Novel vertebrate features identified in the rudimentary eye of the Pacific hagfish (*Eptatretus stoutii*)
by

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ABSTRACT

A disparate range in eye complexity exists between vertebrates and their sister group, tunicates. In tunicates, "eyes" take the shape of simple shadow-detecting photoreceptive patches, while vertebrates possess one of the most intricate and complex eyes seen in the animal kingdom. We do not yet know how the complex vertebrate eye could have arisen from a highly simplified eye structure in the shared ancestor with tunicates. With no extant transitionary forms between tunicates and vertebrates, we must use the earliest diverging vertebrates to better understand the evolutionary history of vertebrate eyes.

Hagfish are one of two surviving jawless fish lineages, and so represent one of the earliest examples of vertebrate eyes. Despite being vertebrates, hagfish lack many of the conserved vertebrate eye features that are conserved from lamprey to humans. Their eye is unpigmented, obscured by transparent epidermis, and lacks a three-layered retina, lens, and ocular musculature. This makes hagfish a unique case in vertebrate evolution. The cause of this simplicity has been explained by several different potential histories. One school of thought is that the rudimentary features can be thought of as ancestral, meaning that the hagfish eye represents an early stage in vertebrate eye evolution that existed prior to the evolution of the typical vertebrate camera-style eye. However, this hypothesis indirectly suggests that lamprey evolved their eye in parallel with jawed vertebrates, which is in opposition to a number of molecular phylogenetic studies. Our competing hypothesis conversely suggests that these rudimentary features are a result of regression from an ancestrally complex eye.

Very little molecular data exist to characterize the eyes of hagfish, and so we set out to test our hypothesis by defining the molecular characters of the retina of the Pacific hagfish (*Epatatretus stouii)*. In order to do this, we produced a hagfish eye transcriptome from which we pulled a

number of novel transcripts including homologs of retinoid cycling genes (*RPE65, LRAT,* and others) and interneuron markers (*PKC-α, calbindin, Pax6* and others). By *in situ* hybridization, we were able to localize the expression of several of these novel transcripts to regions of the hagfish retina homologous with those in other vertebrates. Identification of these novel transcripts revealed the presence of a greater diversity of cell types within the retina than previously characterized, including the first evidence of interneurons and a supporting functional retinal epithelium. The presence of these features in conjunction with the presence of these features in lamprey and jawed vertebrates, suggests that hagfish eyes share a highly conserved eye with other vertebrates. Our work to identify novel features of the hagfish retina has opened the door to a number of future studies, and provided substantial evidence to support the degenerative eye hypothesis.

PREFACE

This thesis is an original work by Emily M. Dong.

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LIST OF ABBREVIATIONS

aPKC	Atypical protein kinase C
BGI	Beijing Genomics Institute
BMSC	Bamfield Marine Sciences Centre
c-opsin	Ciliary opsin
cDNA	Complementary DNA
CDS	Coding sequence
CHOP	Children's Hospital of Philadelphia
Ci-CRALBP	Ciona intestinalis CRALBP
Ci-RPE65	Ciona intestinalis RPE65
CMZ	Ciliary marginal zone
CRALBP	Cellular retinaldehyde-binding protein
CRBP	Cellular retinol binding protein
DAPI	4',6-diamidino-2-phenylindole
DIG	Digoxigenin
DNA	deoxyribonucleic acid
E. burgeri	*Eptatretus burgeri*
E. cirrhatus	*Eptatretus cirrhatus*
E. stoutti	*Epatatretus stoutii*
EtOH	Ethanol

FLR	Fluorescein
GCL	Ganglion cell layer
GNAT1	Guanine nucleotide-binding protein G(t) subunit alpha-1
GO	Gene ontology
INL	Inner nuclear layer
IPL	Inner plexiform layer
ipRGC	Intrinsically photosensitive retinal ganglion cell
IRBP	Interphotoreceptor retinoid-binding protein
LRAT	Lecithin-retinol acytransferase
LWS	Long wavelength sensitive
M. garmani	*Myxine garmani*
M. glutinosa	*Myxine glutinosa*
MITF	Melanogenesis Associated Transcription Factor
moe	*mosaic eyes*
Nrl	Neural retina-specific leucine zipper protein
ONL	Outer nuclear layer
OPL	Outer plexiform layer
pac	*parachute*
PAX6	Paired box protein 6
PCR	polymerase chain reaction

