, retinal ganglion cells and Müller glia) and a larger undifferentiated peripheral retina. Completion of retinal

differentiation and the formation of horizontal and amacrine cells occurs during metamorphosis. D) The hagfish retina is morphologically distinct from lamprey and gnathostome retinae, which is intriguing considering the position of hagfish in the vertebrate phylogeny (Figure 1-1A). The retinal epithelium is unpigmented, and the lamination between the presumptive inner nuclear layer and the retinal ganglion cell layer is poor. Despite the reduced organization, molecular studies support that the hagfish retina contains all four cellular layers seen in lampreys and gnathostomes (Dong and Allison, 2021). Graphics created using BioRender.com.

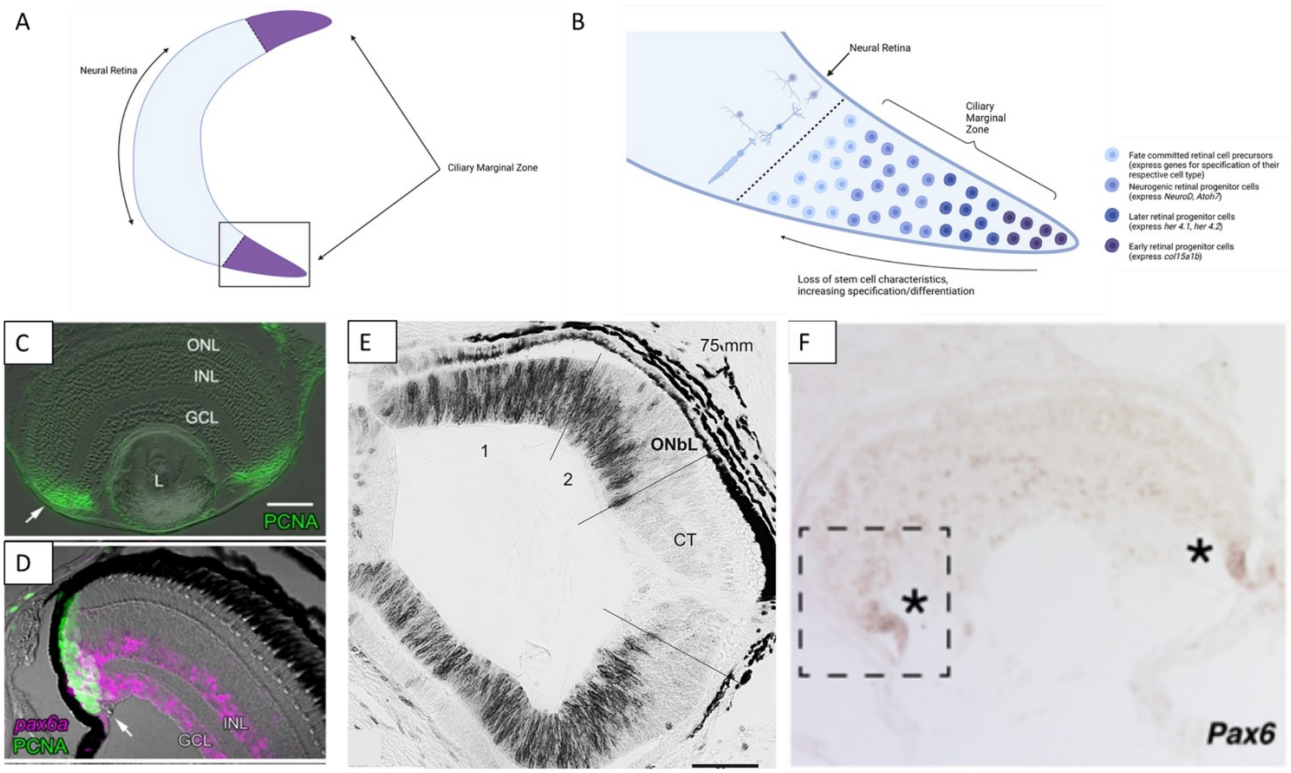


Figure 1-3: Structure of the ciliary marginal zone (CMZ) in gnathostomes and cyclostomes. A) The ciliary marginal zone is a proliferative region that occurs at the periphery of the neural retina in multiple vertebrate groups (though it is reduced/absent in mammals). B) The boxed section of panel 1-3A. Within the ciliary marginal zone cells closest to the retinal periphery are multipotent stem cells that divide to generate new retinal cells. Moving towards the center of the retina the cells of the CMZ begin to express markers of neurogenesis and ultimately become specified and differentiated as one of the main retinal cell types. C) Presence of PCNA (a marker of proliferative cells) in the ciliary marginal zone of zebrafish. D) In zebrafish *Pax6* is expressed at the very peripheral margin of the CMZ (*Pax6* marks the retinal stem cells and a subset of differentiated INL and GCL cells). E) In sea lamprey (*Petromyzon marinus*) PCNA (black) labelling indicates that cell proliferation also occurs throughout the more peripheral sections of the retina. Once the lamprey complete metamorphosis the proliferative region of the retina loses

PCNA expression (the CMZ-like tissue is not maintained into adulthood). F) The hagfish retina has expression of *Pax6* at the most peripheral retinal margin. This expression pattern is similar to the *Pax6* expression seen in the CMZ of other vertebrates and supports the interpretation that hagfish have a CMZ (or a CMZ-like tissue) at the margin of the retina. Panels C and D were adapted from Raymond et al., (2006) (Copyright © 2006, Raymond et al; licensee BioMed Central Ltd.). Panel E was adapted from Villar-Cheda et al., (2008) (Reprinted from Brain Research, Volume 1201, Begoña Villar-Cheda, Xesús Manoel Abalo, Verona Villar-Cerviño, Antón Barreiro-Iglesias, Ramón Anadón, María Celina Rodicio, Late proliferation and photoreceptor differentiation in the transforming lamprey retina, Page 61, 2008, with permission from Elsevier). Panel F was adapted from Dong and Allison, 2021 (Copyright © 2021, Dong and Allison, 2021; The Royal Society (U.K.)). Graphics in Panels A and B were created using BioRender.com.

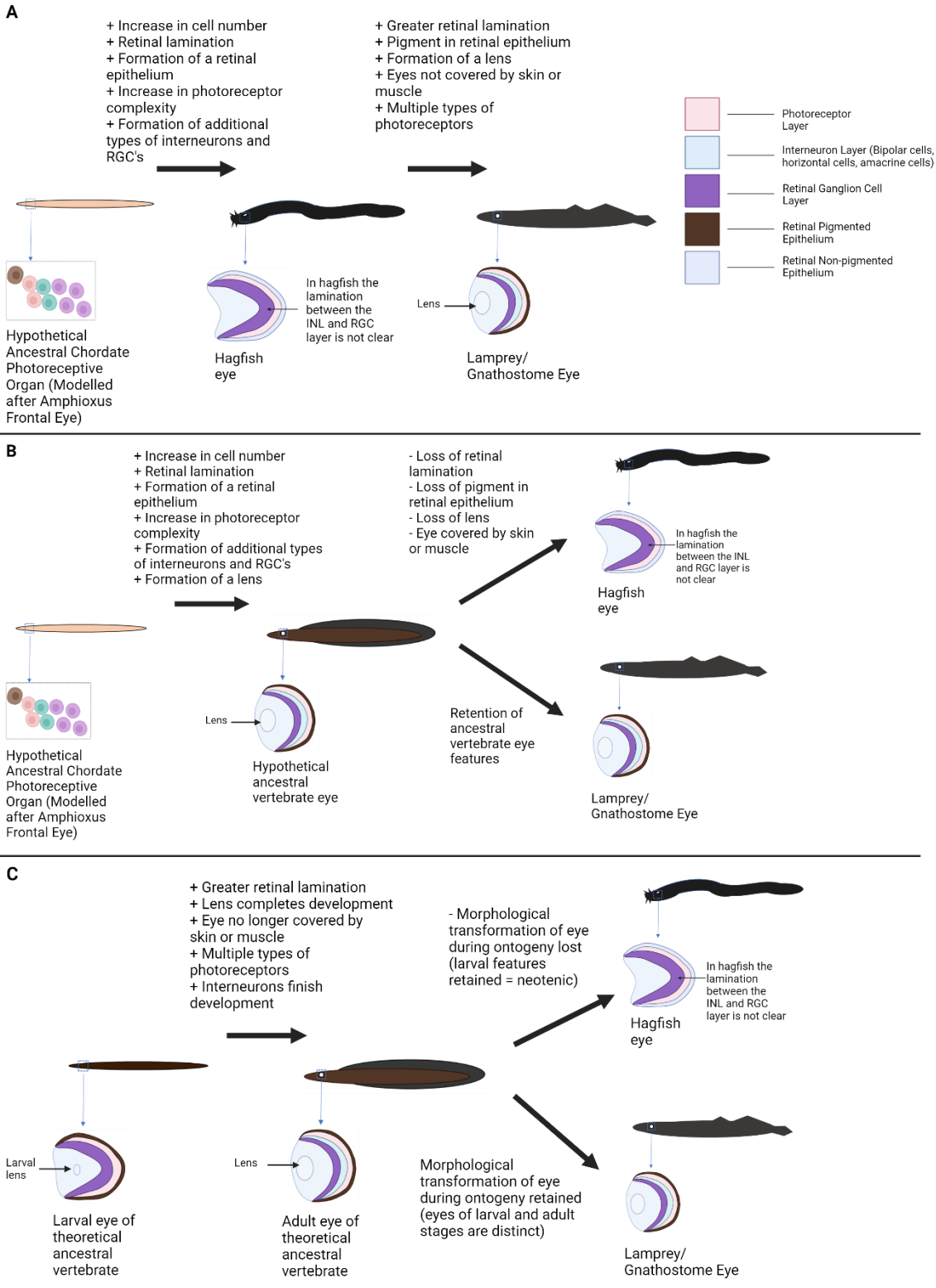

A
+ Increase in cell number
+ Retinal lamination
+ Formation of a retinal epithelium
+ Increase in photoreceptor complexity
+ Formation of additional types of interneurons and RGC's
+ Greater retinal lamination
+ Pigment in retinal epithelium
+ Formation of a lens
+ Eyes not covered by skin or muscle
+ Multiple types of photoreceptors
Photoreceptor Layer
Interneuron Layer (Bipolar cells, horizontal cells, amacrine cells)
Retinal Ganglion Cell Layer
Retinal Pigmented Epithelium
Retinal Non-pigmented Epithelium
Hypothetical Ancestral Chordate Photoreceptive Organ (Modelled after Amphioxus Frontal Eye)
In hagfish the lamination between the INL and RGC layer is not clear
Hagfish eye
Lens
Lamprey/ Gnathostome Eye
B
+ Increase in cell number
+ Retinal lamination
+ Formation of a retinal epithelium
+ Increase in photoreceptor complexity
+ Formation of additional types of interneurons and RGC's
+ Formation of a lens
- Loss of retinal lamination
- Loss of pigment in retinal epithelium
- Loss of lens
- Eye covered by skin or muscle
In hagfish the lamination between the INL and RGC layer is not clear
Hagfish eye
Hypothetical Ancestral Chordate Photoreceptive Organ (Modelled after Amphioxus Frontal Eye)
Lens
Hypothetical ancestral vertebrate eye
Retention of ancestral vertebrate eye features
Lamprey/ Gnathostome Eye
C
+ Greater retinal lamination
+ Lens completes development
+ Eye no longer covered by skin or muscle
+ Multiple types of photoreceptors
+ Interneurons finish development
- Morphological transformation of eye during ontogeny lost (larval features retained = neotenic)
In hagfish the lamination between the INL and RGC layer is not clear
Hagfish eye
Larval lens
Larval eye of theoretical ancestral vertebrate
Lens
Adult eye of theoretical ancestral vertebrate
Morphological transformation of eye during ontogeny retained (eyes of larval and adult stages are distinct)
Lamprey/ Gnathostome Eye

Figure 1-4

Figure 1-4: Alternative models of vertebrate eye evolution. A) The hagfish eye as the ancestral condition of the vertebrate eye. In this scenario the extant hagfish eye is representative of the ancestral vertebrate eye state (this 'primitive' eye still has greater complexity than the photoreceptive structures of non-vertebrate chordates). The lamprey converged upon a more complex eye condition alongside the gnathostomes. B) The hagfish eye as a degenerate/regressed condition. In this scenario the last common ancestor of cyclostomes and gnathostomes had a relatively sophisticated eye. Lampreys and the lineage leading to gnathostomes maintained this eye condition whereas the hagfish eye degenerated resulting in extant hagfish possessing rudimentary visual structures. C) The hagfish eye as a paedomorphic/neotenic condition. In this hypothesis, the last common ancestor of gnathostomes and cyclostomes would have undergone a shift in eye morphology during ontogeny, with larvae having more rudimentary features and adults having complex eyes. Lampreys (and gnathostomes) maintained this transition to a more complex state. The hagfish lost the transition and the eye seen in adult hagfish represents 'juvenile' vertebrate eye features. It is also possible that a combination of these scenarios could have contributed to the state of the extant hagfish eye (see **Section 1.5**). Graphics created using BioRender.com.

Table 1-1. Presence of retinal homeobox genes across representative vertebrates

Gene	Hagfish	Lamprey	Zebrafish	Mouse	Function
Pax 6	Present (Dong and Allison, 2021; Feiner et al., 2014)	Present (Ravi et al., 2019)	Present (Feiner et al., 2014)	Present (Feiner et al., 2014)	Acts as a master regulator of eye development, specifies eye field, regulates timing of retinogenesis, regulates retinal cell multipotency and contributes to specification of multiple retinal cell types (Chow et al., 1999; Marquardt et al., 2001; Kozmik, 2005; Philips et al., 2005; Oron-Karni et al., 2008; Remez et al., 2017)
Otx1	Present * (*OtxC*)	Present (*OtxC*) (Yamamoto et al., 2020)	Present (Lane and Lister, 2012)	Present (Martinez-Morales et al., 2001)	*Otx1* contributes to the formation of distinct eye regions (i.e., neural retina vs. RPE) and the proper formation of the neural retina (Martinez-Morales et al., 2001; Lane and Lister, 2012)
Otx2	Present * (*OtxA*)	Present (*OtxA*) (Yamamoto et al., 2020)	Present (Lane and Lister, 2012)	Present (Martinez-Morales et al., 2001; Nishida et al., 2003)	*Otx2* contributes to specification of the eye field in the neural plate, formation of distinct eye territories, and proper formation of photoreceptors and bipolar cells (Martinez-Morales et al., 2001; Nishida et al., 2003; Lane and Lister, 2012)
Otx5/Crx	Present *(*OtxB*)	Present (*OtxB*)	Present** (Shen and	Present (*Crx*)	Aids in terminal differentiation of photoreceptors

		(Yamamoto et al., 2020)	Raymond, 2004)	(Furukawa et al., 1997)	(Chen et al., 1997; Furukawa et al., 1997; Viczian et al., 2003)
Six3	Present *** (Oisi et al., 2013)	Present ***	Present (Kumar, 2009)	Present (Kumar, 2009)	Promotes eye field formation, promotes neural retina fate over RPE and together with *six6* promotes neural retinal progenitor fate (Carl et al., 2002; Kumar, 2009; Diacou et al., 2018)
Six6	Present? ***	Present? ***	Present (Kumar, 2009)	Present (Kumar, 2009)	Promotes neural retinal progenitor fate alongside *six3* (Jean et al., 1999; Kumar, 2009; Diacou et al., 2018)
Rx/Rax	Present (Kon and Furukawa, 2020)	Present (Kon and Furukawa, 2020)	Present (Mathers et al., 1997)	Present (Furukawa et al., 2000)	Necessary for optic vesicle formation; promotes proliferation of retinal progenitor cells, maintains *Pax6* expression, helps specify Müller glia (Mathers et al., 1997; Furukawa et al., 2000; Nelson et al., 2009b)

*Yamamoto et al., (2020) argue lamprey *OtxA, OtxB*, and *OtxC* are homologous to gnathostome *Otx2*, *Otx5/Crx*, and *Otx1* respectively. Higuchi et al., (2019) provide support for the homology of lamprey *OtxA*, *OtxB* and *OtxC* to hagfish *Otx* paralogs. This suggests the hagfish *Otx* genes are also homologs to gnathostome *Otx2*, *Otx5/Crx*, and *Otx1*.

**Mammalian *Crx* is a highly divergent orthologue of *Otx5* whereas the zebrafish *Crx* gene is believed to be from an independent duplication event (zebrafish have both an *Otx5* gene and a *Crx* gene) (Plouhinec et al., 2003; Shen and Raymond, 2004)

***Hagfish (*Eptatretus burgeri*) and lamprey (*Petromyzon marinus*) appear to have *Six3/6* paralogs, but it is difficult to assign the homologs as closer to a *Six3* or *Six6* identity. Oisi et al., (2013) identified a *Six3/6* homolog in hagfish. A TBLASTN search against the hagfish and lamprey genomes in Ensembl identified three possible *Six3/6* homologs in hagfish and three possible homologs in lamprey (when reciprocally blasted each of these sequences were the closest match to mouse and zebrafish *Six3* or *Six6*).

Table 1-2. Presence of retinal bHLH genes across representative vertebrates

Gene	Hagfish	Lamprey	Zebrafish	Mouse	Function
Atoh7	None found *	Present (Lara-Ramirez, 2013 (unpublished))	Present (Miesfeld et al., 2020	Present (Miesfeld et al., 2020)	Retinal ganglion cell specification (Brown et al., 2001; Kay et al., 2001; Wang et al., 2001)
Ascl1	Present**	Present (Häming et al., 2011)	Present (Jorstad et al., 2017)	Present (Jorstad et al., 2017)	Regulation of Notch signalling during retinogenesis, Müller glia reprogramming to multipotency, specification of bipolar cells (Hatakeyama et al., 2001; Nelson et al., 2009a; Gao et al., 2021)
NeuroD1	Present (Higuchi et al., 2019)	Present (Higuchi et al., 2019)	Present (Ochocinska and Hitchcock, 2009)	Present (Cherry et al., 2011)	Photoreceptor and amacrine cell differentiation (Inoue et al., 2002; Ochocinska and Hitchcock, 2009)
NeuroD4	None found	None found	Present (*Zath3*) (Wang et al., 2003)	Present (*Math3*) (Cherry et al., 2011)	Bipolar cell and amacrine cell specification (Hatakeyama et al., 2001; Inoue et al., 2002)
NeuroG	Present (Higuchi et al., 2019)	Present*** (Higuchi et al., 2019)	Present *** (Korzh et al., 1998; Jeong et al., 2006; Hufnagel et al., 2010)	Present (Hufnagel et al., 2010)	Helps to drive the initial wave of retinogenesis in the retina (in mammals) (Hufnagel et al., 2010)

* An *Atoh1* homolog was the top result for a TBLASTN search of mouse and zebrafish *Atoh7* sequences against the hagfish (*Eptatretus burgeri*) genome in Ensembl. A reciprocal blast of hagfish *Atoh1* matched mouse and zebrafish *Atoh1* more closely than *Atoh7*.

** Three hagfish *ascl1* homologs were identified via a TBLASTN search using mouse and zebrafish *ascl1* homologs as query sequences against the hagfish (*E. burgeri*) genome. The reciprocal best hits for each of the three hagfish sequences was *ascl1* sequences from mouse and zebrafish.

*** *NeuroG* homologs are present in zebrafish but their function in eye/retina development is unclear compared to mammals. Similarly, *NeuroG* was identified in the lamprey genome but does not appear to be expressed in the eye (Lara-Ramirez et al., 2015).

Chapter 2:

The retina of an early-branching vertebrate reveals deep conservation of mechanisms for retinogenesis within the vertebrate lineage

2.1 Abstract

The retina is a complex photosensory structure that is crucial for light mediated behaviors. However, the evolutionary origins of the vertebrate retina are currently a mystery. Comparative morphology has revealed fascinating structural and organizational differences in the eyes of cyclostomes (jawless vertebrates) and gnathostomes (jawed vertebrates). Here we examine retinal development to complement that approach and have begun to characterize retinogenesis in a representative cyclostome, the Pacific hagfish (*Eptatretus stoutii*). Knowledge of hagfish neurodevelopment is very limited because hagfish embryos are difficult to acquire, but evidence suggests they may have continued retinal neurogenesis late into their ontogeny. We applied a brief pulse of EdU to label proliferating cells, if any, in the retina. We also utilized bioinformatics and *in situ* hybridization to reveal if homologs of gnathostome retinal genes also drive retinogenesis in a jawless vertebrate. We observed EdU+ cells within the retinal periphery of the hagfish, a region reminiscent of the ciliary marginal zone of gnathostomes, in addition to within the central retina. We found that hagfish possess homologs of several key genes required for retinal neurogenesis in other vertebrates, and that these genes are expressed within the hagfish eye. We also demonstrated through *in situ* hybridization that two of these genes, *OtxA* and *Rx* (retinal homeobox), are expressed in the hagfish retina, including within the proliferative retinal periphery. These findings support that there are deeply conserved mechanisms for retinogenesis within the vertebrate lineage.