PDE6G	Gamma subunit of cyclic GMP-phosphodiesterase
PFA	Paraformaldehyde
PKC-α	Protein kinase C alpha
PKD	Polycystic Kidney Disease domain
PMEL	Pre-melanosome protein
POD	Horseradish peroxidase
r-opsin	Rhabdomeric opsin
RACE	Rapid amplification of cDNA ends
RDH5	11-*cis*-retinol dehydrogenase 5
RGCs	Retinal ganglion cells
RH1	Rhodopsin
RH2	Green opsin
RNA	Ribonucleic acid
RnPE	Retinal (non)pigment epithelium
RPC	Retinal progenitor cell
RPE	Retinal pigment epithelium
RPE65	Retinoid Isomerohydrolase
Rx	Retinal homeobox protein
Sdk	*Sidekick*
SV2	Synaptic vesicle protein 2

SWS	Short wave sensitive
SWS1	Short wavelength sensitive opsin 1
SWS2	Short wavelength sensitive opsin 2
TEM	Transmission electron microscopy
UV	Ultraviolet

1 INTRODUCTION

Part of this chapter contains works previously published in:

Dong EM & Allison WT (2017) *The Eye.* Encyclopedia of Evolutionary Psychological Science. Editors: V Weekes-Shackelford & TK Shackelford. Springer. doi: 10.1007/978-3-319-16999-6. (Invited Book Chapter). *Written by EMD, edited by WTA.*

1.1 What is an eye

The glint of a predator's gaze, plumage of a potential mate, or an ominous dark cloud announcing the approach of a violent storm all represent visual signals imperative to an organism's survival, and all necessitate the use of vision. Vision was one of the major sensory innovations to arise during the Cambrian Explosion. It provided animals with the means to interpret and react to light information, a driving force in the growing divergence of life strategy and corresponding body plan (Parker 1998; Conway-Morris 2003; Plotnick, Dornbos and Chen 2010). The first appearance of eyes coincides with the first observed examples of complicated behavioral strategies such as predation (Parker 1998; Conway Morris 2000). Across the animal kingdom, the prevalence of eyes in over 90% of known fossilized and extant species demonstrates that eyes have undoubtedly contributed the massive radiation and diversification of animals (Land and Fernald, 1992).

Light information is multifaceted and can be interpreted based on direction, intensity, wavelength, or polarization, either alone or in combination. As such, there are a multitude of different ways by which animal eyes receive and use this information, leading naturally to a great variety of light receiving strategies. Herein is described a broad overview of eyes found throughout the animal kingdom, with a focus on vertebrates. This chapter will briefly explore the

simultaneous diversity in structure, function and development of animal eyes, in balance with similarities and deep conservation.

1.1.1 Image formation

The basis of sight is a capacity for detection of photons by specialized sensory cells called photoreceptors. These cells transduce light information into cellular signals through the action of a group of light sensitive photopigments – the opsins. When photons interact with opsins, a chain of signals is induced that culminates in the production of electrochemical signals that are subsequently sent to the brain (Wald 1968). In order to improve the efficiency of photon capture, photoreceptors have membranes with high surface area to maximize the amount of opsin that can be housed within a single cell. By efficiently packing opsins in high density, the photoreceptors provide more visual information for the brain to produce high quality images

Photoreceptors have been recruited to a variety of structures and organs including some that are non-visual. Accordingly, the definition of "eye" can be quite broad, encompassing anything from the simplest photoreceptor patch to the high resolution image-forming camera-style eye. For the purposes of this text, an "eye" is defined as a light sensing organ capable of image formation. Simpler photoreceptive structures that are not considered eyes by this definition include the "frontal eye" of the cephalochordate, amphioxus or the vertebrate circadian rhythm entraining pineal-complex (Ekstrom and Meissl, 2003; Lamb et al., 2007; Vopalensky et al., 2012). These structures, while able to detect light intensity, are unable to collect sufficient light information to form images, and by this definition are not true eyes.

An image is defined here as an integration of light information received by the eye from multiple directions into a signal which, when carried to the central nervous system, is interpreted as a dimensional representation of the environment. Image formation requires more than a

single photoreceptive cell, it requires a group, and this grouping must be arranged in such a way that directionality can be perceived (Fig. 1.1). The basic components of any eye involve an opening through which light enters, and a photosensory retina that transduces light energy into an electrochemical signal. By arranging photoreceptors in a cup shape, the amount of light that can be captured is drastically improved (Fig. 1.1). In being able to capture directional information, detection of such things as texture, shape and trajectory of movement is made possible. The evolution of an eye with these capabilities is what permitted animals to pursue a larger variety of food sources, higher energy food, provided another means of species recognition, and most importantly allowed for immediate reactions to potential threats (Lamb et al. 2007).

1.2 Eye diversity in the animal kingdom

We recognize that the eye of a great horned owl is vastly different than that of a house fly – and yet, both are recognized under the same umbrella of "eyes". Within the category of "image forming" visual organs, we find a great number of eyes that have evolved among different animal lineages and elaborated in vastly different ways. Among all eye and eye-like organs, two features are highly conserved: phototransduction by a photosensory cell, in association with light screening pigment (Arendt and Wittbrodt, 2001). Melanins, pterins, carotenoids and iridiophores have all been reported in association with photosensory organs of animals (Vopalensky and Kozmik, 2009). This partnership has served to improve directional sensing, and has given way to the evolution of complex image forming eyes that appear in several animal phyla including: molluscs, chordates, annelids, and arthropods. In order to better appreciate the forms that exist, eyes can be generally categorized by a few major criteria: (1) if the eye is compound or simple, and (2) the type of photoreceptors that comprise the retina.

1.2.1 Compound vs. simple eyes

Compound eyes combine multiple individual units of light collection into a single light detecting organ. Crustaceans are canonical examples of compound eyes. These eyes are comprised of many single units of photoreception (ommatidia) grouped into a large multi-faceted eye. Individual ommatidium possess their own individual photoreceptors and lenses, and can either work together to bring light onto a shared photoreceptive structure (superposition eyes), or may act independently, with each facet optically isolated from its neighbors (apposition eyes). Apposition and superposition eyes are dispersed throughout crustacean groups (Nilsson, 1983). The scattered and seemingly random co-existence of both superposition and apposition eyes across crustaceans suggests a strong evolutionary link between the two not easily explained as two independently evolved eyes. Apposition eyes, due to their comparatively simpler optics, have been regarded as the more primitive of the two. This is supported by some evidence that superposition eyes may arise from apposition eyes during ontogeny. In decapod and euphausiid crustaceans, both eye types are present during different life stages of the animal: apposition during larval stages and superposition in adulthood (Nilsson, 1983; Nilsson et al., 1986). In at least one crustacean, *Spongicoloides koehleri*, the presence of apposition eyes in the adult has been proposed to be a result of neoteny, or retained juvenile features (Gaten, 2007).

A "simple" or "single chambered" eye relies on the action of a single unit that focuses light through a single opening onto a photosensory retina. The term "simple eye" can encompass a number of morphological types with varying degrees of complexity. Pinhole eyes are among the most basic of single chambered eyes, and are most famously found in *Nautilus*. The pinhole eye of *Nautilus* is comprised of a retina housing cup, with a small or "pinhole" anterior opening.

Though it's optics allow for the focusing of light from great distances, the pinhole eye has a limited capacity for capturing a large field of view. The pinhole is contracted to improve resolution, at the cost of light information that can reach the retina (Hurley et al., 1978). This style of eye offers a highly restrictive field of view, as only light from a limited number of angles will reach the retina through the pinhole. It also means that the inner environment of the eye is continuous with the environment.

Camera-style eyes are the most complex of the simple eyes and are the most capable in terms of high resolution of image formation (Land and Fernald, 1992). Lens bearing camera-style eyes build upon the basic principles of a pinhole eye, with the addition of a light focusing element (lens), accompanied by musculature and a cornea. These combined features allow for the dynamic capture of images across varying focal planes, and expand the field of view that can be focused onto the retina. Camera-style eyes are notably found in both vertebrates and cephalopods.