2.2 Introduction

The vertebrate eye and retina are highly conserved sensory structures that are remarkable for their morphological complexity, capacity for image formation, and also the stark mystery surrounding their evolutionary origins. Paired eyes and a sophisticated retina arose early in vertebrate evolution and are present in all extant representatives of this group (Bradshaw and Allison, 2022). In comparison, the light sensitive organs of the non-vertebrate chordates are composed of simpler clusters of photoreceptor and pigment cells that are only capable of light detection and not image formation (Lamb, 2013). To understand how the visual sense organs of vertebrates gained such complexity, previous work has employed the study of fossils or comparative morphology to explain the evolution of the eye and retina (Collin et al., 2009; Collin 2010; Davies et al., 2012; Lamb, 2013; Gabbott et al., 2016). These studies have revealed that the eyes of the early-branching jawless vertebrates (Cyclostomata) contain striking differences in structure and organization to other vertebrates. However, it is difficult to determine if these differences are due to retention of ancestral traits or secondary loss (Fernholm and Holmberg, 1975; Bradshaw and Allison, 2022).

Therefore, we propose taking an evolutionary and developmental (evo-devo) approach to illuminate the evolutionary history of the retina. We have begun to explore retinogenesis in an obscure but phylogenetically important vertebrate species, the Pacific hagfish (*Eptatretus stoutii*). These organisms are deep-sea marine scavengers, and little is known about the development of their visual system (or their development in general) (Locket and Jørgensen, 1998; Martini, 1998). As a representative cyclostome, characterizing the hagfish retina would be highly informative and create a basis of comparison for the data available from the well-studied jawed vertebrates (Gnathostomata) (Bradshaw and Allison, 2022). A comparison of the retinal

developmental pathways between cyclostomes and gnathostomes will help to infer what the state of retinal development would have been like in the last common ancestor of jawed and jawless vertebrates.

When compared to other vertebrates, the hagfish eye is strikingly small and rudimentary. The eye is buried under a translucent layer of skin (or even buried beneath muscle in certain species) and lacks a lens (Fernholm and Holmberg, 1975). The hagfish retina is unpigmented and based on morphology the retinal layers appear poorly laminated (Dong and Allison, 2021). These observations have led to the very reasonable interpretation that the hagfish eye is representative of the ancestral or proto-vertebrate eye condition (Collin et al., 2009; Lamb, 2013). However, recently our research group has helped to revise this understanding by providing compelling evidence that the hagfish retina is not a transitional form but is instead a rudiment that remains following regression of a once elaborate organ (Dong and Allison, 2021; Bradshaw and Allison 2022). Careful examination of the hagfish eye and use of molecular markers reveals the presence of photoreceptors, interneurons and retinal ganglion cells within the retina (Dong and Allison, 2021). The existence of the interneurons was previously obscured by the disorganized lamination of the inner nuclear layer. Compared to gnathostome eyes which have the interneurons and retinal ganglion cell bodies organized into two distinct layers, these two cell populations are intertwined in the hagfish retina (Dong and Allison, 2021). In addition, although the retinal epithelium layer is non-pigmented, this layer does appear to contain machinery required to complete the retinoid cycle, suggesting the hagfish retina may be more functional (and share a greater affinity with gnathostome eyes) than previously thought (Dong and Allison, 2021).

Phylogenetic studies based on molecular data support the monophyly of hagfishes with the other living cyclostome clade, the lampreys (see Figure 1-1A) (Mallatt and Sullivan, 1998; Kuraku et

al., 1999; Heimberg et al., 2010; Ota et al., 2011; Miyashita et al., 2019). This is very informative for interpreting the history that produced the rudimentary state of the hagfish eye, as the lamprey eye is similar morphologically to the gnathostome eye. The lamprey eye contains a lens, a pigmented RPE, and elaborate lamination of the interneuron and retinal ganglion cell layers (Suzuki and Grillner, 2018; Bradshaw and Allison, 2022). If the hagfish eye represents the state of the proto-vertebrate eye, lampreys would have converged onto an extremely similar retinal morphology to jawed vertebrates, a scenario that is highly unlikely. This provides further support that the rudimentary morphological features of the hagfish eye are due to evolutionary loss (regression) rather than retention of an ancestral state (Fernholm and Holmberg, 1975; Dong and Allison, 2021; Bradshaw and Allison, 2022). However, given hagfish diverged from jawed vertebrates 600 million years ago (Blair and Hedges, 2005) the retina of this group is still highly important for inferring the state of the eye in the last vertebrate common ancestor. Exploring cyclostome eye development will reveal either deeply shared homology with gnathostomes or divergent mechanisms driving vertebrate retinal formation.

Although hagfish are in an excellent phylogenetic position to answer questions about vertebrate eye evolution, it is challenging to utilize an evo-devo approach for this organism because hagfish embryos are difficult to acquire (Gorbman, 1997; Holland, 2007; Kuratani and Ota, 2008a; Ota and Kuratani, 2006; Ota and Kuratani, 2008). Therefore, we turn our attention to the possibility of examining retinal development in the form of ongoing tissue morphogenesis in post-embryonic hagfish. In fish and amphibians, adult retinogenesis recapitulates many aspects of retinal embryonic development (Perron et al., 1998; Xu et al., 2020). Recent work suggests the hagfish eye continues to grow late into ontogeny (Dong and Allison, 2021). The hagfish eye increases in size with body size (and presumably age) of hagfish. In addition, the peripheral

region of the hagfish retina also expresses *Pax6*, a marker of retinal progenitor cells (Dong and Allison, 2021). The occurrence of *Pax6* at the hagfish retinal periphery is reminiscent of the *Pax6* expression in the gnathostome ciliary marginal zone (CMZ). The ciliary marginal zone (CMZ) is a retinal stem cell niche found at the retinal periphery in several vertebrate groups, including teleost fish and amphibians (Raymond et al., 2006). If the hagfish retinal margin is equivalent to a CMZ-like zone, then this region could be extremely useful for investigating mechanisms of retinal development/growth in adult animals.

Finding evidence of a proliferative region in the hagfish retina would be extremely surprising given the rudimentary nature of the eye. In addition, limited information from lampreys suggests that the only other living cyclostome group does not have adult retinal neurogenesis (Villar-Cheda et al., 2008). The possibility of a CMZ in hagfish suggests that post-embryonic retinogenesis could be an ancestral characteristic that has been secondarily lost in several vertebrate groups. Based on the available evidence, we hypothesize that the hagfish retinal periphery functions similarly to the gnathostome CMZ and drives continued retinal neurogenesis past embryonic development. The aims of this chapter are to determine where (if anywhere) proliferation occurs in the adult hagfish retina and to utilize this ongoing growth to study the genetic mechanisms producing new retinal tissue. We have identified several genes known to have highly conserved roles in retinogenesis of other vertebrates that we expect to be expressed in the hagfish retinal margin. These genes include the *Otx* genes (Plouhinec et al., 2003; Yamamoto et al., 2020), and the homeobox genes *Rx* (Mathers et al., 1997) and *Six3* (Carl et al., 2002). If the retina of adult hagfish displays similar cell proliferation and gene expression patterns to the gnathostome CMZ, this would provide support that there are shared (conserved) developmental mechanisms driving retinal growth in cyclostomes and gnathostomes. The

presence of a CMZ in hagfish also suggests post-embryonic neurogenesis in the retina may have originated early in the evolution of the retina.

2.3 Materials and Methods

2.3.1 Animal collection and care

This study was conducted under the approval of the BioSciences Animal Care and Use Committee at the University of Alberta (Animal Use Protocol number: AUP00000077) which adheres to the Canadian Council for Animal Care guidelines. Animals were caught and held according to a Department of Fisheries and Oceans Canada collection permit (XR 225 2021) and the animal use protocol approved by the Bamfield Marine Sciences Center (BMSC) (RS-21-06). Hagfish were acquired near BMSC in Barkley Sound on the west coast of Vancouver Island, British Columbia, Canada. BMSC staff caught the hagfish using a Korean cone trap baited with dead fish (BMSC Animal Care Standard Operating Procedure, Hagfish (*Eptatretus stoutii*), v.3 (Bartlett and Janusson, 2019)). The traps were deployed for 8-12 hours and then brought to the surface. Hagfish were held in large tanks with circulating seawater until tissue was collected (1-3 days). Hagfish were euthanized by administration of excess MS-222 (tricaine methanesulfonate). Eye and brain tissues were collected from euthanized specimens and were preserved in 4% paraformaldehyde /5% sucrose/0.1M phosphate buffer (referred to as 4% PFA from here on) or RNAlater (Invitrogen, Cat. No. AM7020).

2.3.2 EdU administration and tissue collection

Ten hagfish were anesthetized via MS-222 administration until they no longer responded to physical stimuli (i.e., touching, poking). The animals ranged in size from 26.99 g to 110.17g. An

intraperitoneal injection was completed by inserting a needle into the ventral side of the hagfish abdomen with the skin held off the wall of the abdomen. 20 ul of 10mM EdU (5-ethynyl-2'-deoxyuridine) (EdU in vivo kit, baseclick GmbH, Cat. No. BCK488-IV-IM-S) diluted in PBS (phosphate buffered saline, pH 7.4) was injected per gram of body weight (Zhang et al., 2014). Fish were then allowed to recover in a bucket with circulating seawater. After 24 hours the fish were sacrificed, and eye and brain tissues were collected and preserved in 4% PFA as described previously.

2.3.3 Cryopreservation and cryosectioning of fixed tissue

After fixation with 4% PFA, hagfish eye samples were cryopreserved in a series of sucrose washes. The eyes were washed for 1 hour in 12.5% sucrose//0.1M phosphate buffer (room temperature), 1 hour in 20% sucrose//0.1M phosphate buffer (room temperature), and then overnight (at 4 °C) in 30% sucrose//phosphate buffer. The following day, the eyes were placed in a 1:1 solution of 30% sucrose//phosphate buffer and OCT (optimal cutting temperature) embedding compound (hereafter referred to as OCT) (VWR® Premium Frozen Section Compound, Cat. No. 95057-838) for 1 hour (4C). The samples were then flash frozen in a mold (made by taking the bottom half of a 1.5mL centrifuge tube and fixing it to a glass slide with nail polish) filled with OCT using dry ice. The frozen samples in the molds ('cryostat chucks') were stored at -80°C at least overnight prior to sectioning. The samples were sectioned at 10 μM thickness using a Leica CM 1900 UV cryostat (chamber temperature: -20°C). The sections were placed onto Superfrost Plus microscope slides (ThermoFisher Scientific, Cat. No. 12-550-15) and kept at -80°C at least overnight until use.

2.3.4 EdU detection assay

An EdU cell proliferation detection kit (EdU in vivo kit, baseclick GmbH, Cat. No. BCK488-IV-IM-S or BCK647-IV-IM-S) was used to detect EdU+ cells in retinal sections from six of the ten hagfish individuals treated with EdU. Sectioned eye tissue was thawed and fixed in 4% PFA for 15 min prior to the EdU detection assay. The samples were washed thrice for 5 min in 3% bovine serum albumin in PBS (pH 7.4) (hereafter 3% BSA). The samples were re-hydrated with PBS + 0.1% Tween (PBS-T) for 30 min. The sections were then permeabilized by adding a solution of PBS with 0.1% Triton X-100 onto the samples for 20 min (and then rinsed 3X 5 min in 3% BSA). The EdU detection cocktail was prepared according to the manufacturer's instructions (EdU in vivo kit, baseclick GmbH, Cat. No. BCK488-IV-IM-S or BCK647-IV-IM-S) and applied to the slides for 30 min. The samples were kept in the dark during this period and gently rocked/agitated every 5 min. Once the detection solution was removed, the samples were rinsed twice for 5 min in 3% BSA and 2 X 5 min in PBS-T. Nuclei were stained using 1ug/ml DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) (Invitrogen™, Cat. No. D1306) diluted 1:1000 in PBS-T. The nuclear stain was applied for 10 min. The DAPI solution was then removed, and the samples washed 3 X 15 min in PBS. The completed slides were covered with 80% glycerol and sealed with nail polish. The slides were imaged using an LSM 710 confocal microscope (Zeiss) or an Olympus FV3000 confocal microscope. Of the samples from the six individuals imaged, the sections from three individuals (EdU 5, EdU 6, EdU 8) were intact enough to be used for quantification.

2.3.5 EdU cell quantification and statistical analysis

To determine if EdU + cells were occurring in specific regions of the hagfish retina, the length of the RnPE layer of each retinal section was measured in ImageJ (Schneider et al., 2012). This

length was then used to divide the retinal sections into six equal regions (regions 1-6). Region 1 was the most peripheral whereas region 6 was the most central. The number of EdU+ cells was then quantified in each region for each of the three individuals. This data was analyzed by a 2-way ANOVA followed by a Tukey's multiple comparisons test (GraphPad Prism version 9.5.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). In a second analysis, the same retinal section images were divided into a peripheral 'CMZ-like' zone (based on the hagfish retinal morphology) and a 'central' zone (the remainder of the retina). The number of EdU+ cells were then counted in the two regions. This data was also analyzed by a 2-way ANOVA.

2.3.6 Sox2 immunohistochemistry

Sectioned zebrafish (n=1 eye) and hagfish (n=1 eye) tissues were thawed to room temperature and blocked in a mix of 10% donkey serum (Sigma-Aldrich, Cat. No. D9663-10ML)//PBS- T for 1 hour. Anti-sox2 (raised in goat, human Sox2 immunogen (Accession number: P48431) (R&D systems, Cat. No.AF2018)) was diluted 1:100 in PBS-T with 2% donkey serum and applied to the sections overnight at 4°C (negative controls were treated with only 2% donkey serum//PBS-T without the primary antibody). The samples were rinsed 2X and washed 2X (30 min each) in PBS-T before the secondary antibody solution was applied (1:1000 dilution of anti-goat 488 (Invitrogen, Cat. No. A-11055) or anti-goat 555 (Invitrogen, Cat. No. A-21432) in 2% donkey serum//PBS-T) overnight at 4°C. The samples were then rinsed 2 X and washed 3X (30 min each) in PBS-T before being labelled with 1:1000 DAPI//PBS-T for 30 min. The samples were washed 3 X 15 min in PBS-T and then covered with 80% glycerol and sealed with nail polish. The slides were imaged using an LSM 710 confocal microscope (Zeiss).

2.3.7 Synteny analysis

Two approaches were used to visualize the syntenic relationships between the three gnathostome *Otx* paralogs (*Otx1*, *Otx2*, and *Otx5/Crx*) and the four cyclostome paralogs (*OtxA*, *OtxB*, *OtxC*, *OtxD*). First, Genomicus (v.106) (Muffato et al., 2010; Nguyen et al., 2022) was used to visualize the relationships between the *Otx* genes across representative vertebrate species (mouse (*Mus musculus*), Western clawed frog (*Xenopus tropicalis*), spotted gar (*Lepisosteus oculatus*), coelacanth (*Latimeria chalumnae*), elephant shark (*Callorhinchus milii*), sea lamprey (*Petromyzon marinus*), and inshore hagfish (*Eptatretus burgeri*). The results generated were compared to genomic data in the NCBI (National Center for Biotechnology Information) Genome Data Viewer database (Sayers et al., 2022) (when this data was available - the NCBI database does not contain an annotated hagfish genome).

For synteny analysis using Simple Synteny (Veltri et al., 2016) a tBLASTn (translated nucleotide BLAST) search with a mouse query sequence (the proteins of a mouse *Otx* gene and the 10 genes directly to either side) was used to identify syntenic genomic regions in the area surrounding each *Otx* paralog (the 1 M base pairs directly adjacent on either side of each *Otx* gene) for the lamprey (*P. marinus*) and hagfish (*E. burgeri*). This was repeated using the proteins for each of the three mouse *Otx* paralogs (*Otx1*, *Otx2*, *Crx*) and their adjacent genes as query sequences. The e-value threshold used was 0.0001 and the minimum query coverage cut-off was left at one. The results generated were compared to the NCBI Genome Data Viewer database and hits that were not supported by both the database and the Simple Synteny analysis were excluded (for hagfish an annotated genome assembly is not available on NCBI, so only the syntenic analysis was completed). Query and genomic sequences were retrieved from NCBI for most

species examined. Genomic sequences for the hagfish (*E. burgeri*) were retrieved from the Ensembl database (Ensembl Genome Browser v. 108, Cunningham et al., 2022).

2.3.8 Phylogenetic analysis

A protein alignment of *Otx* paralogs (from representative chordates) was generated using Geneious Prime (Geneious Prime v. 2022.0.1, Biomatters Ltd.) with the Geneious alignment setting and inputted into the IQ-tree webserver (Nguyen et al., 2015; Trifinopoulos et al., 2016). A maximum-likelihood tree was generated using IQ-tree default settings (and ultrafast bootstrap-1000 iterations) (Hoang et al., 2018). Protein sequences were retrieved from NCBI (most organisms) or Ensembl (hagfish).

2.3.9 cDNA production

Hagfish RNA previously extracted by Emily Dong was used to generate cDNA for riboprobe production. cDNA was made as per manufacturer's instructions (Quantabio, Cat. No. 95048-100).

2.3.10 Riboprobe production

An antisense riboprobe to label the *E. stoutii* homolog of *Rx* was produced from a plasmid template using published methods (Barthel and Raymond, 1993; Barratt and Arkell, 2020; Dong and Allison, 2021). The template was generated by creating a gene block (IDT - Integrated DNA Technologies) based on the sequence from CL3100.Contig2_All (*Rx*) (Table 2-1). Restriction enzyme sites added to either end of the gene block to facilitate ligation into a PCS2+ plasmid. The plasmid was linearized with BamHI and used in a transcription reaction overnight with a T3 RNA polymerase (Roche, Cat. No.11031171001) and DIG (digoxigenin) conjugated dNTPs (Roche, Cat. No. 11277073910) to generate an antisense *Rx* probe. The RNA from the reaction

was precipitated and the probe was suspended in 50uL of RNase free water to assess concentration and quality via Nanodrop 2000 (Thermo Scientific, Cat. No. ND-2000). The probe was diluted to its final concentration in Hauptmann's hybridization solution and kept at -80°C before use. The final *Rx* riboprobe was 1206 bp long and spanned three exons of the gene (Table 2-1).

A riboprobe to label the homolog of hagfish (*E. stoutii*) *OtxA* was generated from a template created via PCR reaction on hagfish eye cDNA (Phusion high-fidelity polymerase kit (ThermoFisher Scientific, Cat. No. F530S)) (Barthel and Raymond, 1993; Barratt and Arkell, 2020; Dong and Allison, 2021). Primers were designed to amplify *E. stoutii OtxA* with a T7 RNA polymerase site added to the 5' end of the reverse primer (Table 2-2). The amplicon underwent a transcription reaction similar to the plasmid derived probes (except using a T7 RNA polymerase (Roche, Cat. No. 10881767001)) and the RNA was then precipitated and quantified using the same methods as above. The *OtxA* riboprobe was 591 bp long and spanned three exons of the gene (Table 2-2).

2.3.11 *In situ* hybridization

In situ hybridization was completed as per Dong and Allison (2021). Slides were thawed to room temperature and fixed with 4% PFA for 10 min. After removing the PFA and rinsing the slides with RNase free 2X SSC (saline-sodium citrate buffer), sections were treated with proteinase K (Roche, Cat. No. 3115836001) for 2-10 min (probe dependent – see Table 2-3). They were then rinsed with RNase free 2X SSC and fixed again in PFA for an additional 10 min. After removing PFA and rinsing with 2X SSC, the slides were placed into a mixture of 0.3% acetic anhydride and 0.1M TEA (triethanolamine) for 10 min before being dehydrated through a series of ethanol washes (50%, 75%, 95%, 100% ethanol diluted in DEPC treated water). The slides were then

dried for an hour at room temperature. The slides were incubated with Hauptmann's hybridization solution for an hour at room temperature before the appropriate riboprobe (diluted in Hauptmann's solution) was applied (concentration used was probe dependent - see Table 2-3). For negative control slides, Hauptmann's hybridization solution without an added probe was used. The sections were then placed in a hybridization chamber overnight at 55°C.

The following day the probe solution was removed, and the slides underwent a series of post hybridization washes at 55 °C – 10 min in 2/3 2X SSC: 1/3 post-hybridization buffer, 5 min in 1/3 2X SSC: 2/3 post-hybridization buffer, 5 min in 2X SSC, 20 min in 0.2X SSC+0.1% Tween, and 2 X 20 min in 0.1X SSC+0.1% Tween. The slides were blocked in maleate buffer for an hour before being incubated with an alkaline phosphatase conjugated anti-DIG antibody (Roche, Cat. No. 11093274910) diluted 1:1000 in the maleate block overnight (4°C). The slides were then stained with a solution made using NBT (nitro blue tetrazolium)/BCIP (5-Bromo-4-chloro-3-indolyl phosphate) Ready-to-Use Tablets (Roche, Cat. No.11697471001) following the manufacturer's instructions to visualize the bound riboprobe. For fluorescent *in situ* hybridization (FISH), after the post-hybridization washes the slides were incubated in an alkaline phosphatase conjugated anti-DIG POD (peroxidase) conjugated antibody (Roche, Cat. No. 11207733910) (antibody diluted 1:100 in the maleate block). The slides were incubated with the antibody solution in the dark overnight (4°C). The following day the slides were rinsed 3 X 15 min with PBS-T. The slides were then incubated in an Alexa fluor 488 tyramide detection solution (length of incubation was signal dependent) prepared according to the manufacturer's instructions (Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, Invitrogen™, B40943). The reaction was stopped with application of a 1X stop reaction solution (Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, Invitrogen™, B40943) for 10 min. Slides were rinsed 3 X with PBS-T to remove any residual

detection and stop solutions. The slides were then stained with DAPI as previously described and sealed with 80% glycerol and nail polish. The slides were imaged on a LSM 710 Confocal (Zeiss) or a FV3000 Confocal (Olympus).