1.2.2 Rhabdomeric vs. ciliary photoreceptors

Among the retinas of animals, one of two photoreceptor types dominate the retina. Photoreceptors can be separated into one of two major groups based on their morphology and mechanism of cellular signaling: ciliary and rhabdomeric photoreceptors (Eakin 1965). These two broad characterizations arise by two different means of enlarging the opsin housing membranes within the photoreceptor outer segment. In ciliary photoreceptors, opsin bearing membrane is increased by a specialization of primary cilia. Rhabdomeric photoreceptors increase membrane surface area by forming microvilli on the cell surface (Eakin 1982; Eakin 1965). The dichotomy of ciliary and rhabdomeric photoreceptor cell types is not only defined by morphology, but also by the intracellular signaling of opsins in response light. Opsins found in rhabdomeric

photoreceptors are termed “r-opsins”, whereas opsins found in ciliary photoreceptors are termed “c-opsins”. In ciliary photoreceptors c-opsins transduce light information by a hyperpolarizing phosphodiesterase pathway, whereas in rhabdomeric photoreceptors, depolarization is initiated by phospholipase C (Arendt 2003; Fernald 2000). The families of opsins found in each ciliary and rhabdomeric photoreceptors are unique to each cell type, though are part of a larger family which is ubiquitous across metazoans (Arendt, 2003; Fain et al., 2010). Most invertebrate retinas are dominantly populated by rhabdomeric photoreceptors, while vertebrate retinas primarily use ciliary photoreceptors.

1.2.3 Deep homology in the evolution of animal eyes

Researchers have been engaged in the study of eye evolution for centuries, including Charles Darwin who was most famously intrigued by these “organs of extreme perfection” (Darwin, 1859). The history of interpreting and understanding the evolutionary origin of eyes across the animal kingdom has proven to be nearly as dynamic and complex as the eyes themselves. In a survey of photoreceptor and eye morphology across the animal kingdom, von Salvini-Plawen & Mayr (1977) suggested that eyes (defined by a looser definition, not restricted by a capacity for image formation) have evolved independently upwards of 40 times.

Years later, a transcription factor (*Pax6)* was found to be both necessary and sufficient to drive the development of eyes in both vertebrates and invertebrates. Expression of the mouse Pax6 ortholog in the developing eye field of *Drosophila melanogaster* produced fully functional eyes (Gehring and Ikeo, 1999; Onuma et al., 2002; Quiring et al., 1994). This finding was unexpected because the eyes of mammals and flies are so fundamentally different in their development, organization at maturity, and in the photoreceptors that dominate their retinas. The discovery that one single master gene from a vertebrate was sufficient to drive eye

development in flies was paradigm shifting, and brought the idea of independent evolution into question. A new family of hypotheses surrounding the shared origin of animal eyes was born following this great discovery.

Rather than a completely independent evolution, it is now proposed that animal eyes have arisen by the contributions of *Pax6*, which likely predated image forming eyes in a metazoan ancestor (Nilsson, 1996). The shared underlying developmental signal (*Pax6*) has been passed down from a shared ancestor of the different photoreceptor across metazoans. It is proposed that both rhabdomeric and ciliary photoreceptors were present in Urbilateria (the theorized shared common ancestor of all bilaterians). A large scale appraisal reveals a general pattern, in which a majority of animal groups have retained the rhabdomeric style, while vertebrates use ciliary photoreceptors, though ciliary and rhabdomeric types do co-occur in some instances (Arendt 2003).

The eye of the scallop *Pecten maximus*, for example is comprised of a distal and proximal retina. The distal retina is comprised of ciliary photoreceptors, and the proximal of rhabdomeric photoreceptors (Barber et al., 1967). Recent sequencing of the scallop genome has also confirmed that both rhabdomeric and ciliary opsins are present (S. Wang et al., 2017). In vertebrates, where ciliary photoreceptors are the image forming photoreceptive cells in the retina, homologues of rhabdomeric photoreceptors have also been identified. Retinal ganglion cells (RGCs), the neurons that form the optic nerve and are responsible for relaying light information to the brain, have been proposed as retained rhabdomeric photoreceptors in vertebrates (Arendt, 2003). Based on the RGCs use of developmental cues shared with rhabdomeric cells (*brn3, atonal, Pax6*) and the expression of a rhabdomeric-type opsin (melanopsin), RGCs are considerably homologous to rhabdomeric photoreceptors (Arendt,

2003). However, the phototransduction cascade whereby melanopsin transduces light signals has yet to be identified.

1.2.4 Convergent evolution in animal eyes: vertebrates and cephalopods

It is not surprising to see that multiple lineages implement similar strategies in deciphering light information considering the necessary role that vision has played in the evolution of metazoans. Though photoreception in metazoans is nearly ubiquitous across groups, image formation by way of a camera-style eye is achieved in two immensely different groups: vertebrates and cephalopods (Packard, 1972). Incredibly, though the eyes found in each group are composed by disparate cell types that use different phototransduction mechanics, and arise by divergent mechanisms in development, they form one incredibly similar structure.