2.4 Results

2.4.1 The post-embryonic hagfish retina contains proliferating cells, including at the retinal margin (the putative CMZ)

To assess whether the adult hagfish retina contains proliferating cells, several adult hagfish were treated with a brief 24 h pulse of EdU (a thymidine analog incorporated into DNA of proliferating cells (Chehrehasa et al., 2009)) and sacrificed the next day. Sectioned eye tissue from those individuals underwent a ClickIt EdU detection reaction to label EdU positive (newly born) cells. The majority of retinal sections examined contained some cells positively labelled for EdU (Figure 2-1). EdU-positive cells (EdU+) were detected in the eyes of all animals treated with EdU (six individuals examined). The EdU+ cells often occurred in clusters at the peripheral edges of the retina. Some hagfish retina sections also contained several EdU positive cells in the more central portions of the retina. No such labeling was observed in hagfish that did not receive EdU (Figure 2-1C).

Qualitatively, the abundance of EdU+ cells was concentrated at the peripheral margins of the retina, nearest to the iris, consistent with the location of a CMZ in teleosts and other species (Raymond et al., 2006). To support this qualitative observation, we counted the EdU positive cells in various retinal regions: images of hagfish retina slices were divided into 6 equal sections distributed across the peripheral to central retina and EdU+ cells were enumerated (Figure 2-2).

The majority of EdU+ cells were peripherally located (based on 21 retinal sections from three EdU treated individuals – EdU 5, EdU 6, EdU 8) (Figure 2-2B). Over half (57%) of the EdU positive cells occurred in the most peripheral region (region 1) in the three individuals we examined (Figure 2-2C). On average, 3.7 EdU+ cells were found in region 1. Moving centrally, the number of EdU+ cells decreased (on average less than one cell per region for regions 2-6). A two-way ANOVA showed the retinal region had a significant effect on the number of EdU+ cells observed ($p<0.0001$) whereas the individual the retinal samples were taken from did not ($p=0.06$). There was a significant interaction between the effects of individual and region ($F(10, 108) = 6.133$, $p<0.0001$). The number of EdU+ cells in region 1 was significantly higher than the number of cells occurring in any of the other five regions (Tukey's multiple comparisons test, $p<0.0001$).

When the retina was separated into the peripheral region that morphologically resembles the gnathostome CMZ (the area between where the ONL and INL layers merge and the edge of the retina (Raymond et al., 2006)) and the central retina the result differed. In this analysis (two-way ANOVA for region ('CMZ' vs. 'central retina') and individual) there was no significant difference between the number of cells observed in the CMZ region and the rest of the retina ($p=0.73$) (Figure 2-2D). Although many proliferative cells occur in the hagfish retinal CMZ-like zone, there is a substantial number of EdU+ cells occurring in other parts of the retina as well.

Attempts to label proliferating cells with antibodies such as PCNA or pH3 were not successful, but we attribute this to technical problems (non-optimal tissue fixative and/or challenges optimizing antigen retrieval). Attempts to label hagfish retinal tissue with a Sox2 antibody (a marker for neuronal progenitor cells) also failed. In zebrafish tissue, Sox2 clearly labelled cells in the INL whereas in hagfish no noticeable labelling was observed (Figure 2-3). The antibody

was designed against the human Sox2 protein, so we believe the lack of label could be due to low protein conservation between humans and hagfish rather than an absence of neuronal progenitor cells in the retina.

2.4.2 Bioinformatic and phylogenetic analysis supports homology of hagfish retinal transcripts with critical retinal development genes in lampreys and jawed vertebrates

The bioinformatics analyses reveal that hagfish have homologs for several genes known to be required for gnathostome retinal development. These homologs were initially identified via tBLASTn searches of the hagfish genome (*E. burgeri* genome assembly available on Ensembl v.108) and an available RNAseq dataset of *E. stoutii* (generated by Emily Dong; Dong and Allison, 2021) with query sequences from representative gnathostomes (mouse, spotted gar, elephant shark) (Table 2-4, Table 2-5, Table 2-6). tBLASTn searches of the hagfish genome were completed through the Ensembl BLAST search interface, whereas searches against the RNA sequencing dataset were performed with the BLAST function available in Geneious Prime (v. 2022.0, Biomatters). For reciprocal BLAST searches, RNA transcript sequences were queried against the NCBI Nucleotide collection (nr/nt) database.

The analyses identified one hagfish retinal homeobox (*rx*) homolog in the *E. burgeri* genome and the *E. stoutii* RNA sequencing dataset (Table 2-4). The *E. burgeri Rx* sequence was annotated in Ensembl (v. 108) and tBLASTn searches support its homology with gnathostome *Rx*, even if protein sequence conservation is low (Figure 2-4). The *E. burgeri Rx* genomic sequence and mouse and spotted gar *Rx sequences* matched closely with one of the *E. stoutii* transcript sequences in a tBLASTn search (CL3100.Contig2_All) (Table 2-7). The *E.stoutii Rx* transcript sequence had higher expression in eye tissue than the brain, suggesting that this gene may play

an important role in the eye. Therefore, this gene was selected as a target for *in situ* hybridization.

The analyses also identified three *Six3/6* homologs in the *E. burgeri* genome (the sequences could not be assigned as the direct ortholog of either the *Six3* or *Six6* gnathostome paralogs) (Table 2-5). One of these sequences (ENSEBUG00000012950) was annotated as the hagfish *Six3* homolog in Ensembl (v. 108). Comparing the percent identity of the hagfish (*E. burgeri*) *Six3/6* protein sequences to gar (*L. oculatus*) and mouse (*M. musculus*) *Six* proteins suggests the hagfish *Six3/6* is closer to gnathostome *Six6* (Figure 2-5). However, when mouse and gar *Six gene* sequences were used as query sequences in a tBLASTn search against the hagfish genome, the *Six3* sequences were stronger matches with the hagfish *Six3/6* paralog than the *Six6* sequences (Table 2-5). The three *E. burgeri Six3/6* genomic sequences matched closely with three *E. stoutii* transcript sequences from the RNA sequencing dataset. The query gnathostome *Six3* and *Six6* paralogs matched most closely to one of these *E. stoutii Six3/6* transcripts (Unigene16248_All) (Table 2-5). This transcript also had much higher expression in eye tissue than in the brain, suggesting it may have a specific function in the eye (Table 2-7). Therefore, this paralog sequence was identified as a possible candidate for *in situ* hybridization (although this probe was not fully optimized – see Supplementary Figure 1 and Supplementary Table 1).

The tBLASTn searches also identified four potential *Otx* homologs in the *E. burgeri* genome. Two of these genes were previously annotated in Ensembl (v. 108) (*OtxA* and *OtxD*). The tBLASTn searches support a strong relationship between gnathostome and cyclostome *Otx* genes although protein sequence conservation is low (Figure 2-6, Table 2-6). It is difficult to assign direct orthology relationships between gnathostome and cyclostome *Otx* homologs based on this data, so synteny and phylogenetic analysis was employed to elucidate these relationships.

A phylogenetic analysis supports the overall homology of cyclostome *Otx* paralogs with gnathostome *Otx* genes, but the individual paralogs do not line up clearly with gnathostome *Otx1*, *Otx2*, or *Otx5* (Figure 2-7). Lamprey and hagfish *OtxB* and *OtxC* were placed into a sister group with gnathostome *Otx1* (lamprey *OtxD* was also placed in this group). Cyclostome *OtxA* formed a sister group to hagfish *OtxD*. The group containing the cyclostome *OtxA* and lamprey *OtxD* sequences did not form a sister group with one of the gnathostome *Otx* paralogs. Rather, the node containing both gnathostome *Otx1* and cyclostome *OtxB*/*OtxC* was the sister group to these sequences, leaving the orthology relationships unclear. The Drosophila *Otx* homolog (*oc*) did not cluster with the vertebrate sequences, as expected.

Synteny analysis of cyclostome *Otx* genes support hagfish (and lamprey) *OtxC* are homologous to gnathostome *Otx1* (Figure 2-8A, Figure 2-9). In gnathostomes *Otx1* is almost always directly adjacent to *Ehbp1*, *Wdpcp*, *Mdh1* and *Peli1*. Homologs of these genes occur near cyclostome *OtxC*. Similarly, syntenic relationships support hagfish *OtxA* and *OtxD* as being homologous to gnathostome *Otx2*. *Otx2* is generally found near *Tmem260*, *Peli2*, *Slc35f4* and *Naa30* (Figure 2-8B, Figure 2-10). Hagfish and lamprey *OtxA* are located in between *Slc35f4* and *Tmem260* homologs. For both lamprey and hagfish, *OtxD* is found near an *Naa30* and a *Peli2* homolog. Homologous relationships between cyclostome *OtxB* genes and gnathostome *Otx* homologs were harder to determine with synteny. The genomic regions surrounding hagfish and lamprey *OtxB* did not share many genes in common with those surrounding gnathostome *Otx* genes (Figure 2-8C, Figure 2-11). It should be noted that *UGP2* (a gene commonly located near *Otx1* in the gnathostome genomes examined) did occur near both hagfish and lamprey *OtxB*, but no other potentially syntenic genes could be identified in this analysis. Based on the synteny data it was decided that hagfish *OtxA* and *OtxD* (as likely homologs of gnathostome *Otx2*) would be chosen

as targets for *in situ* hybridization. The *E. stoutii OtxA* (Unigene14754_All) and *OtxD* (CL6481.Contig1_All) transcript sequences were identified via tBLASTn searches (Table 2-6) and used to generate riboprobes. Only the *OtxA* probe was used for *in situ* hybridization (the *OtxD* probe was created, but not fully optimized (see Supplementary Table 2)). RNA sequencing data showed that both genes have higher expression in the eye than in the brain, supporting they may have specific roles in the eye and/or retina (Table 2-7).

2.4.3 The hagfish retina expresses markers for retinal progenitor cells and neurogenesis

In *situ hybridization* on hagfish retinal sections reveals expression of *OtxA* throughout multiple areas of the retina (Figure 2-12). Several cell populations appear to label for the *OtxA* probe including cells in the RnPE (retinal non-pigmented epithelium), the retinal periphery (CMZ), the inner nuclear layer, and possibly the photoreceptor layer (Figure 2-12A, B, D, E). Expression in the RnPE is particularly strong. In *situ hybridization* performed for the *Rx* probe reveals labelling within cells of the inner nuclear layer and perhaps the retinal periphery (Figure 2-13).

2.5 Discussion

2.5.1 The adult hagfish retina continues to proliferate despite its rudimentary features

Although the hagfish retina is small and has reduced organization compared to other vertebrates, EdU cell labeling reveals that the adult hagfish retina contains actively proliferating cells (Figure 2-1). Retinal sections examined from EdU treated animals consistently contained EdU+ cells with over 50% of these cells being located at the retinal margin (Figure 2-2). This finding mirrors a retinal stem cell niche found in other vertebrates, the ciliary marginal zone (CMZ) (Raymond et al., 2006). Other data also supports the hagfish retina grows throughout ontogeny.

Eye size increases with hagfish body size, suggesting continuous retinal growth through the animal's lifespan (Dong and Allison, 2021). In addition, Dong and Allison (2021) demonstrated that the periphery of the hagfish retina expresses *Pax6*, a marker for retinal progenitor cells and the CMZ. Together with this study's findings that the hagfish retinal periphery expresses *OtxA* and *Rx* (markers of developing retinal neurons and neuronal progenitors respectively – see **Section 2.5.3**) these results support the EdU+ cells at the hagfish retinal periphery may be part of a CMZ-like structure (Dong and Allison, 2021).

The vertebrate CMZ is a stem cell niche that is best known from studies on teleost fish and amphibians (Raymond et al., 2006). The niche is less prominent in adult birds and is essentially absent in elasmobranchs (sharks and rays) and mammals (Fischer et al. 2014; Hernández-Núñez et al., 2021). Interestingly, sharks do exhibit continual retinal growth past sexual maturity (Harahush et al., 2009; Hernández-Núñez et al., 2021). Hernández-Núñez et al., (2021) suggest the continued growth could be driven by changes to retinal cell size and morphology rather than continued proliferation from the CMZ. One paper suggests the niche may also be absent in the lamprey retina (Villar-Cheda et al., 2008). Finding active growth of the retina in adult hagfish is surprising given the hagfish eye is so rudimentary in form. Why maintain and actively grow a tissue which cannot be used for vision? This raises the possibility that there are other functions for this tissue (i.e., regulation of circadian rhythm) or continued proliferation could be a paedomorphic trait where developmental retinal growth continues into adulthood. As mentioned, no CMZ-like tissue has been identified so far in other early branching vertebrate groups such as lampreys and sharks (Villar-Cheda et al., 2008; Hernández-Núñez et al., 2021). These organisms appear to lose neurogenesis in the eye once adulthood is reached. This suggests several possible evolutionary scenarios. One possibility is constitutive retinal neurogenesis was the ancestral

vertebrate condition and lampreys, sharks and mammals have lost this ability. Another scenario is that ancestrally vertebrates lost retinal proliferation after the embryonic stage but hagfish, teleost fish, and amphibians exhibit paedomorphosis for this particular trait. Alternatively, certain gnathostome groups may have gained the CMZ via a different mechanism altogether.

When quantified by position from the edge of the retina, roughly 40% of the EdU labelled cells were found in the more central regions (Figure 2-2C). In addition, when cells were assigned a 'CMZ' or 'central retina' identity based on morphology of the retinal sections, similar numbers of proliferative cells were observed in the main body of the retina as in the presumptive CMZ region (Figure 2-2D). These central EdU+ cells do not occur in the correct location to be considered part of a (peripheral) CMZ-like stem cell niche. The identity of these cells is currently unknown, but their presence is consistent with proliferating cells in the mature retina of teleost fish. It is possible these cells could be a Müller glia like cell (a retinal glial cell that can de-differentiate to produce new retinal neurons). Similar to the distribution of the centrally located EdU+ cells in the hagfish retina, Müller glia are normally located throughout the inner nuclear layer in jawed vertebrates. Müller glia proliferation can be activated in response to retinal injury (Raymond et al., 2006). In certain vertebrates (i.e., teleosts) the glia also proliferate under normal physiological conditions to produce certain retinal cell types (i.e., rod photoreceptors) (Lenkowski and Raymond, 2014). For this study, in order to collect hagfish from their natural environment the animals were captured from a depth of 80 m and were abruptly brought to the surface. This event likely exposed the fish to far brighter light than they were acclimated to and could have induced retinal injury. Therefore, if hagfish do have a Müller glia-based regeneration mechanism, the collection event could drive more glia to dedifferentiate and proliferate than under natural conditions. There is one report of Müller glia in a species of lamprey (based on

immunohistochemistry markers) (Fernández-López et al., 2016), but no studies have examined the hagfish retina for the presence of Müller glia as of yet. Alternatively, the unidentified EdU+ cells could be a different cell type such as microglia (Silverman and Wong, 2018) or even blood cells. Further work is needed to confirm the identity of the centrally located EdU+ cells.

The findings also raise additional questions about adult neurogenesis in hagfish. If continued retinal growth is a form of paedomorphosis would there be greater (relative) retinal proliferation in young hagfish than in older hagfish? As the animals age do they lose the ability to produce new retinal cells? Another factor to consider would be the rate of retinal proliferation. Future studies should expose hagfish to different durations of EdU administration (both shorter and longer periods than the 24 h pulse used in this study) to characterize how quickly new cells are produced and incorporated into the hagfish retina. Furthermore, the EdU labelled cells from hagfish treated with long durations of EdU exposure (several days to weeks) could be labelled with additional markers (i.e., for neuronal progenitors, for mature retinal cells). This would make it possible to confirm if the proliferating cells of the hagfish retina differentiate and join the mature retina as in the gnathostome CMZ. An attempt was made in this study to label neuronal progenitors in the hagfish retina with an antibody for Sox2 (Taranova et al., 2006; Gorsuch et al., 2017). This antibody did not label the retina in hagfish (although it clearly labelled cells in zebrafish) (Figure 2-3). However, this result is likely due to poor protein conservation between the target protein of the antibody (human Sox2) and the hagfish Sox2 homolog rather than an absence of neuronal progenitor cells in hagfish retina. There are other markers for which hagfish and gnathostomes have higher protein sequence conservation. An *in-situ* hybridization using probes designed against genes such as *Notch1* or *Olig2* (highly conserved neuronal progenitor

markers) could be very informative for determining the nature of the retinal periphery in hagfish (Perron and Harris, 2000; Nakamura et al., 2006; Dvoriantchikova et al., 2015).

2.5.2 Hagfish homologs of genes critical for gnathostome retinal development are expressed in the eye

Examination of hagfish genomic and RNAseq datasets revealed the hagfish eye expresses transcripts of several genes critical for vertebrate eye and retinal development. Some genes of particular interest (as markers of retinogenesis) include the retinal homeobox (*Rx*) gene, the *Six3/6* genes (hagfish have 3 paralogs), and the *Otx* genes (hagfish have 4 paralogs – *OtxA*, *OtxB*, *OtxC*, *OtxD*) (Figures 2-4 to 2-6, Tables 2-4 to 2-6). In gnathostomes these genes are required for proliferation and maintenance of retinal progenitor cells (*Rx* and *Six3* (Nelson et al., 2009b; Diacou et al., 2018) and differentiation of several neuronal cell types (*Otx* genes - photoreceptor and bipolar cell specification (Nishida et al., 2003; Viczian et al., 2003)).

The homology of hagfish (both *E. burgeri* and *E. stoutii*) *Rx* to gnathostome *Rx* homologs was strongly supported by data from tBLASTn searches, although the protein sequence conservation was low (Figure 2-4, Table 2-4). The homology of hagfish and gnathostome *Rx* is also supported by a synteny analysis from Kon and Furukawa (2020). This in combination with the available RNA sequencing data which shows a high expression of *Rx* in the *E. stoutii* eye (relative to brain tissue) (Table 2-7) support our prediction that *Rx* will also be important for eye/retina formation in hagfish. To further characterize *Rx* expression an *in-situ* hybridization was completed on hagfish retinal sections (see **Section 2.5.3**). Similar analyses support the homology of the three hagfish *Six3/6* paralogs with both gnathostome *Six3* and *Six6* (Figure 2-5, Table 2-5). It was not possible to assign any of the three hagfish paralogs to a 1:1 orthologous relationship with either gnathostome *Six3* or *Six6*. It is possible the hagfish *Six3/6* paralogs may be a result of a separate

duplication event from the gnathostome *Six* genes. Regardless even if a 1:1 orthologous relationship between the hagfish and gnathostome sequences cannot be assigned, *Six3* and *Six6* are both genes known to be required in the maintenance of retinal progenitor cells (Diacou et al., 2018). The hagfish *Six3/6* homologs are likely involved in the cyclostome retinal development and their expression in the hagfish eye and retina should be explored further. Two hagfish *Six3/6* transcript sequences are of particular interest. One (Unigene16248_All) matched mostly closely to the gnathostome *Six3* and *Six6* sequences in the BLAST searches and had high expression in the retina (relative to brain tissue) (Table 2-5, Table 2-7). The other (Unigene5558_All) had high expression in both retina and brain tissue (Table 2-7).

Finally, the bioinformatics and phylogenetic analyses suggested hagfish have four *Otx* gene homologs (this is consistent with other studies (Higuchi et al., 2019)) (Figure 2-6, Figure 2-7, Table 2-6). However, assigning 1:1 orthology relationships of the cyclostome *Otx* paralogs to gnathostome *Otx* genes is difficult. The phylogenetic analysis did not show the hagfish *Otx* sequences clustering with specific gnathostome *Otx* paralogs, although it did suggest the cyclostome *Otx* genes are most closely related to gnathostome *Otx1* and *Otx2* (Figure 2-7). The synteny analysis supports homology between gnathostome *Otx1* and cyclostome *OtxC* (Figure 2-8A, Figure 2-9). The synteny analysis also suggests homology between gnathostome *Otx2* to both cyclostome *OtxA* and *OtxD* (Figure 2-8B, Figure 2-10). However, no syntenic relationship could be identified between gnathostome *Crx*/*Otx5* with any of the hagfish *Otx* genes (Figure 2-8C, Figure 2-11). A previous publication (Yamamoto et al., 2020) suggested lamprey *OtxB* was homologous with gnathostome *Crx* (*Otx5*). Hagfish (*E. burgeri*) *OtxB* is a 1:1 ortholog to lamprey (*P. marinus*) *OtxB*, so this finding would suggest a link between hagfish *OtxB* and gnathostome *Crx*. However, the genes used to support the syntenic relationship by Yamamoto et

al., (2020) are located farther from the cyclostome *OtxB* gene than the genes used in this synteny analysis. This is partially due to the limited genomic data available for hagfish (the *E. burgeri* contig containing *OtxB* only holds 8 genes). Interestingly, both the hagfish (*E. burgeri*) and lamprey (*P. marinus*) contigs containing *OtxB* also contain *UGP2*. *UGP2* is a gene located near *Otx1* in gnathostome genomes. Based on this data it is unclear if cyclostome *OtxB* shares a closer orthologous relationship to gnathostome *Otx1* or *Crx/Otx5*. There is also the possibility that *OtxB* is a cyclostome specific paralog.

All three gnathostome *Otx* genes have important roles in eye and retina development. *Otx1* and *Otx2* help establish the identity of the early neural retina. In the context of post-embryonic retinogenesis, *Otx2* is also required for the specification of photoreceptor cells and bipolar cells (Nishida et al., 2003; Viczian et al., 2003). *Otx5/Crx* is required for terminal photoreceptor differentiation (Furukawa et al., 1997). Based on the synteny and bioinformatics analyses, hagfish *OtxC* appears to be homologous to *Otx1* and *OtxA* and *OtxD* are homologs of gnathostome *Otx2*. The RNA sequencing data supports that some of these paralogs could have functions in the adult eye. The *E. stoutii* transcripts that are the best match for *OtxA* and *OtxD* have high expression in the hagfish eye (compared to the brain) (Table 2-7). In contrast, the *E. stoutii OtxC* sequence has some expression in the eye, but not as high as the other three *Otx* paralogs. If *OtxC* coordinates early eye formation in cyclostomes, like *Otx1* in gnathostomes, perhaps this paralog is expressed during embryonic eye development rather than in the adult eye. Therefore, we decided to focus on *OtxA* and *OtxD* for *in situ* hybridization on adult hagfish retinal sections (the *OtxD* probe was created but not fully optimized – see Supplementary Table 2). We expected *OtxA* to have a similar expression profile to gnathostome *Otx2* (expressed in RPE (retinal pigmented epithelium) cells, photoreceptor cells and bipolar cells (Viczian et al.,

2003; Béby et al., 2010; Béby and Lamonerie, 2013)) as it consistently matched closely with *Otx2* in tBLASTn searches (data not shown) (see **Section 2.5.3**). It is unclear which (if any) hagfish *Otx* paralog would take on the function of *Crx/Otx5* to complete differentiation of hagfish photoreceptors. One possibility is the *OtxB* gene, as suggested by Yamamoto et al., (2020). This gene is expressed in the adult hagfish eye and could not be detected in the brain, suggesting it could have an eye specific function (Table 2-7). Another potential candidate would be the *OtxD* sequence, which also has high relative expression in the hagfish eye. The expression of both genes in the hagfish eye and/or retina should be characterized further (i.e., with *in situ* hybridization) in future studies.

2.5.3 Expression of *OtxA* and *Rx* in the hagfish retina parallels the expression of gnathostome *Otx2* and *Rx*

In situ hybridization confirmed that several putative retinogenesis genes identified via genomic and RNA database searches are expressed in the hagfish (*E. stoutii*) retina. The expression of *OtxA* was particularly prominent (Figure 2-12). *OtxA* labels several cell populations within the hagfish retina. Interestingly, many of these cell types express *Otx2* in the gnathostome retina. The RnPE (retinal non-pigmented epithelium) cellular layer of the hagfish retina was heavily labelled by the *OtxA* probe (Figure 2-12A'). This expression pattern matches closely with the *Otx2* expression in mammalian retinae at the RPE (retinal pigmented epithelium) where it is vital for RPE cell specification and maintenance (Martínez-Morales et al., 2003; Rath et al., 2007; Béby and Lamonerie, 2013). *OtxA* was also expressed in other areas of the hagfish retina including directly adjacent to the RnPE (potentially the photoreceptor layer), the inner nuclear layer and the retinal periphery (Figure 2-12A'', A''', B, D, E). Expression in the photoreceptor layer corresponds to the gnathostome *Otx2* expression in photoreceptors. *Otx2* is critical for

photoreceptor specification and in some vertebrates *Otx2* expression is maintained in the photoreceptors and is required for these cells to remain healthy (Nishida et al., 2003). *Otx2* is also utilized by gnathostomes to specify bipolar cells (Yamamoto et al., 2020). If the inner nuclear layer cells labelled by *OtxA* correspond to hagfish bipolar cells, this again would match the gnathostome profile. Performing a double labelling *in situ* hybridization experiment with the *OtxA* probe and a marker for hagfish bipolar cells (such as PKC-α) would be an important next step to confirm the identity of the labeled INL cells (Dong and Allison, 2021). Although the similar expression profiles of *OtxA* and *Otx2* suggest that these genes may serve similar functions in hagfish and gnathostomes respectively, we cannot conclusively state that hagfish *OtxA* is promoting retinal development/retinogenesis based on the gene expression data alone. As mentioned, in other vertebrates *Otx2* also functions in the maintenance of mature retinal neurons in addition to the specification of developing neurons. However, the labeling of *OtxA* at the retinal periphery (the putative CMZ zone) suggests that at least some of the *OtxA* expression is occurring in newly forming retinal neurons.

There is one other study that demonstrated *OtxA* expression in the retina of a cyclostome. Yamamoto et al., (2020) showed that *OtxA* is present in the retina of the Japanese lamprey (*Lethenteron camtschaticum*). However, in the adult lamprey *OtxA* was only observed in the photoreceptor layer (ONL). Based on this expression pattern, the authors suggest that *Otx2* may have taken on its bipolar specification role only in the gnathostomes, and in cyclostomes it contributes solely to photoreceptor formation. However, the hagfish *OtxA in situ* hybridization data suggests otherwise. In adult hagfish *OtxA* expression occurred in multiple retinal layers including ONL and INL cells. Therefore, the absence of *OtxA* in lamprey bipolar cells compared to jawed vertebrates is likely not generalizable to cyclostomes overall.

Another remaining curiosity is that in jawed vertebrates *Otx5/Crx* helps drive terminal photoreceptor differentiation (Furukawa et al., 1997), but it is unclear if cyclostomes have a direct ortholog of this gene. It has already been shown that *Maf* (a homolog of the gnathostome rod-specifying gene *Nrl*) is expressed in the hagfish retina (Dong, 2018 (unpublished)). In gnathostomes, *Nrl* interacts with *Crx* to drive proper photoreceptor differentiation (Mitton et al., 2000; Montana et al., 2011). It would be interesting to determine if there is an *Otx* homolog in hagfish that is expressed strictly in the photoreceptors and/or if any *Otx* genes interact with *Maf*/*Nrl*. As synteny analyses with the available genomic data could not identify a clear hagfish ortholog of *Otx5*, it is possible that *Otx5* is a gnathostome specific paralog and one of the four hagfish *Otx* paralogs performs a similar role. *OtxD* is a potential candidate gene as it has high relative expression in eye tissue compared to brain tissue and appears to be homologous to gnathostome *Otx2*. Alternatively, if hagfish *OtxB* is homologous to *Otx5/Crx* (as suggested by data from Yamamoto et al., (2020)) and this relationship was missed by the synteny analysis because of incomplete hagfish genomic data, then perhaps this gene could regulate photoreceptor differentiation. RNA sequencing data shows *OtxB* is expressed in the adult hagfish eye at levels comparable to *OtxD* (Table 2-7), further supporting this gene could have a function in the eye and/or retina.

As *Rx* is a marker of retinal progenitor cells, expression of this gene was expected at the hagfish retinal periphery, but a stronger *Rx* signal was detected in the inner nuclear layer (INL) (Figure 2-13). *Rx* is a gene required for the maintenance of retinal progenitors in gnathostomes (Nelson et al., 2009b). As a result, during early development this gene has a broad expression pattern that gets restricted to specific areas (e.g., CMZ) once the retinal layers are established (Chuang et al., 1999). In adult zebrafish *Rx1* and *Rx2* are expressed in the CMZ (and cone photoreceptors) and

Rx3 is expressed in cells in the inner nuclear layer (Chuang et al., 1999). The hagfish *in situ* hybridization showed greatest *Rx* expression across the INL layer (Figure 2-13B) which is comparable to the expression pattern of zebrafish *Rx3*. The expression was not consistent across the whole layer but rather occurred in scattered cells. There was some *Rx* expression near the CMZ zone on one side of the retina (Figure 2-13C). However, the expression was unclear on the opposite side. There are reports of *Rx* being expressed in the INL cells and the photoreceptor cells of both zebrafish and rats (Chuang et al., 1999; Rohde et al., 2011). This expression pattern matches with what was observed in the hagfish retinal sections. As *Rx* is normally associated with retinal progenitor cells it does raise the possibility that the EdU+ cells located in the central retina and the *Rx*+ cells could be associated with each other. An important future experiment would be to perform *Rx in situ* hybridization on EdU treated samples. If the EdU and *Rx* labels co-localized in the same cells, that would support that the *Rx* riboprobe was labelling progenitor cells in the INL of the hagfish retina.

2.6 Conclusion

The hagfish is a poorly understood early-branching vertebrate with a bizarre, yet fascinating eye morphology. Investigating the eyes of this organism may reveal previously hidden clues to the evolutionary origins of the vertebrate eye and retina. One of the greatest limitations to studying hagfish is the logistical difficulty in acquiring embryos. However, careful examination of the hagfish retina in this study (and a previous study (Dong and Allison, 2021)) has revealed the presence of a CMZ-like zone in the hagfish retina. This creates a potential avenue to explore retinal neurogenesis in adult hagfish. The presence of a CMZ also suggests post-embryonic neurogenesis may have a more ancient origin in the vertebrate lineage than previously believed.

Further work exploring neurogenesis in other cyclostomes and early-branching gnathostomes will be crucial for elucidating the evolutionary history of this trait.

This study also presents data supporting there are shared developmental mechanisms driving retinogenesis in the hagfish and gnathostomes. Homologs of critical gnathostome retinal development genes (e.g., *Rx*, *Otx2*, *Six3*) are present in the hagfish genome and expressed in the eye. In addition, *in situ* hybridization reveals the expression patterns of two of these genes (*OtxA*/*Otx2* and *Rx*) are similar in the hagfish and the gnathostome retina. This suggests potentially conserved functions of these genes in vertebrate retinogenesis. However, it is still unknown if the cyclostome genes fulfill the same functional roles as the gnathostome homologs. In order to determine if shared mechanisms drive retinal formation in hagfish and jawed vertebrates, more work needs to be done to characterize the expression of putative neurogenesis genes in the hagfish retina. Ideally work should also be done to assess the function of these genes. Although hagfish are not an organism that is amenable to laboratory studies, there is the potential to insert hagfish homologs of retinal genes into transgenic lines of other vertebrate species (i.e., zebrafish) to infer gene function. Moving forward, it will also be vital to compare data from the continuously proliferative adult hagfish retina to the retina of embryos (once those embryos become more widely available). Getting access to data from embryonic hagfish will be very informative for understanding conserved mechanisms driving eye and retinal development in vertebrates.

Although several studies have investigated the morphology of cyclostome eyes, data on eye and retinal development are extremely limited. Neurodevelopmental data could provide new insights on eye evolution where morphological data from extant organisms or fossil data are lacking. Functional work to determine if these genes operate during retinogenesis similarly to other

vertebrates will be incredibly valuable for dissecting shared developmental mechanisms between gnathostomes and cyclostomes. By taking advantage of a putative proliferative zone at the hagfish retinal margin, this study has laid the groundwork for further investigations of retinal development and evolution in the early-branching cyclostomes.

Acknowledgements

We thank the staff at the Bamfield Marine Sciences Center (BMSC) who collected and housed the live hagfish for this study. A special thanks to Dr. Juhno Eom (University of British Columbia) who assisted with the administration of the intraperitoneal EdU injections. We also thank Dr. Zachary Hall who provided advice for the EdU labelling protocol. We are grateful for Dr. Guobin Sun and Dr. Kacie Nortion who assisted with the microscopy. Finally, we thank Emily Dong who generated the RNA sequencing dataset and the hagfish RNA used in this project. This work was funded by an NSERC Discovery grant and an NSERC studentship.

Figures and Tables

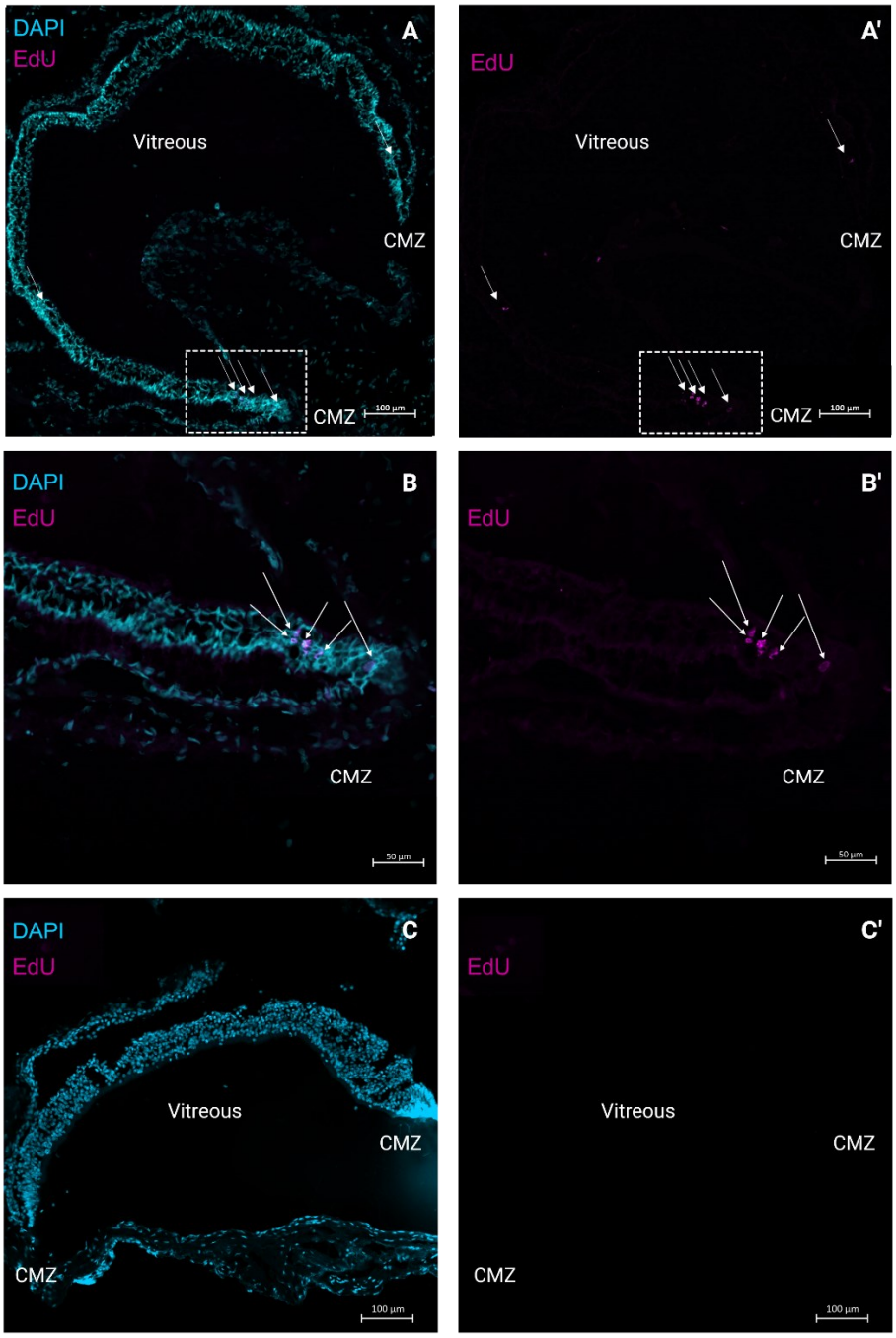


Figure 2-1: EdU (5-ethynyl-2'-deoxyuridine) incorporation reveals proliferating cells in the post-embryonic hagfish retina. An abundance of EdU-positive (EdU+) cells at the retinal

margins indicate that ongoing proliferation likely contributes to adult retinal growth in this otherwise very rudimentary tissue. A) an overview of a hagfish retinal section from an adult hagfish treated with EdU 24 hours prior to sacrifice. The white arrows indicate cells that are EdU+. Nuclei are stained with DAPI. A') the same image with only EdU labelling shown. B) a closer view of the periphery of the retinal section in A (boxed region). White arrows indicate there are multiple EdU+ cells in this region. B') the same image as panel B with only EdU labeling shown. C) A retinal section from an animal not treated with EdU (negative control). No EdU+ cells are observed. C') The same image as panel C with only EdU labelling shown. Vitreous = vitreous side of retina. CMZ= putative ciliary marginal zone.

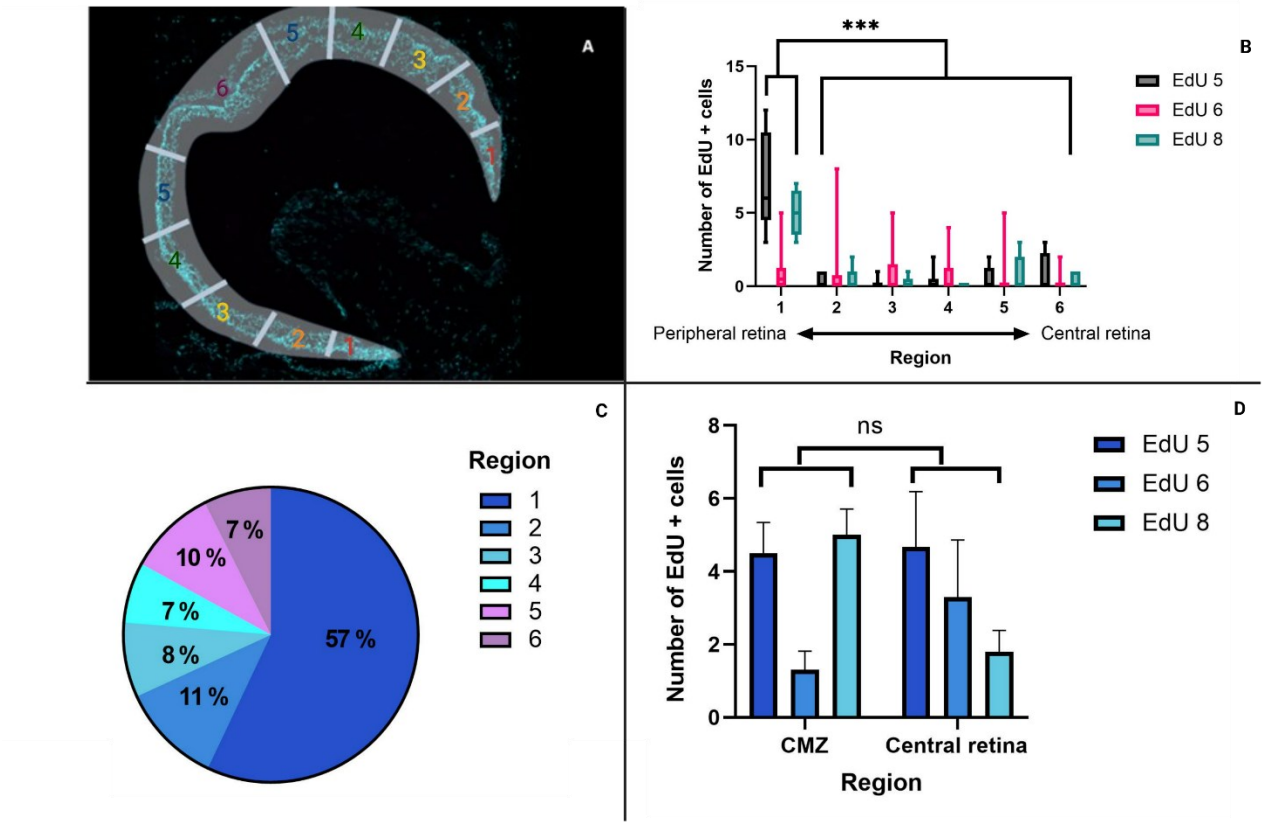


Figure 2-2: Quantification of EdU-positive (EdU+) cells in the hagfish retina suggests proliferating cells are concentrated at the peripheral retina. A.) Division of the retina for EdU+ cell quantification. Region 1 is the most peripheral and region 6 the most central. B.) Number of EdU+ cells per region across 21 retinal sections (from three EdU treated individuals – EdU 5, EdU 6, EdU 8). The maximum, minimum and average number of EdU+ cells per individual and per each region were plotted. The number of EdU+ cells in region 1 was significantly higher than the number found in the other 5 regions ($P<0.001$). C.) Proportion of the total number of EdU+ cells found in each retinal region. D.) The average number of EdU+ cells found in the 'CMZ' (defined by morphology) zone compared to the rest of the retina for each individual. The overall number of EdU+ cells (across all three individuals) located in the CMZ zone does not differ significantly from the number of cells located in the rest of the retina (ns = not significant). Error

bars represent standard error. Panels B-D were created in GraphPad Prism version 9.5.1 for Windows.