Side-by-side, the camera-style eye in both vertebrates and cephalopods possesses parallel features including an iris, lens, vitreous compartment, pigment cells, and a retina comprised of opsin-packed photosensitive neurons. Both eyes use a lens to focus light onto the retina, though the mechanism by which the plane of focus is altered differs between the two. In cephalopods and vertebrates, the lens is used to change the focal plane by use of associated musculature (Beer, 1897). Photoreceptors of the cephalopod retina are arranged tangentially in the eye in a regular mosaic pattern, as in vertebrates (Engström, 1963; Young, 1960). Functionally, these two eyes both produce high quality images, best exemplified by the common cuttlefish *Sepia officinalis* which uses visual cues to induce drastic changes to body camouflage to match the surrounding environment with incredible accuracy (Chiao et al., 2015; Marshall and Messenger, 1996). However, at the cellular, molecular and genetic levels, it becomes clear that

though they appear similar, the shared morphology between vertebrates and cephalopods is a result of uncanny convergence.

One feature of the cephalopod eye that clearly distinguishes it from that of the vertebrates, is the retina. In cephalopods, photoreceptors are directed into the vitreal space of the eye, with axons that project out of the back of the retina behind the photoreceptors rather than passing in front of the photoreceptors as they do in vertebrates. These photoreceptors are rhabdomeric photoreceptors, while vertebrate retinas are comprised of ciliary photoreceptors. Accompanying these vastly different cell types are a number of developmental signals and functional components which also differ greatly. Cephalopod eyes develop from an ectodermal eye placode on the dorsal surface of the developing head that, by internalization of the optic cup, forms the neural retina (Koenig et al., 2016; Tomarev et al., 1997; Yamamoto et al., 1985). Ectodermal tissue from another source later surrounds the optic vesicle and by projection and infolding, develop the lens, cornea and iris (Harris, 1997). In vertebrates, the eye instead begins as an evagination of the neural tube. This outpocket forms the optic cup, and makes contact with the overlying ectoderm to induce the lens and cornea formation.

1.3 Chordate eyes

Key homologous features unite some features of the "eyes" across Chordata, but the intricacy of the vertebrate camera-style eye eclipses that of the simple eye-spot or eye-patch tissues in the other two extant chordate lineages: cephalochordates and tunicates. The disparate complexity between vertebrate eyes and those of non-vertebrate chordates, with very little information about intermediate forms, presents an intriguing evolutionary gap in our understanding of camera-style eyes in vertebrates. Tunicates, cephalochordates, and vertebrates all share a common ancestor, thus evolution of the eyes of vertebrates necessitates an understanding of

the eyes of early diverging chordates. The visual systems of these other two chordate groups are less complex in comparison, and are composed of a much less diverse cast of cells.

In cephalochordates, there are several light sensitive organs – Joseph's cells, dorsal ocelli, organs of Hesse, lamellar body, and the frontal eye. Joseph cells and dorsal ocelli utilize rhabdomeric photoreceptors, the corresponding r-opsins and associated phototransduction cascade (Lacalli, 2001). Joseph's organ and dorsal ocelli are unique among animal photoreceptors in that they appear not to require Pax6 expression in order to develop (Glardon et al., 1998). These are proposed to have appeared by a separate origin without the use of Pax6. Very little is known about the development of these Pax6-independent photoreceptors.

The cephalochordate photoreceptive organ considered homologous to vertebrates is the frontal eye, which is made up of ciliary photoreceptors, expressing a ciliary opsin (Pergner and Kozmik, 2017; Vopalensky et al., 2012). The proposed function of the frontal eye is to detect shadow, and aid the orientation of the body in the water column during feeding (Stokes and Holland, 2016).

The photoreceptors of the unpaired frontal eye are found in a single-row horiztonal array, and lack the capacity for forming a two dimensional image (Lacalli et al., 1994). The frontal eye resembles the vertebrate retina in the absolute simplest terms. Photoreceptors are associated with adjacent pigment cells (Lacalli et al., 1994). Photoreceptors make contact with neighbor secondary nerve cells, representing an early form of the complex messaging relay of the vertebrate photoreceptors to interneurons, followed by ganglion cells (Lacalli et al., 1994; Pergner and Kozmik, 2017). Though in the case of the frontal eye, the cells are arranged laterally, rather than layered horizontally. This retina-like structure is also supported by glial-like cells (Lacalli et al., 1994). Retinal pigment epithelium genes (*Mitf*) and melanin synthesis genes have

also been identified in the pigment cell of the frontal eye, and *Otx* was shown to be expressed in the photoreceptors as well as *Rx* (Vopalensky et al., 2012). As the earliest diverging chordate lineage, cephalochordates provide the best representation of ancient chordate vision. Though it is a simple tissue, it possesses homologs of vertebrate retinal markers.