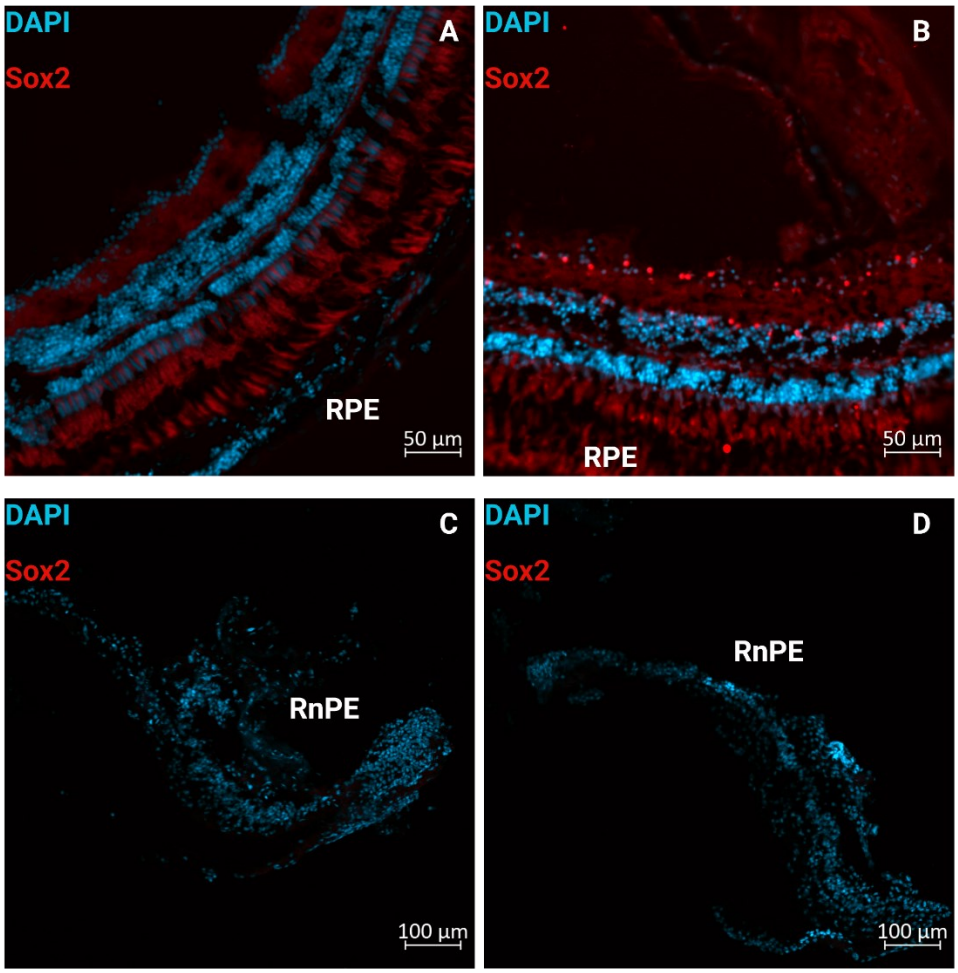
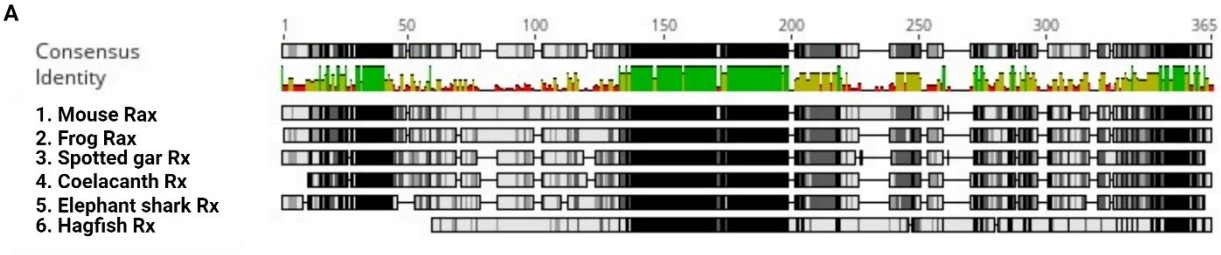


Figure 2-3: Sox2 immunohistochemistry (IHC) labels the zebrafish retina but not the hagfish retina. A.) Negative control (no primary antibody) for Sox2 in zebrafish retina. B.) Sox2 IHC in the zebrafish retina. Sox2 positive cells are present in the inner nuclear layer and the retinal ganglion cell layer. C.) Negative control for Sox2 in hagfish retina. D.) Sox2 IHC in hagfish retina. No Sox2+ cells were observed. RPE = retinal pigmented epithelium. RnPE = retinal non-pigmented epithelium).

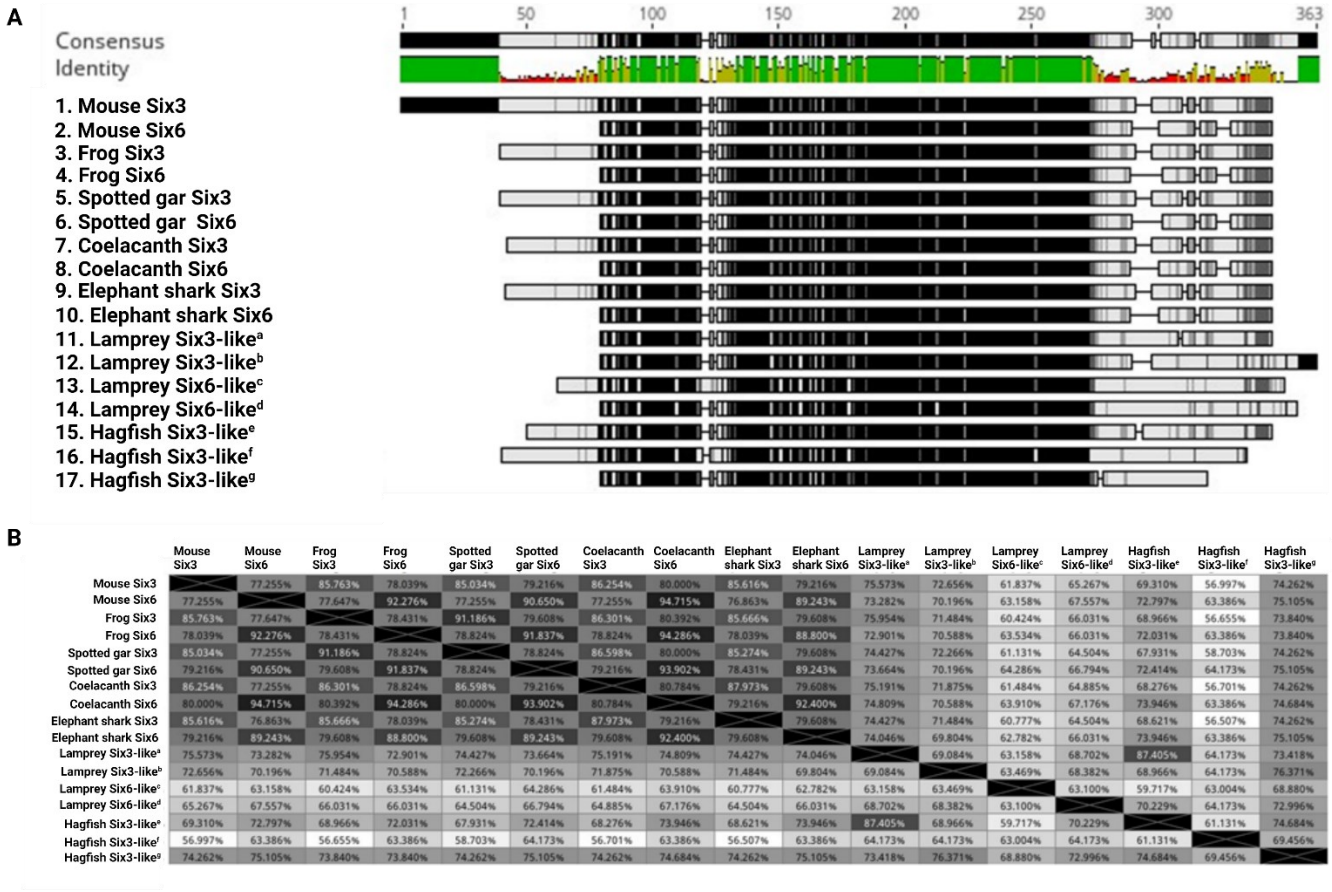
A



B

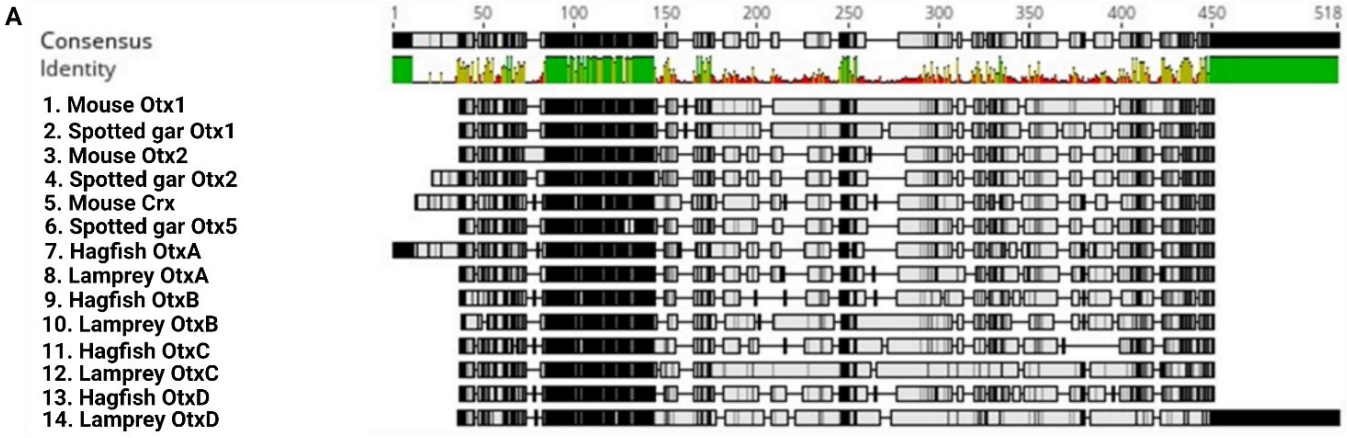
	Mouse Rax	Frog Rax	Spotted gar Rx	Coelacanth Rx	Elephant shark Rx	Hagfish Rx
Mouse Rax		57.391%	55.233%	54.599%	50.437%	34.641%
Frog Rax	57.391%		59.021%	65.409%	54.321%	32.353%
Spotted gar Rx	55.233%	59.021%		67.857%	60.062%	34.653%
Coelacanth Rx	54.599%	65.409%	67.857%		62.581%	33.007%
Elephant shark Rx	50.437%	54.321%	60.062%	62.581%		31.353%
Hagfish Rx	34.641%	32.353%	34.653%	33.007%	31.353%	

Figure 2-4: Hagfish retinal homeobox (Rx) protein shares low sequence identity with gnathostome Rx. A.) Protein alignment of hagfish (*Eptatretus burgeri*) Rx with representative gnathostome Rx homologs. B.) Protein % identity matrix for hagfish Rx compared to representative gnathostome Rx homologs. Image was created in Geneious Prime (v. 2022.0, Biomatters) and was edited in BioRender (Biorender.com).



	Mouse Six3	Mouse Six6	Frog Six3	Frog Six6	Spotted gar Six3	Spotted gar Six6	Coelacanth Six3	Coelacanth Six6	Elephant shark Six3	Elephant shark Six6	Lamprey Six3-like[a]	Lamprey Six3-like[b]	Lamprey Six6-like[c]	Lamprey Six6-like[d]	Hagfish Six3-like[e]	Hagfish Six3-like[f]	Hagfish Six3-like[g]
Mouse Six3		77.255%	85.763%	78.039%	85.034%	79.216%	86.254%	80.000%	85.616%	79.216%	75.573%	72.656%	61.837%	65.267%	69.310%	56.997%	74.262%
Mouse Six6	77.255%		77.647%	92.276%	77.255%	90.650%	77.255%	94.715%	76.863%	89.243%	73.282%	70.196%	63.158%	67.557%	72.797%	63.386%	75.105%
Frog Six3	85.763%	77.647%		78.431%	91.186%	79.608%	86.301%	80.392%	85.666%	79.608%	75.954%	71.484%	60.424%	66.031%	68.966%	56.655%	73.840%
Frog Six6	78.039%	92.276%	78.431%		78.824%	91.837%	78.824%	94.286%	78.039%	88.800%	72.901%	70.588%	63.534%	66.031%	72.031%	63.386%	73.840%
Spotted gar Six3	85.034%	77.255%	91.186%	78.824%		78.824%	86.598%	80.000%	85.274%	79.608%	74.427%	72.266%	61.131%	64.504%	67.931%	58.703%	74.262%
Spotted gar Six6	79.216%	90.650%	79.608%	91.837%	78.824%		79.216%	93.902%	78.431%	89.243%	73.664%	70.196%	64.286%	66.794%	72.414%	64.173%	75.105%
Coelacanth Six3	86.254%	77.255%	86.301%	78.824%	86.598%	79.216%		80.784%	87.973%	79.608%	75.191%	71.875%	61.484%	64.885%	68.276%	56.701%	74.262%
Coelacanth Six6	80.000%	94.715%	80.392%	94.286%	80.000%	93.902%	80.784%		79.216%	92.400%	74.809%	70.588%	63.910%	67.176%	73.946%	63.386%	74.684%
Elephant shark Six3	85.616%	76.863%	85.666%	78.039%	85.274%	78.431%	87.973%	79.216%		79.608%	74.427%	71.484%	60.777%	64.504%	68.621%	56.507%	74.262%
Elephant shark Six6	79.216%	89.243%	79.608%	88.800%	79.608%	89.243%	79.608%	92.400%	79.608%		74.046%	69.804%	62.782%	66.031%	73.946%	63.386%	75.105%
Lamprey Six3-like[a]	75.573%	73.282%	75.954%	72.901%	74.427%	73.664%	75.191%	74.809%	74.427%	74.046%		69.084%	63.158%	68.702%	87.405%	64.173%	73.418%
Lamprey Six3-like[b]	72.656%	70.196%	71.484%	70.588%	72.266%	70.196%	71.875%	70.588%	71.484%	69.804%	69.084%		63.469%	68.382%	68.966%	64.173%	76.371%
Lamprey Six6-like[c]	61.837%	63.158%	60.424%	63.534%	61.131%	64.286%	61.484%	63.910%	60.777%	62.782%	63.158%	63.469%		63.100%	59.717%	63.004%	68.880%
Lamprey Six6-like[d]	65.267%	67.557%	66.031%	66.031%	64.504%	66.794%	64.885%	67.176%	64.504%	66.031%	68.702%	68.382%	63.100%		70.229%	64.173%	72.996%
Hagfish Six3-like[e]	69.310%	72.797%	68.966%	72.031%	67.931%	72.414%	68.276%	73.946%	68.621%	73.946%	87.405%	68.966%	59.717%	70.229%		61.131%	74.684%
Hagfish Six3-like[f]	56.997%	63.386%	56.655%	63.386%	58.703%	64.173%	56.701%	63.386%	56.507%	63.386%	64.173%	64.173%	63.004%	64.173%	61.131%		69.456%
Hagfish Six3-like[g]	74.262%	75.105%	73.840%	73.840%	74.262%	75.105%	74.262%	74.684%	74.262%	75.105%	73.418%	76.371%	68.880%	72.996%	74.684%	69.456%	

Figure 2-5: Hagfish have three six3/6 paralogs which share some protein sequence identity with gnathostome Six3 and Six6 sequences. A.) Protein alignment of hagfish (*Eptatretus burgeri*) Six3/6 paralogs with representative gnathostome and lamprey Six3 and Six6 homologs. B.) Protein % identity matrix for hagfish Six3/6 paralogs compared to representative gnathostome and lamprey Six3 and Six6 homologs. Superscript letters (a-g) refer to different cyclostome Six3/6 sequences (see Supplementary Table 4). Image was created in Geneious Prime (v. 2022.0, Biomatters) and was edited in BioRender (Biorender.com).



B

	Mouse Otx1	Spotted gar Otx1	Mouse Otx2	Spotted gar Otx2	Mouse Crx	Spotted gar Otx5	Hagfish OtxA	Lamprey OtxA	Hagfish OtxB	Lamprey OtxB	Hagfish OtxC	Lamprey OtxC	Hagfish OtxD	Lamprey OtxD
Mouse Otx1		68.156%	49.326%	50.685%	37.634%	48.189%	45.879%	46.575%	35.772%	39.889%	40.822%	38.501%	46.260%	34.430%
Spotted gar Otx1	68.156%		54.902%	55.556%	38.440%	54.046%	49.570%	50.000%	36.415%	43.840%	39.831%	39.370%	47.278%	35.844%
Mouse Otx2	49.326%	54.902%		91.000%	45.342%	66.343%	59.748%	58.044%	41.121%	45.821%	45.231%	37.306%	52.960%	32.808%
Spotted gar Otx2	50.685%	55.556%	91.000%		43.636%	67.550%	58.896%	59.807%	42.222%	47.801%	46.395%	37.008%	54.603%	33.156%
Mouse Crx	37.634%	38.440%	45.342%	43.636%		47.771%	37.752%	44.099%	35.535%	36.522%	37.037%	28.385%	39.623%	28.191%
Spotted gar Otx5	48.189%	54.046%	66.343%	67.550%	47.771%		56.230%	56.731%	40.127%	47.463%	44.860%	35.884%	52.733%	33.067%
Hagfish OtxA	45.879%	49.570%	59.748%	58.896%	37.752%	56.230%		70.740%	39.557%	42.775%	44.343%	35.696%	54.574%	32.632%
Lamprey OtxA	46.575%	50.000%	58.044%	59.807%	44.099%	56.731%	70.740%		41.083%	43.732%	42.638%	36.148%	51.735%	33.867%
Hagfish OtxB	35.772%	36.415%	41.121%	42.222%	35.535%	40.127%	39.557%	41.083%		40.351%	37.736%	30.366%	40.317%	27.321%
Lamprey OtxB	39.889%	43.840%	45.821%	47.801%	36.522%	47.463%	42.775%	43.732%	40.351%		39.650%	35.958%	40.469%	33.594%
Hagfish OtxC	40.822%	39.831%	45.231%	46.395%	37.037%	44.860%	44.343%	42.638%	37.736%	39.650%		40.157%	45.342%	29.708%
Lamprey OtxC	38.501%	39.370%	37.306%	37.008%	28.385%	35.884%	35.696%	36.148%	30.366%	35.958%	40.157%		36.148%	34.772%
Hagfish OtxD	46.260%	47.278%	52.960%	54.603%	39.623%	52.733%	54.574%	51.735%	40.317%	40.469%	45.342%	36.148%		34.492%
Lamprey OtxD	34.430%	35.844%	32.808%	33.156%	28.191%	33.067%	32.632%	33.867%	27.321%	33.594%	29.708%	34.772%	34.492%	

Figure 2-6: Hagfish have four Otx paralogs which share low overall protein sequence identity with other vertebrate Otx homologs. A.) Protein alignment of hagfish (*Eptatretus burgeri*) Otx paralogs with representative gnathostome and lamprey Otx homologs. B.) Protein % identity matrix for hagfish Otx paralogs compared to representative gnathostome and lamprey Otx homologs. Image was created in Geneious Prime (v. 2022.0, Biomatters) and was edited in BioRender (Biorender.com).

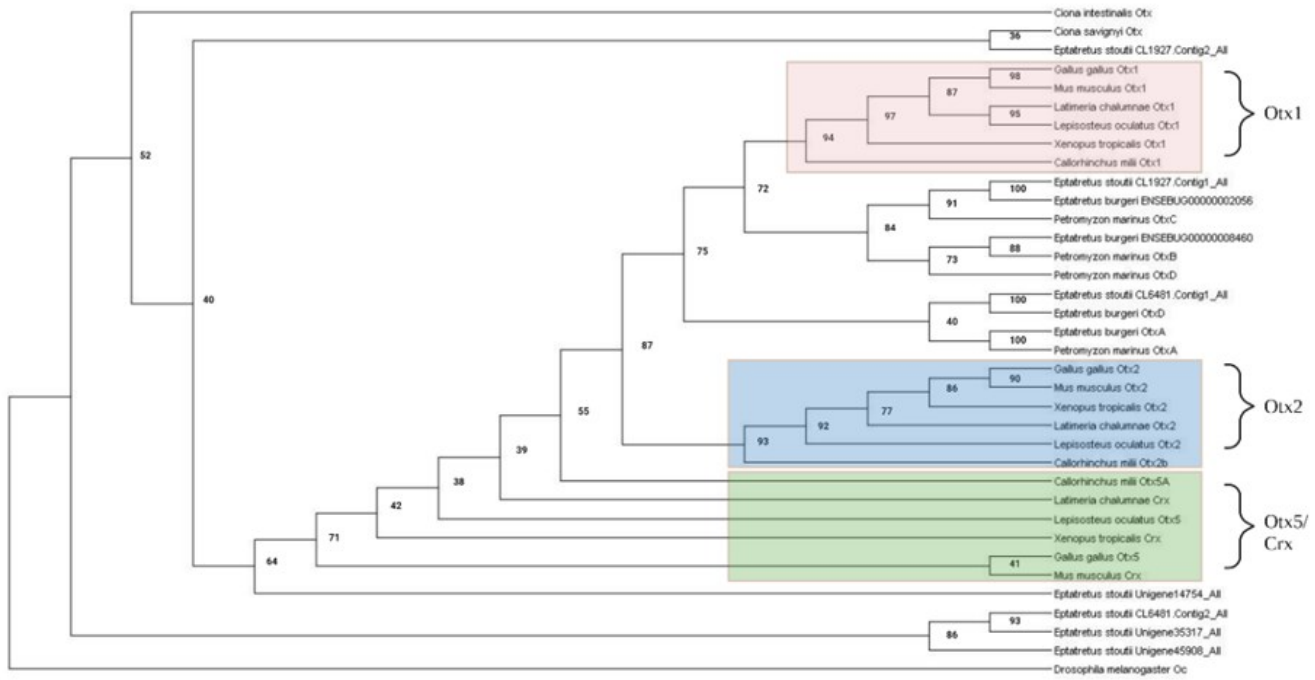


Figure 2-7: Maximum likelihood tree of cyclostome and gnathostome Otx protein sequences shows vertebrate sequences cluster together. Hagfish (*Eptatretus burgeri*) Otx paralogs (OtxA, OtxB, OtxC, and OtxD) fall between gnathostome Otx1 and Otx2 sequences on the tree. The hagfish sequences group closely with the lamprey (*P. marinus*) Otx paralogs (hagfish OtxA, OtxC and OtxD form sister groups with the equivalent lamprey Otx homologs). The numbers at each node represent the bootstrap support. Chordate (*Ciona intestinalis* and *Ciona savigyni*) and *Drosophila melanogaster* Otx sequences were used as outgroups for comparison.