Tunicates, not cephalochordates, have been determined to be the closest extant relatives of vertebrates (Delsuc et al., 2006). The features of their eye also support this relationship. Tunicate life-history is biphasic, and only the larval free-swimming stages possess a light sensitive organ called an "ocellus". This organ is significantly simplified in comparison with vertebrate eyes, though more complex than that of cephalochordates. The ocellus is comprised of approximately 10-20 photoreceptive modified ciliary cells that are surrounded by a single cup-shaped pigment cell (Eakin and Kuda, 1971). These cilia are more highly modified than those of cephalochordates, with structures that the approximate inner and outer segment observed in vertebrate photoreceptors (Eakin and Kuda, 1971). The tunicate photoreceptive cilia express ciliary opsin, Ci-opsin1 (Kusakabe et al., 2001). These photoreceptors have been further characterized to produce hyperpolarizing potentials, like vertebrates (Gorman et al., 1971). During phototransduction, light absorption changes the conformation of retinal, the opsin bound chromophore responsible for transducing light to an electrochemical signal. In order for an opsin to function, it requires the isomerization of this retinal back to its original conformation. In *Ciona intestinalis*, Ci-CRALBP, a homolog of a key component of this visual cycle, is expressed in the larval photoreceptor cell (Tsuda et al., 2003). Adults express a homolog of another key member of the vertebrate visual cycle in their photosensitive neural complex: Ci-RPE65 (Ohkuma et al., 2000). The presence of retinoid cycling homologs suggests that the tunicate visual cycle is homologous with vertebrates, and may represent an early stage in the evolution of the

dedicated visual cycle tissue of vertebrates, the retinal pigment epithelium (RPE). Further, developmental studies of the ocellus revealed the involvement of a homolog of *Rx,* a family of genes that have been shown to be necessary in retina formation of jawed vertebrates (D'Aniello et al., 2006). The high degree of homology between the features of the "ocellus" and vertebrate eyes are a likely representation of a primitive precursor in vertebrate eye evolution.

Vertebrate eyes are the most complex of those in any chordates, and possess several accessory features that distinguish them from eye-like structures found in tunicates and cephalochordates (Fig. 1.2). Despite the wide array of body plans, adapted to suit aquatic or terrestrial and nocturnal or diurnal life histories, vertebrate eyes are strikingly similar. The eye is organized into several layers. Most exterior are the sclera and cornea, involved in providing structural support and protection for the eye, and focusing light onto the retina respectively. Next is the choroid, a tissue that contains a network of vasculature responsible for supplying all other layers with blood, and the ciliary body, which includes the intraocular muscles as well as the ciliary epithelium which produces the fluid of the aqueous humor, the liquid that fills the space between the cornea and ciliary body. The innermost layer is the 3-layered retina comprised of a duplex of photoreceptors (rods and cones), interneurons (such as bipolar, amacrine, and horizontal cells), and RGCs that form the optic nerve and are responsible for transferring information to visual processing regions of the brain.

Vertebrate eye development begins as a lateral bulging-out of the anterior portion of the neural tube, an embryonic structure of neural ectoderm origin that will give rise to the central nervous system. These out-pockets (optic vesicles) come into contact with epithelial ectodermal tissue overlying the neural tube, which induces invagination of the neural tube to form an optic cup, and the epithelial ectoderm to form the lens placode. From here, the optic cup develops the

major sensory tissue of the eye (the retina), while contact with the ectodermal tissue induces the formation of the lens from the lens placode. In this sense, the eye can be considered a direct extension of the brain. In simple terms, the eye can be imagined as a photoreceptive layer which has, over the course of evolutionary time, elaborated its structure and organization as well as adopted accessory structures to improve vision. The lens, cornea and other non-sensory components of the eye all work to improve acuity, efficiency and longevity of eye function.