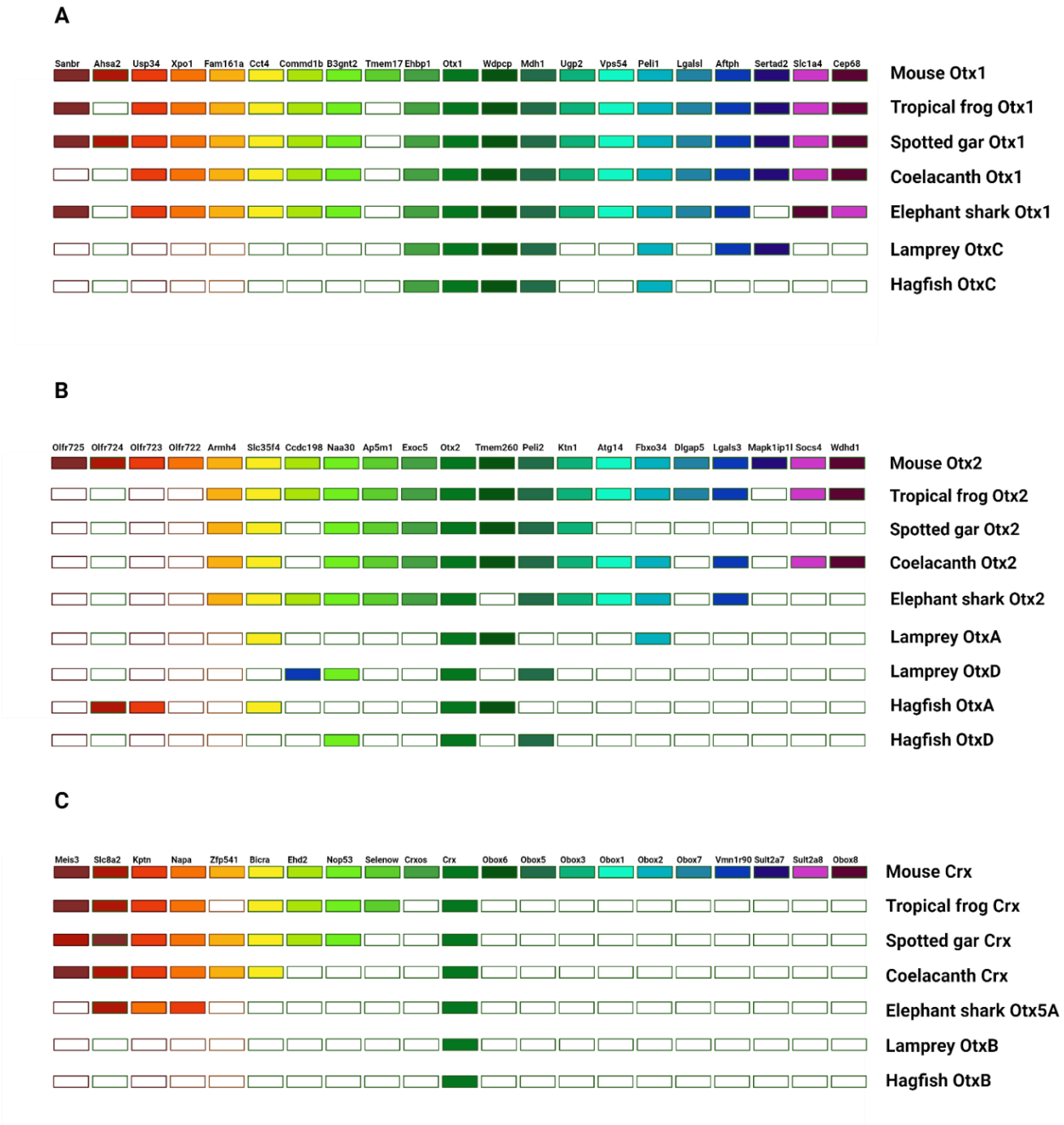


Figure 2-8: Homology of *Otx1* to *OtxC* and *Otx2* to *OtxA/OtxD* is supported by a comparison of the genomic arrangement of mouse *Otx* paralogs and surrounding genes to cyclostome and gnathostome genomes. A) The 20 genes directly adjacent to mouse *Otx1* were compared to the

genes surrounding *Otx1* (or *OtxC* for hagfish and lamprey) for other vertebrates. This data shows there is synteny between gnathostome *Otx1* and cyclostome *OtxC*. B) The 20 genes directly adjacent to mouse *Otx2* were compared to the genes surrounding *Otx2* (or *OtxA* and *OtxD* for hagfish and lamprey) for other vertebrates. These data support a syntenic relationship between gnathostome *Otx2* and cyclostome *OtxA/OtxD*. C.) The 20 genes directly adjacent to mouse *Crx* were compared to the genes surrounding *Otx5/Crx* (or *OtxB* for hagfish and lamprey) for other vertebrates. This data did not support a syntenic relationship between gnathostome *Otx5/Crx* and cyclostome *OtxB*. This figure was created in BioRender using data from Genomicus v. 106 (Nguyen et al., 2022).

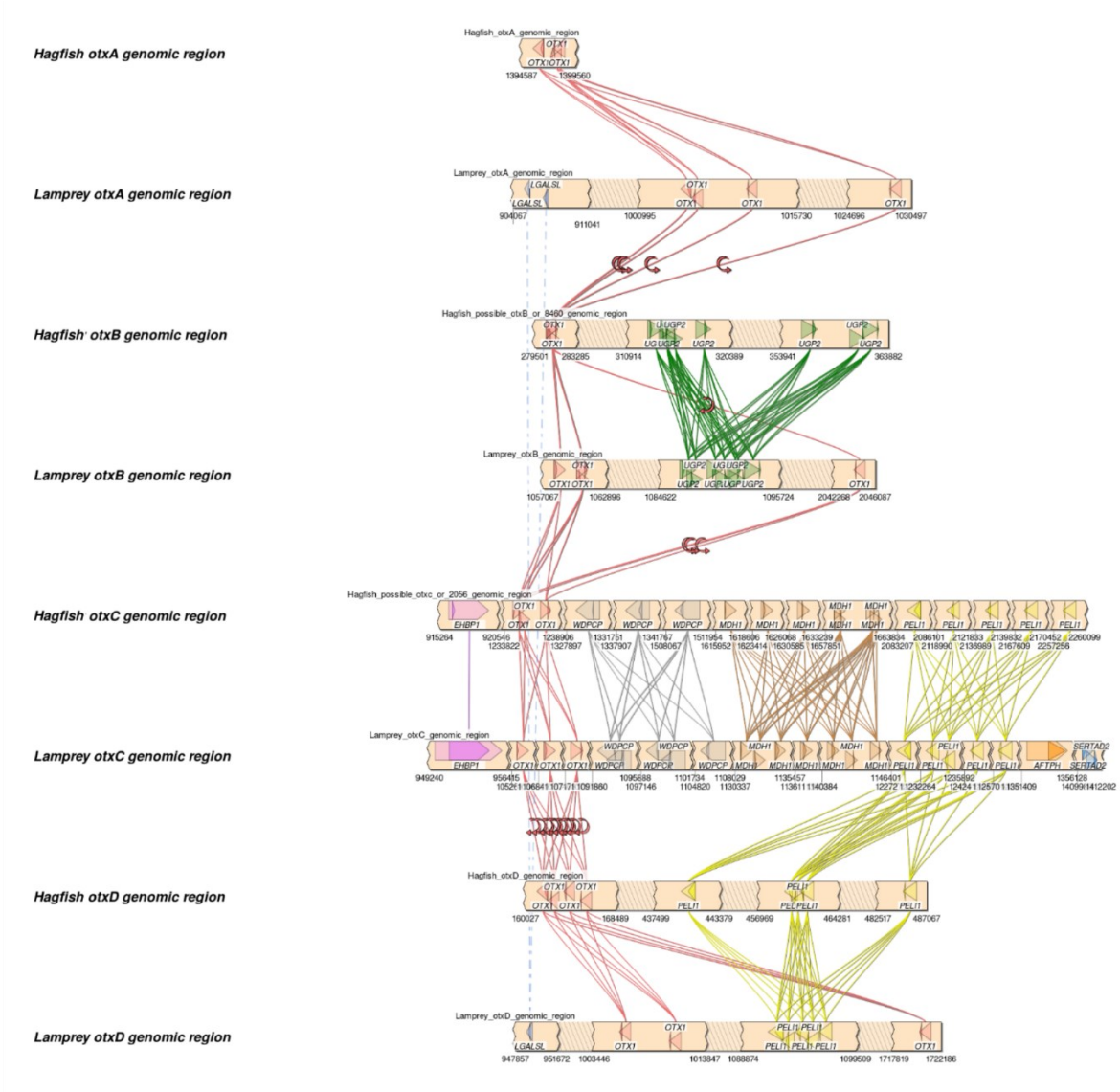


Figure 2-9: Homology between gnathostome *Otx1* and cyclostome *OtxC* is suggested by a synteny analysis of mouse *Otx1* to cyclostome genomes. Mouse *Otx1* and the 20 genes adjacent to it were used as query sequences against the genomic region (2M bases) surrounding the hagfish and lamprey *Otx* paralogs. Blast searches were used to map the query sequences to the genomic regions. For the *Otx1* query, the majority of search hits were centered on the hagfish and lamprey contigs containing *OtxC*. One query gene (*UGP2*) also matched closely to genes on

the hagfish and lamprey *OtxB* containing contigs. This figure was made using the Simple Synteny web server (Veltri et al., 2016).

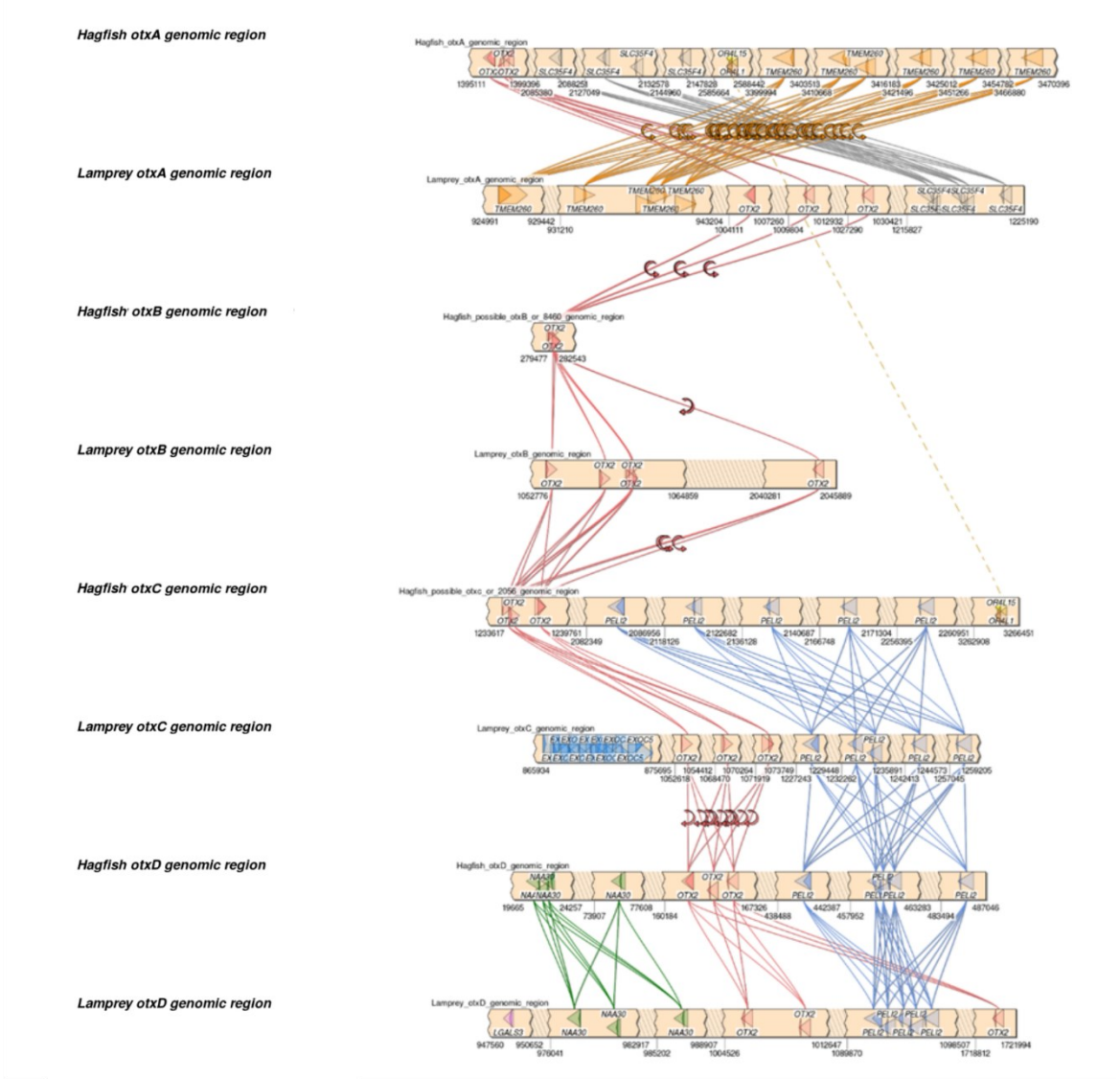


Figure 2-10: Synteny analysis of mouse *Otx2* to cyclostome genomes supports homology of gnathostome *Otx2* to cyclostome *OtxA* and *OtxD*. Mouse *Otx2* and 20 adjacent genes were used as query sequences against the genomic region (2M bases) surrounding the hagfish and lamprey *Otx* paralogs. A blast search was used to map the query sequences to the genomic regions. For the *Otx2* query sequences, the majority of matches were centered on the hagfish and lamprey contigs containing *OtxA* and *OtxD*. This figure was made using the Simple Synteny web server (Veltri et al., 2016).

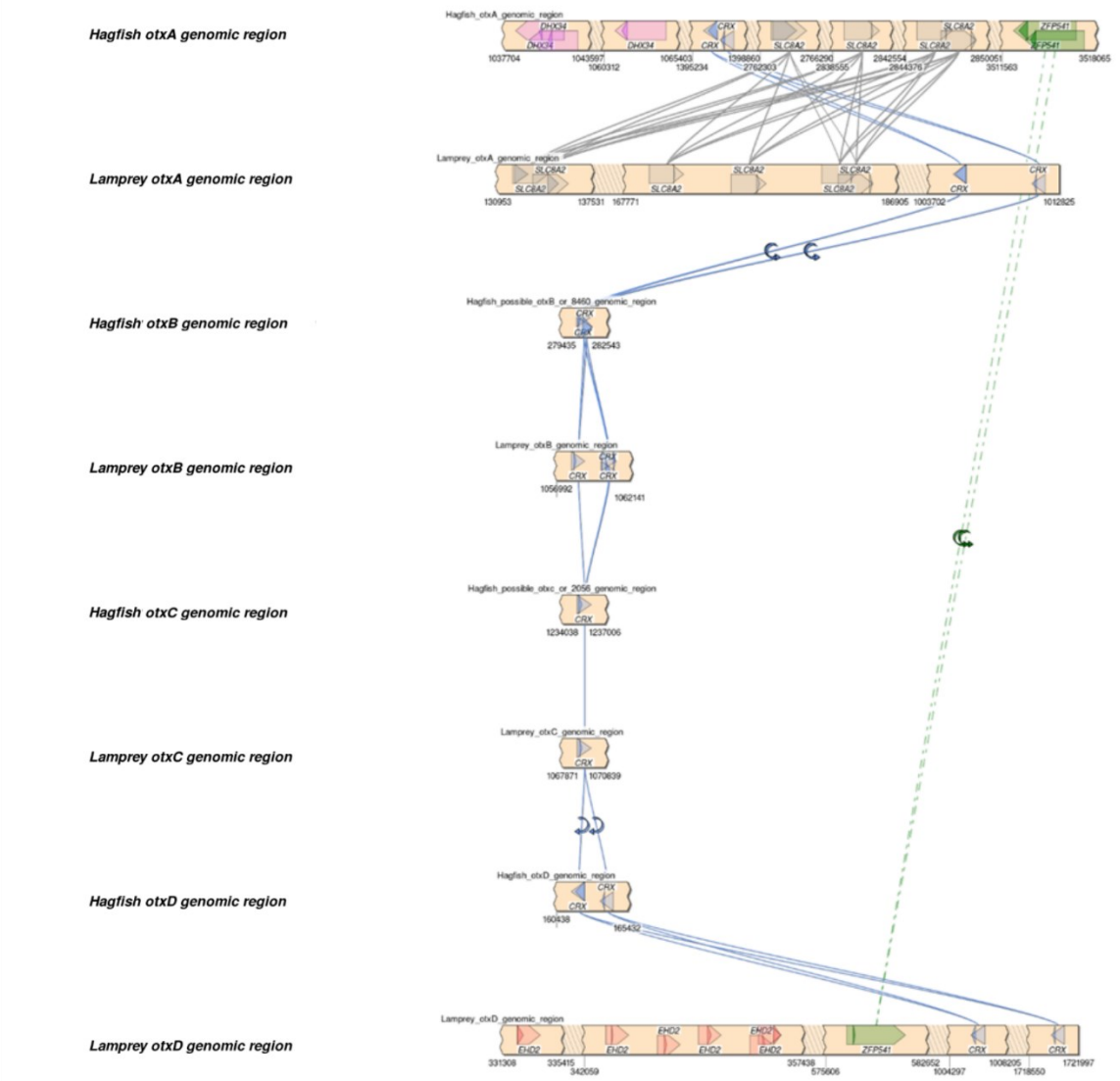


Figure 2-11: Synteny analysis of mouse *Crx* (*Otx5*) to cyclostome genomes does not support homology between gnathostome *Otx5* and cyclostome *Otx* homologs. Mouse *Crx* and 20 adjacent genes were used as query sequences against the genomic region (2M bases) surrounding the hagfish and lamprey *Otx* paralogs. A blast search was used to map the query sequences to the genomic regions. For the *Crx* query, the best search hit was on the lamprey *OtxD* contig and the hagfish *OtxA* contig. In both cases the cyclostome gene identified by the search hit did not match perfectly back to the original mouse query gene (i.e., mouse *EDH2* matched most closely to

lamprey *EDH1* and mouse *DHX34* matched to hagfish *DHX57)* and the support for these matches was relatively weak compared to the *Otx1* and *Otx2* synteny analyses. This figure was made using the Simple Synteny web server (Veltri et al., 2016).

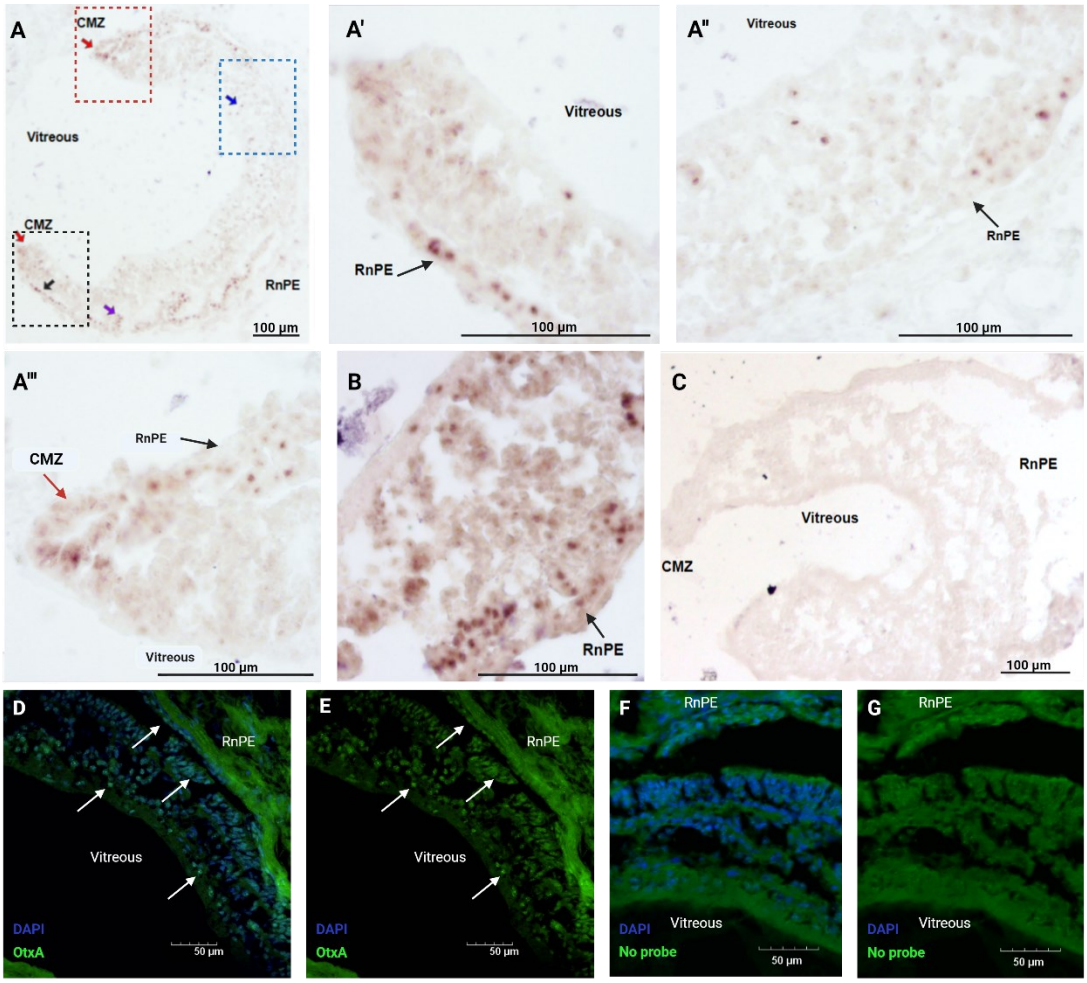


Figure 2-12: *OtxA i*s expressed throughout multiple cell types in the Pacific hagfish (*Eptatretus stoutii*) retina. In situ hybridization probes were visualized with colorimetric detection (panels A-C) or fluorescent detection (panels D-G). A) Overview of a retinal section labeled with *OtxA*. A') Magnified image of the black box in panel A showing *OtxA* labelled cells in the RnPE layer. A'') Magnified image of the blue box in panel A showing *OtxA* labelled cells in the inner nuclear layer. A''') Magnified image of the red box in panel A showing *OtxA* labelled cells in the CMZ region. B) *OtxA* labelled cells in the photoreceptor layer and inner nuclear layer of the *E. stoutii* retina (from a different retinal section than panel A). C) Negative control section with no probe

applied. D) Retinal section fluorescently labelled with *OtxA* and stained with DAPI. E) Same image as panel D, but only showing the *OtxA* label. F,G) Negative control section with no riboprobe applied, with (panel F) and without (panel G) DAPI respectively. RnPE = retinal non-pigmented epithelium, vitreous = vitreous side of the hagfish retina, CMZ=ciliary marginal zone. Red arrows = CMZ. Blue arrows = inner nuclear layer. Purple arrows = photoreceptor layer. Black arrows = RnPE layer. White arrows = fluorescently labeled *OtxA*+ cells.

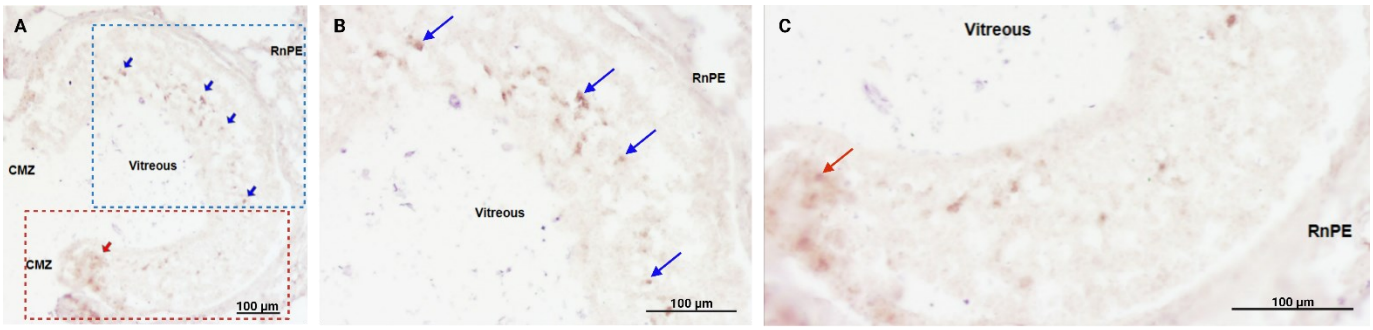


Figure 2-13: *Rx* is expressed in the inner nuclear layer and (perhaps) the periphery of the hagfish (*Eptatretus stoutii*) retina. An *Rx in situ* hybridization probe was visualized with NBT/BCIP detection of alkaline phosphatase. A) Overview of a retinal section labeled with *Rx*. B) Magnified image of the blue box in panel A showing *Rx* labelled cells in the inner nuclear layer (blue arrows). C) Magnified image of the red box in panel A showing *Rx* labelled cells in the CMZ (red arrow). RnPE = retinal non-pigmented epithelium layer, vitreous = vitreous side of the hagfish retina, CMZ=ciliary marginal zone. Blue arrow = inner nuclear layer. Red arrow = CMZ.

Table 2-1: Gene block sequence for the *Rx* (plasmid based) riboprobe

Riboprobe	Length (bp)	Gene Block Sequence 5' to 3' (red text = EcoRI cut site, blue text = XbaI cut site)
Rx	1206	GAT CGA ATT CAT GGA GCG TGA AGA TTC TTG TGG TAC TTC GCA TGC ACA CAG CAT CGA CTC GAT TCT GGG ATT CCA GAG GGA TGA TTC CTT CTT CGA TTC CTC CTT GAT CCA GGA ACA TTT GCC AGT GGA AGA AAG GGA AGC TTC CCA AGA ACA CTC TGA GTG CGA CGG CAG CGC CTT TGC GTC TCT GAA AGA GCT CCC TTC ACC CGA GCA GGA GTT GGA TGT GCG AGT CCC ATG TGG GAA ACC GCG GCG AAA TCG AAC CAC GTT CAC CAC GTT CCA GCT GCA TGA ACT TGA GCG TGC CTT TGA GCG ATC ACA CTA TCC AGA CGT GTA CAG CCG AGA GGA ACT GGC ACT CAA GGT CAA CCT TCC CGA AGT CCG CGT GCA GGT GTG GTT TCA GAA TCG TCG TGC TAA ATG GCG ACG TCA AGA AAG GCT TGA ACC AGT AAC ATC TTG CCT TCC TGG CCA AAT TGG CCA GCC ACC TTG CCA ACG TCC CAC TCC ACC ACT ACC TCT TGA GCC ATG GTT GCC CCC TGC CAT CTG TGG AGG TGC AGG AAC AGG CTC TGG TGG CAG TCC AGG AGT GCC AGT TAC TCC TCC AGT GTC TTC ACC CAG TCT GTC GTG CCT GTT GGG CTG CAC CGG CGG ACT GCA TGG ACC CCA TAG CCT GGC AGG TCT TAT ACC TCA GGG AGG GCA AGC TCT AAC TTA TGT TCC ATC TAC TCC TAC AAT AGG ACA CAC AAC GCA TCA CAC GTT GGG CAC TGC TCA GGG TCA AAT GAC TGT GAC AGC TTA CCA ACC AGC ATT ATC TGG ACT GCA GTT ATC TGG GCC GCC ATA TGA AGA GAC TTA CGG GCT AGG TGA GGT GCG GGT ACC TCG ATC AGG ATT GGC TAC CCT ACG TGT CAA AGC CAA GGA GCA TCT TCA AGT GTT AGG CAC CGG TTG GCA TCA AAT CTA GAG ATC

Table 2-2: Primer sequences used to generate the *OtxA* (PCR based) riboprobe

Probe	Forward Primer (5' to 3')	Reverse Primer with T7 Polymerase Site (underlined) (5' to 3')	Amplicon Size (bp)
OtxA	GTG TGG ATC CAT CAC TCC GG	TAA TAC GAC TCA CTA TAG GGG GC TCC AGA TAG AAA CGG G	591

Table 2-3: *In situ* hybridization conditions per each riboprobe

Riboprobe	Proteinase K Digestion Time (min)	Probe concentration (μg/ml)	Hybridization temperature (°C)
Rx	6	2	55
OtxA	4	1	55

Table 2-4: Best tBlastn results for various vertebrate *Rx* homologs to the *Eptatretus stoutii* transcriptome dataset

Query Rax/Rx Sequence (protein)	Best transcript match	% identity	e-value	Does the transcript sequence reciprocally blast (blastn) to the query sequence?
Mouse Rax	CL3100.Contig_ All	45.5%	9.43e-33	Yes (best hit)
Spotted gar Rx	CL3100.Contig2 _All	54.8%	3.78e-39	No (it is the best hit for the other spotted gar paralog – Rx1)
Inshore hagfish Rx	CL3100.Contig2 _All	99.0%	2.82e-155	Yes (best hit)

Table 2-5: Best tBlastn results for various vertebrate *Six3* and *Six6* homologs to the *Eptatretus stoutii* transcriptome dataset

Query Six3 or Six6 Sequence (protein)	Best transcript match	% identity	e-value	Does the transcript sequence reciprocally blast (blastn) to the query sequence?
Six3 (mouse)	Unigene16248_All	91.1%	3.88e-122	Yes (best hit)
Six3 (spotted gar)	Unigene16248_All	92.7%	6.03e-122	Yes (best hit)
Six6 (mouse)	Unigene16248_All	92.3%	2.43e-120	Yes (2nd best hit)
Six6 (spotted gar)	Unigene16248_All	77.1%	4.25e-123	Yes (2nd best hit)
Six-3 like[a] * (sea lamprey)	Unigene16248_All	88.2%	9.98e-145	Yes (best hit)
Six-3 like[b] * (sea lamprey)	Unigene5558_All	93.2%	8.74e-129	Yes (best hit)
Six6-like[c] * (sea lamprey)	Unigene22484_All	71.7%	1.95e-125	Yes (best hit)
Six6-like[d] * (sea lamprey)	Unigene16248_All	89.2%	2.60e-117	Yes (2nd best hit)
Six3-like[e] * (inshore hagfish)	Unigene16248_All	99.7%	0	Yes (best hit)
Six3-like[f] * (inshore hagfish)	Unigene22484_All	98.0%	3.49e-141	Yes (best hit)
Six3-like[g] * (inshore hagfish)	Unigene5558_All	100.0%	7.42e-137	Yes (best hit)

*Superscript letters (a-g) refer to different cyclostome Six3/6 sequences (see Supplementary Table 4).

Table 2-6: Best tBlastn results for various vertebrate *Otx* homologs to the *Eptatretus stoutii* transcriptome dataset

Query Otx Sequence (protein)	Best transcript match	% identity	e-value	Does the transcript sequence reciprocally blast (blastn) to the query sequence?
Otx1 (mouse)	CL6481.Contig1_All	82.1%	3.11e-56	Yes (best hit)
Otx1 (spotted gar)	CL6481.Contig1_All	83.2%	7.87e-54	Yes (3rd best hit)
Otx2 (mouse)	Unigene14754_All	65.2%	1.09e-104	Yes (2nd best hit)
Otx2 (spotted gar)	Unigene14754_All	63.5%	1.44e-92	Yes (2nd best hit)
Crx (mouse)	CL6481.Contig1_All	42.5%	3.77e-50	Yes (3rd best hit)
Crx (spotted gar)	Unigene14754_All	55.5%	9.90e-91	Yes (best hit)
OtxA (sea lamprey)	Unigene14754_All	67.7%	1.97e-108	Yes (best hit)
OtxB (sea lamprey)	CL6481.Contig1_All	72.7%	3.05e-48	Yes (3rd best hit)
OtxC (sea lamprey)	CL1927.Contig1_All	88.3%	1.04e-51	Yes (best hit)
OtxD (sea lamprey)	CL6481.Contig1_All	83.2%	2.32e-46	Yes (2nd best hit)
OtxA (inshore hagfish)	Unigene14754_All	99.7%	0	Yes (best hit)
OtxB (inshore hagfish)	CL1927.Contig1_All *	46.5%	1.02e-60	Yes (4th best hit)
OtxC (inshore hagfish)	CL1927.Contig1_All*	98.3%	3.60e-171	Yes (best hit)
OtxD (inshore hagfish)	CL6481.Contig1_All	99.3%	0	Yes (best hit)

* CL1927.Contig1_All was assigned as the *E. stoutii OtxC* homolog based on the tBLASTn results. Unigene45908_All was designated as the *E. stoutii OtxB* homolog based on a reciprocal BLAST search of the *E. stoutii* transcripts to the *Eptatretus burgeri* genome (data not shown).

Table 2-7: Relative expression of select *Eptatretus stoutii* gene transcripts in eye vs. brain tissue.

FPKM = Fragments Per Kilobase of transcript per Million mapped reads.

Gene	Transcript ID	FPKM (brain)	FPKM (eye)	Ratio of eye/brain FPKM
OtxA	Unigene14754_All	2.78	18.78	6.76
OtxB	Unigene45908_All	N/A	11.54	N/A
OtxC	CL1927.Contig1_All	2.99	1.75	0.59
OtxD	CL6481.Contig2_All	0.3	13.12	44.47
Six3/6[1]*	Unigene16248_All	0.34	8.88	26.51
Six3/6[2]*	Unigene22484_All	12.2	3.63	0.30
Six3/6[3]*	Unigene5558_All	22.16	47.99	2.17
Rx	CL3100.Contig2_All	0.47	25.29	53.81
RPE65	Unigene9362_All	0.78	525.43	673.63
Pax6	CL2037.Contig3_All	4.06	12.90	3.18

*Three *Six3/6* transcripts were identified in the hagfish (*E. stoutii*) RNA sequencing dataset and are denoted by [1], [2], and [3].

Chapter 3

Discussion

3.1 The organization of the hagfish retina is not representative of the ancestral vertebrate condition, but other aspects of this structure (i.e., development) could still be informative for the study of eye and retinal evolution across vertebrates

Morphology hints that the hagfish eye has more shared features with the eyes of gnathostomes (and lampreys) than previously believed. However, there are still differences in morphology (and potentially development) that could be informative for understanding the origins of the vertebrate eye. One example is the types of cells occurring in the hagfish retina. Although hagfish appear to have the same retinal cell types as other vertebrates (RnPE, photoreceptors, interneurons, retinal ganglion cells) both the morphology and organization of these cells is distinct from jawed vertebrates (Bradshaw and Allison, 2022). The hagfish RnPE has similar machinery to the gnathostome RPE to drive the retinoid cycle (i.e., expression of RPE65), but it contains no pigment (Dong and Allison, 2021). Loss of pigment is a feature that is likely due to loss rather than retention of an ancestral character (both lampreys and the non-vertebrate chordates contain pigment in their photosensory organs). However, it would still be worthwhile to investigate if the same developmental processes regulate RnPE cell specification in hagfish and RPE formation in gnathostomes. The expression of *OtxA* in the hagfish RnPE suggests that at least some of the developmental machinery is shared, as *Otx2 i*s also critical in the development and maintenance of the jawed vertebrate RPE (Martínez-Morales et al., 2003; Béby and Lamonerie, 2013). Hagfish photoreceptors also have dramatic morphological differences compared to jawed vertebrates (and lampreys). Structurally, hagfish photoreceptors look ciliary in nature, but lack

the features that would allow them to be easily classified as rod or cone cells (Lamb, 2013). In addition, hagfish only possess one type of photoreceptor cell, compared to lampreys and gnathostomes which have multiple types of photoreceptors (usually one rod and at least one cone subclass) (Fain et al., 2010; Lamb, 2013; Fain, 2020). Investigating the morphology of hagfish photoreceptors and the developmental pathways that produce them has the potential to reveal how this cell type may have originated and/or changed throughout vertebrate evolution. Finally, the hagfish inner nuclear cell layer could also reveal clues to the evolution of the interneuron and retinal ganglion cells layers in vertebrates. Though disorganized, the hagfish retina does contain cells with markers for several classical gnathostome interneuron subtypes (calbindin, pKC-a, SNCG) (Dong and Allison, 2021). However, more work needs to be done delineating the specific interneuron cell subtypes the hagfish retina contains and how those neurons interact with the photoreceptor and retinal ganglion cell layers. In addition, genomic data suggests that hagfish do not have a direct homolog for *Atoh7* (although this could be due to incomplete genome data). *Atoh7* is a critical factor for specification of the retinal ganglion cells in the vast majority of vertebrates (Brown, et al., 2001; Kay et al., 2001; Wang et al., 2001). If this gene is truly absent in hagfish, it would be interesting to explore if hagfish employ an alternative method to form retinal ganglion cells, and if these alternative pathways are also employed by other vertebrate groups. Comparing the development of hagfish RnPE, photoreceptors, interneurons and retinal ganglion cells to that of lampreys and gnathostomes would be incredibly informative for understanding how conserved the mechanisms of retinogenesis are throughout the vertebrate lineage.

3.2 Proliferation in the hagfish retina

3.2.1 The ciliary marginal zone may be an ancestral vertebrate trait

Although the hagfish retina is rudimentary and small, the retina does continue to proliferate throughout the life of the animal. Hagfish eye size increases with age and the periphery of the retina expresses a marker of retinal progenitor cells *Pax6* (Dong and Allison, 2021). The hagfish retina also shows incorporation of EdU after a short 24 h pulse (see **Sections 2.4.1, 2.5.1**). This data suggests that the hagfish retina may have a region comparable to the gnathostome ciliary marginal zone. This retinal stem cell niche allows for continuous retinal growth in teleost fish and amphibians throughout ontogeny (Raymond et al., 2006). Compared to those groups, it is surprising that hagfish would have ongoing retinal growth, given their small eyes and deep-sea habitat. In addition, adult neurogenesis does not appear to occur in other early branching fish groups, the lampreys and elasmobranchs (Villar-Cheda et al., 2008; Hernández-Núñez et al., 2021). This raises the possibility that the ciliary marginal zone is an ancestral vertebrate characteristic that was subsequently lost in several groups (i.e., lampreys, elasmobranchs, mammals) (Figure 3-1). However, further work is needed to elucidate the nature of this structure in different vertebrate groups. It is also possible that a CMZ-like region evolved in more than one lineage (or embryonic retinal neurogenesis evolved to persist in the adult retina in more than one group - see **Section 3.2.3**) (Figure 3-1). More early-branching cyclostome and gnathostome species (e.g., lampreys, sharks) need to be examined to determine if the CMZ can be considered ancestral or derived for the vertebrate clade. Future studies also need to continue characterizing adult retinogenesis at the hagfish retinal peripheral margin to support this tissue's homology with the CMZ of gnathostomes.

3.2.2 Do hagfish have Müller glia?

Although many EdU labelled cells were observed at the periphery of the hagfish retina in this study (**Sections 2.4.1 and 2.5.1**), a sizeable proportion (~40%) of cells were located throughout the interior retina. The distribution of the EdU+ cells suggests that proliferation in the hagfish retina is not restricted to the peripheral zone. This finding raises the possibility of Müller glia being present in hagfish. Müller glia are glial cells (located in the INL) that can de-differentiate to take on a neural identity after retinal injury in other vertebrates (Raymond et al., 2006). In the hagfish retina, both EdU+ cells and cells expressing *Rx* (a marker for retinal neuronal progenitor cells) were found in the inner nuclear layer. If these markers were labelling retinal cells (as opposed to microglia or blood cells) this would support those cells potentially being Müller glia. To confirm the presence of Müller glia it would be necessary to conduct more studies examining molecular markers in the hagfish retina. This would be particularly informative if performed on hagfish tissue that was light vs. dark adapted. Light exposure is an established method to induce retinal damage (Thummel et al., 2008; Ranski et al., 2018). Exposing hagfish to light to cause retinal damage and then assaying for signs of increased retinal proliferation could provide strong support for the presence of Müller glia (or at least a Müller glia-like mechanism) in the cyclostome eye. There are certain genes which could act as markers. *Ascl1* (a gene involved in the de-differentiation and subsequent re-programming of Müller glia during damage repair) expression would be expected if hagfish EdU + retinal cells are initiating a regenerative response after tissue damage (Gao et al., 2021). Hagfish also have a homolog of CRALBP, another conserved marker of Müller glia (Roesch et al., 2008; Dong and Allison, 2021). However, other common markers of Müller glia (i.e., *GFAP*, *ApoE*, *vimentin*) could not be identified in available hagfish genomic or RNA sequencing datasets. Whether these genes are not present in the hagfish

genome or are just missing due to those genomes being incomplete is unknown. However, if hagfish lack these genes that would create questions about whether Müller glia (or Müller glia-like) cells are specified differently in hagfish compared to gnathostomes.

3.2.3 Could paedomorphosis be linked to continued retinal growth?

Given that the hagfish eye appears to be barely functional, it is surprising that there is evidence of continual retinal growth. One possibility is that this could represent a form of paedomorphosis or retention of larval characteristics. Unlike lampreys which undergo metamorphosis between a larval and adult stage, hagfish have direct development (Ota and Kuratani, 2006). Once embryos hatch, the hagfish emerge as smaller versions of the adult and then continue to grow throughout life. Whereas lampreys appear to lose retinal neurogenesis after metamorphosis (but clearly exhibit it during larval stages) (Villar-Cheda et al., 2008) perhaps the hagfish retina continues to develop because it has lost the signals needed to cease retinogenesis (or the timing of those signals has been altered). This interpretation is supported by the similar morphology of larval lamprey eyes to hagfish eyes (Suzuki and Grillner, 2018). Under this scenario, continuous retinal proliferation could be a remnant from a time when the hagfish eye was more functional (i.e., post-embryonic neurogenesis was retained despite regression of other features of the eye). Alternatively, paedomorphosis could have been selected for if continued retinal growth was somehow useful for the hagfish in their environment. Although not capable of forming images, perhaps the eye is needed as a light detector (Fernholm, 1974). This explanation would account for why hagfish would invest the energy to produce new cells in such a rudimentary sensory structure. There is also the possibility that the genes that promote continued retinal growth also perform other important roles (i.e., perhaps ongoing proliferation of the robust olfactory system)

for the hagfish that are too valuable to lose (i.e., pleiotropy limits what can evolve or what can be lost).

3.3 Hagfish retinal development appears to be conserved (to a degree) with gnathostomes

3.3.1 The (limited) gene expression data available suggests hagfish retinal cell types may be specified through the same pathways as other vertebrates, but further characterization of hagfish retinal development is needed

Although hagfish embryos are not easily accessible, we have been able to explore some aspects of retinal development by examining a proliferative tissue in the adult hagfish. Previous work has already shown that this region expresses *Pax6*, a common marker of retinal progenitor cell pools (Dong and Allison, 2021). This study found that the hagfish retina also expresses *OtxA* (a homolog of gnathostome *Otx2*) and possibly *Rx* at the peripheral CMZ-like zone (**Sections 2.4.3 and 2.5.3**). *Otx2* is vital for the development and maintenance of several cell types within gnathostomes including RPE cells, photoreceptors and bipolar cells (Martínez-Morales et al., 2003; Viczian et al., 2003; Béby and Lamonerie, 2013). Finding the hagfish homolog of this gene expressed in the CMZ (and throughout the hagfish retina) suggests that the cyclostome homolog may have similar functions. *Rx* is a marker of retinal progenitor cells. The *in-situ* hybridization data demonstrated that hagfish *Rx* is expressed throughout the INL and may be expressed at the CMZ. This pattern matched the expression seen in adult gnathostome eyes, again suggesting a shared functional role for this gene in both jawed and jawless vertebrates.

However, this gene expression data is not sufficient to confirm the hagfish homologs are regulating the development of the retina (and if the function of *Otx2/OtxA* and *Rx* are truly conserved between gnathostomes and cyclostomes). It would be highly valuable in the future to perform double-labelling experiments with *OtxA* and EdU or *OtxA* and a marker of retinal progenitor cells (i.e., *Pax6*, *Rx*, *Six3*) to determine if *OtxA* expression occurs in developing retinal cells. This would provide more support for *OtxA* controlling cell specification (as opposed to a maintenance role for mature retinal neurons). It would also be fascinating to treat live hagfish with EdU pulses at different times, and then see if EdU labelled, *OtxA* positive cells are eventually incorporated into the mature retina. If *OtxA* has similar functions in hagfish and gnathostomes one would expect developing *OtxA*+ cells to mature into RnPE, photoreceptor or bipolar cells. Another approach would be to use a transgenic line of animals to label *OtxA* expressing cells and then see what markers those cells co-express at different areas of the retina (i.e., CMZ vs. mature retina). Although this technique is not currently feasible in hagfish due to these organisms being difficult to breed and maintain in captivity, other lab groups have successfully started working on transgenic lines of lamprey (Parker et al., 2014). Work in lamprey could help complement what we have demonstrated in hagfish. Finally, performing double-labelling *in situ* hybridization experiments would provide more information about the nature of the hagfish CMZ zone. If retinal stem cell markers and markers for more committed retinal cells (e.g., neuronal markers) were labelled in the same retina, it would reveal if there were a continuum of cells at different stages of differentiation (i.e., akin to that seen in the gnathostome CMZ).

The ideal experiment would be to directly manipulate genes suspected to play a role in hagfish retinal development. By knocking out genes like *OtxA* and *Rx* and then examining the effect on

the retina, one could test the functional role of these genes. Again, hagfish are not a laboratory organism amenable to these genetic techniques (although it may be possible in lampreys). Another possibility would be to take an organism where genetic manipulation is well-established (i.e., mice or zebrafish) and create transgenic organisms that express the cyclostome homologs of certain retinal genes. These studies could allow one to determine if (and to what extent) retinogenesis gene functions are conserved across vertebrates. If cyclostome genes can drive elements of retinal development in a gnathostome model system, that would support the function of those genes is shared and/or that those genes act within similar pathways in jawed and jawless vertebrates. This study has identified several genes which could be utilized for this purpose. Future experiments comparing (and potentially manipulating) retinal developmental pathways in gnathostomes and cyclostomes would be invaluable for uncovering the evolutionary history of the vertebrate retina.

3.3.2 Absence vs. secondary loss of retinal genes and pathways in the hagfish eye

The hagfish eye is unique from that of other vertebrates in many ways. The existing literature suggests most of these features likely evolved via regression. However, investigation of hagfish retinal development could reveal new characteristics unique to hagfish (i.e., the potential absence of *Atoh7*, *GFAP*, *ApoE*, and *vimentin* in the hagfish genome). If these genes are truly missing in hagfish, this would imply elements of the hagfish retinogenesis pathway must operate differently than in vertebrates that have homologs of these genes. If so, it will be difficult to determine whether the absence of these genes is ancestral for vertebrates or they were secondarily lost in hagfish. Investigating the machinery producing the retina in jawless vertebrates (hagfish and lampreys) and comparing it to the gnathostome condition will be vital for understanding the

evolution of retinogenesis in the vertebrate lineage. Genes and developmental pathways that are shared in hagfish, lampreys and gnathostomes could represent ancestral vertebrate features.

In addition, aspects of retinal development that occur only in hagfish and lampreys can help us understand evolutionary changes unique to the cyclostome lineages. Whereas hagfish may have undergone loss or degeneration of their eyes during adaptation to a deep-sea habitat, lampreys did not undergo this ecological shift. Comparison of eye/retinal development between hagfish and lampreys could help separate whether missing and/or rudimentary elements of the hagfish eye are a result of regression or just never evolved in the first place (Fernholm and Holmberg, 1975; Davies et al., 2012). Another factor to consider is the examination of different species of hagfish and lamprey. Most studies are based on a few species that are easily accessible. However, different species of hagfish possess different retinal and photoreceptor morphologies (likely linked to habitat and light availability) (Fernholm and Holmberg, 1975). Different species of lamprey also have variability in retinal structure, such as the number and classes of photoreceptor subtypes (Davies et al., 2009). By completing a more comprehensive phylogenetic examination of the cyclostome eye and retina, stronger conclusions can be drawn about the state of the cyclostome eye compared to the body of knowledge available for gnathostomes.

3.4 Conclusion

The mysterious and obscure hagfish represent a lineage that could be considered a type of living fossil. These organisms possess certain features that are unchanged from hundreds of millions of years ago (i.e., lack jaws and paired fins) and very little is known about hagfish ecology, development, and behavior. In the context of evolutionary studies, these organisms could contain a wealth of information about features that may be ancestral to the vertebrate lineage (in addition

to derived characteristics that arose solely in cyclostomes). To better facilitate the understanding of evolution of the vertebrate eye and retina, a greater emphasis should be placed on investigating the eyes of these creatures. Although rearing and husbandry of hagfish in captivity is still difficult, these fish are highly valuable from a phylogenetic perspective. Further studies investigating hagfish biology, development and evolution would be extremely informative for unravelling the origins and evolutionary history of key vertebrate innovations, including the eye.

Figures

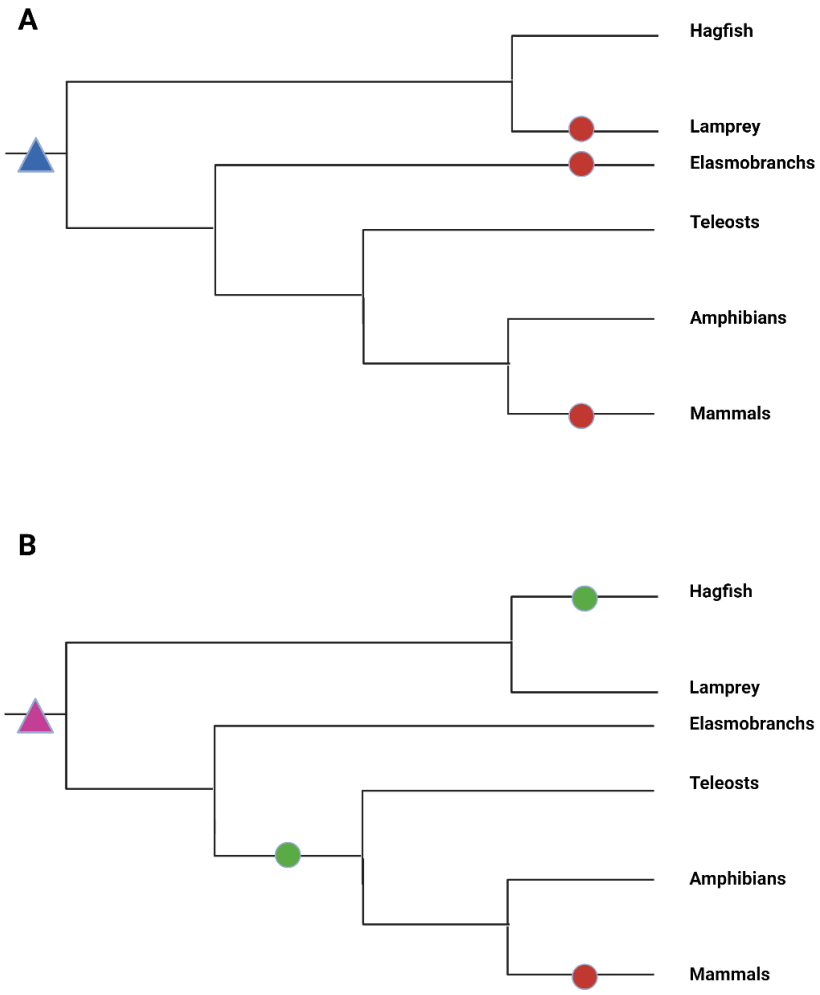


Figure 3-1. Two hypotheses for the evolution of the ciliary marginal zone (CMZ) within the vertebrate lineage. A) Represents a scenario where adult retinal neurogenesis (at the retinal periphery) is the ancestral vertebrate condition (represented by the blue triangle at the base of the tree). In this case, the last common ancestor of vertebrates would have possessed a stem cell niche at the retinal margin (the CMZ) that was active throughout ontogeny. Several extant clades retained this structure (i.e., hagfish, teleost fishes), whereas other groups secondarily lost it (i.e.,

mammals). B) Represents a scenario where the CMZ and adult retinal neurogenesis is a derived condition. In this case, the cessation of retinal neurogenesis after embryonic development would be the ancestral condition (represented by the purple triangle at the base of the tree). Several groups then acquired continued neurogenesis at the retinal margin (CMZ) independently. Red circles indicate clades that secondarily lost the CMZ niche. Green circles represent clades that independently gained a CMZ (relative to the ancestral condition).

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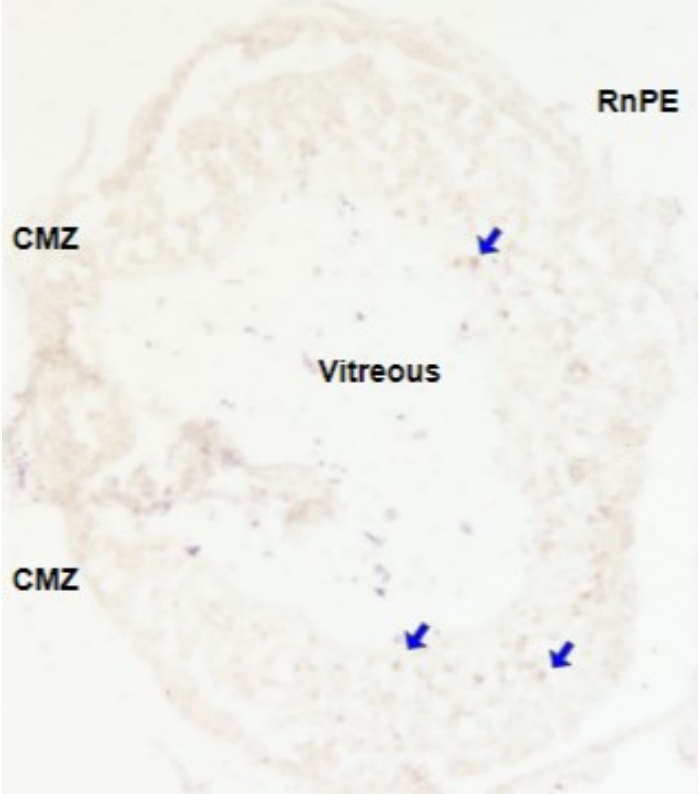
Appendix A – Attempted *In situ* hybridization probes



Supplementary Figure 1. The *Six3 in situ* hybridization probe very weakly labels the inner nuclear layer of the hagfish (*Eptatretus stoutii*) retina. We interpret the very faint labelling as the *in situ* hybridization conditions for the probe not being optimal. This probe should be re-attempted with different hybridization conditions (e.g., more concentrated probe, different proteinase K digestion times) to determine if and where *Six3* is expressed in the hagfish retina.

Supplementary Table 1. Gene block sequence for the *Six3* (plasmid based) riboprobe

Riboprobe	Length (bp)	Gene Block Sequence 5' to 3' (red text = EcoRI cut site, blue text = XbaI cut site)
Six3	1149	GAT CGA ATT CCA TGC TCT CCA TCA TCT CCC CCA GCT CGG AGG GTG CTT CGC TTC TTT ATT ACT CTC TCC AAA GCA CAG TGC CAG CGT TCG GAG GCT CCA TGT TTC ACC TGC CCA TCC TAA GCT TCA CGC CGC AGC AGG TGG CCA GCG TCT GTG AGA CGT TGG AGG AGA GTG GCG ATG TGG AGC GAC TTG GTC GTT TCC TCT GGT CAT TGC CTG TGG CTC CTG GTG CTT GGG AGG CCC TCA ACA AAC ATG AGT CTG TGC TAC GTG CAC GTG CAG TTG TAG CCT TTC ATG CGG GTA ACT TCC GTG ACC TTT ACC ACA TCC TGG AGA ACC ATA AGT TCA CCA AAG AGT CTC ACG GGA AGT TAC AAG CCA TGT GGC TCG AAG CAC ATT ATC AAG AAG CTG AGA AAC TGC GTG GAC GCC CAC TCG GAC CTG TTG ACA AGT ACC GGG TCC GCA AGA AGT TCC CGT TGC CCA AGA CAA TTT GGG ATG GTG AGC AGA AGA CTC ACT GCT TTA AGG AGC GAA CGC GAA ACC TGC TTC GTG AGT GGT ACC TTC AGG ATC CAT ACC CAA ATC CAT CGA AGA AGC GCG AAC TTG CAC AGG CCA CAG GCC TGA CCC CGA CTC AAG TTG GAA ACT GGT TCA AGA ACC GCA GGC AGC GAG ACC GTG CAG CAG CAG CAA AAA ACA GGC TCC AGC AGC CGC TCG CGC TGT GCC CGT CGG AGC GCG CGG TCC AGG CCT GCA CGG CGC GGC CTT ACG ACT CCA AGC ATC GGG GCC TGA GCG AGT CTC CGA CCT GCA CCG TGG CCA GTC CAG GTG CAG GAA GCC TTT CAA GCC TGA GCG ATC GGC CCG GGA TAA GCG CCT GCA CGA CAT CAC CAG TGT GCA GCG AAA ATG ACT GCG AGG ACT TCT AGA GAT C

Supplementary Table 2. Primer sequences used to generate the *OtxD* (PCR based) riboprobe

Probe	Forward Primer (5' to 3')	Reverse Primer + T7 Polymerase Site (underlined) (5' to 3')	Amplicon Size (bp)
OtxD*	GTC TTG TCC TGC GTT TTC CC	TAA TAC GAC TCA CTA TAG GGT TCA GCT TCC AAG AGG AGC C	898

*This probe was created, but optimization of the *in situ* hybridization was not completed due to time constraints.

Appendix B - Gene Accession Numbers

Supplementary Table 3. Accession Numbers for Rx homologs (protein sequences)

Organism	Accession Number
Mouse (*Mus musculus*)	NP_038463.2
Frog (*Xenopus tropicalis*)	XP_002936715.1
Spotted gar (*Lepisosteus oculatus*)	XP_006627202.2
Coelacanth (*Latimeria chalumnae*)	XP_006005850.1
Elephant shark (*Callorhinchus milii*)	XP_007901317.1
Hagfish (*Eptatretus burgeri*)	ENSEBUP00000010652.1

Supplementary Table 4. Accession Numbers for Six3/6 homologs (protein sequences)

Organism	Six3	Six6
Mouse (*Mus musculus*)	XP_017172856.1	NP_035514.1
Frog (*Xenopus tropicalis*)	XP_002944388.2	NP_001093696.1
Spotted gar (*Lepisosteus oculatus*)	XP_006638703.1	XP_015206118.1
Coelacanth (*Latimeria chalumnae*)	XP_005998848.1	XP_005986483.1
Elephant shark (*Callorhinchus milii*)	XP_042189390.1	XP_007902021.1
Lamprey (*Petromyzon marinus*)*	XP_032803937.1 (Six3-like[a]) XP_032820798.1 (Six3-like[b])	XP_032818834.1 (Six6-like[c]) XP_032819592.1 (Six6-like[d])
Hagfish (*Eptatretus burgeri*)*	ENSEBUP00000020951.1 (Six3-like[e]) ENSEBUP00000002483.1 (Six3-like[f]) ENSEBUP00000017317.1 (Six3-like[g])	N/A

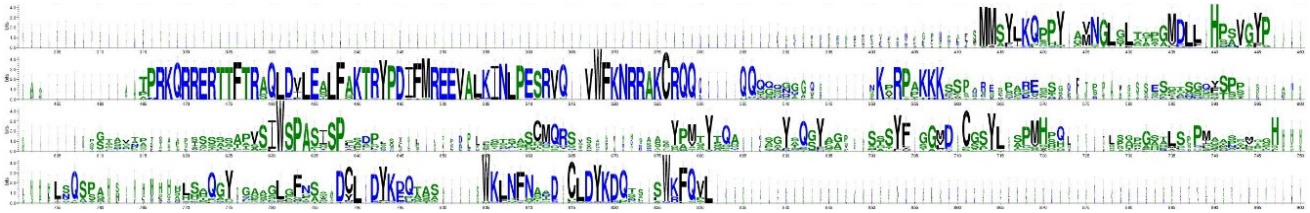
* Both hagfish and lamprey had multiple Six3/6 paralogs, but these paralogs could not be assigned to a Six3 or Six6 identity (the paralogs have been classified here based on tBLASTn search results, but further work is needed to confirm sequence identity). The cyclostome Six3/6 sequences are labeled a-g as per Figure 2-5 and Table 2-5.

Supplementary Table 5. Accession Numbers for Otx homologs (transcript (red) and protein (blue) sequences)

Organism	OtxA	OtxB	OtxC	OtxD	Otx1	Otx2	Otx5/Crx
Mouse (*Mus musculus*)	N/A	N/A	N/A	N/A	ENSMUST00000006071.14 ENSMUSP00000006071.8	ENSMUST00000119070.8 ENSMUSP00000112532.2	ENSMUST00000174318.8 ENSMUSP00000134400.3
Western clawed frog (*Xenopus tropicalis*)	N/A	N/A	N/A	N/A	ENSXETT00000099908.2 ENSXETP00000098844.2	ENSXETT00000034219.5 ENSXETP00000034219.4	ENSXETT00000040682.5 ENSXETP00000040682.4
Spotted gar (*Lepisosteus oculatus*)	N/A	N/A	N/A	N/A	ENSLOCT00000019837.1 ENSLOCP00000019804.1	ENSLOCT00000013768.1 ENSLOCP00000013739.1	ENSLOCT00000017490.1 ENSLOCP00000017459.1
Coelacanth (*Latimeria chalumnae*)	N/A	N/A	N/A	N/A	ENSLACT00000019719.1 ENSLACP00000019581.1	ENSLACT00000021735.1 ENSLACP00000021594.1	ENSLACT00000014227.1 ENSLACP00000014128.1
Elephant shark (*Callorhinchus milii*),	N/A	N/A	N/A	N/A	XM_042333478.1 XP_042189412.1	XM_042338324.1 XP_042194258.1	XM_042345401.1 XP_042201335.1
Sea lamprey (*Petromyzon marinus*)	XM_032956047.1 XP_032811938.1	XM_032964131.1 XP_032820022.1	XM_032948424.1 XP_032804315.1	XM_032962818.1 XP_032818709.1	N/A	N/A	N/A

Inshore hagfish (*Eptatretus burgeri*)	ENSEBUT00000004000.1 ENSEBUP00000003621.1	ENSEBUT00000013972.1 ENSEBUP00000013396.1	ENSEBUT00000003049.1 ENSEBUP00000002692.1	ENSEBUT00000027855.1 ENSEBUP00000027279.1	N/A	N/A	N/A

Appendix C - Protein Alignments for Figure 2-7 *Otx* phylogenetic tree



Supplementary Figure 2. Protein alignment for 36 *Otx* sequences (used to construct the phylogenetic tree in Figure 2-7). Protein alignment completed in Geneious Prime (Geneious Prime, v. 2022.0.1, Biomatters Ltd) using Geneious alignment. Figure created using WebLogo3 (Crooks et al., 2004) https://weblogo.threeplusone.com.