1.3.1 Vertebrate eyes: a case study in evolutionary innovation

Since the same eye plan we see in mammals is present in even one of the most basal vertebrate groups—lamprey— we can infer that the vertebrate eye has remained relatively unchanged since lamprey diverged from jawed vertebrates (Dickson and Collard 1979; Morshedian and Fain 2015). This has led researchers to conclude that the last common ancestor of lamprey and jawed vertebrates possessed a camera-style eye similar to that of extant vertebrates (Lamb et al., 2007). Because of shared common ancestry among chordates, it is likely that the early vertebrate eye evolved from a state similar to extant tunicates and cephalochordates. However, the lack of characterized eyes in the fossils of early diverging vertebrates, and the enormous gap in complexity between the eyes of vertebrates and their closest chordate relatives, leaves a great deal unanswered.

In an ideal case, the entire evolutionary history of a feature can be traced through the fossil record and by interrogating genetic information. However, our account of the evolutionary history of chordate eyes contains large gaps that remain unaccounted for. Much of what is known about vertebrate vision is based on extant jawed vertebrates. The difficulty in finding adequately preserved eye tissue in the fossil record combined with limited (though growing) genomic information for chordate species have been roadblocks in the investigation of early

evolutionary stages of vertebrate eyes. In the absence of transitional fossils, two ancient vertebrate lineages (hagfish and lamprey) can be studied to understand the most ancient condition of vertebrates. In comparison to jawed vertebrates, little has been done to explore the eyes of these jawless vertebrates.

1.4 Vertebrate evolution, through the eyes of agnathans

Naturally, the desire to understand the ancestral state of vertebrates has led to the study of living members of the earliest evolving vertebrate lineages: agnathans. Recent work in lampreys and hagfish have advanced our understanding of several aspects of vertebrate evolution including jaw, immune, and nervous tissue innovations (Kuratani et al., 2001; Rast and Buckley, 2013; Weil et al., 2018).

Lamprey (order Petromyzoniformes) are separated into two southern hemisphere dwelling subfamilies (Mordaciinae and Geotriinae) and one northern hemisphere subfamily (Petromyzoninae) (Kuraku and Kuratani, 2009). Lamprey undergo indirect development, living as larval ammocoetes for several years before metamorphosing into adults.

Adult lamprey eyes demonstrate a high degree of conservation in the retina and general features of the eye with those of jawed vertebrates (Lamb et al., 2007). Much like those of jawed vertebrates, the retinas of post-metamorphic lamprey possess a duplex retina comprised of ciliary photoreceptors, interneurons, and retinal ganglion cells that are organized into three distinct strata (Crescitelli, 1972; Fritzsch and Collin, 1990). While northern hemisphere lampreys (e.g. *Petrymyzon marinus*) have only two classes of photoreceptors (a rod and a single cone), the retina of *Geotria australis* possesses five classes of photoreceptors which individually express all five ciliary opsins homologous with those found in gnathostomes (RH1, RH2, SWS1, SWS2, LWS) (Collin et al., 2003; Govardovskii and Lychakov, 1984). It was only recently that the RH1 (rod

opsin) expressing photoreceptors of lamprey were confirmed to be homologs of vertebrate photoreceptors (Morshedian and Fain, 2015). Early uncertainty stemmed from the external morphology of the RH1 expressing photoreceptors, which shared the cone characteristic of having an outer segment membrane that is continuous with the plasma membrane of the lamellar discs (Dickson and Graves, 1979). Lamprey have provided us with a rich representation of early vertebrate eyes, but only in part. Stronger conclusions about the ancestral state of vertebrate eyes necessitates input from the other living jawless fish—hagfish.

1.4.1 To understand vertebrate evolution, we must understand hagfish

Hagfish represent the other living lineage of jawless vertebrates. Hagfish (Order Myxiniformes) belong to a monophyletic order divided into two major sub families: Myxininae and Eptatreninae (Chen et al., 2005; Kuo et al., 2003). There are 70+ species of hagfish which are found across the world's oceans (Knapp et al., 2011). All species are marine and largely burrowing, and most live at depths of more than 25m (Jørgensen et al., 1998). They occupy a vital role in the nutrient turnover by aiding in the decomposition of fisheries by-catch and large animal carcasses (Martini et al. 1997). Hagfish also present a significant prey source for pinnipeds and other marine mammals. Hagfish are famous for their anti-predatorial slime response in which protein threads and mucins are released from glands along the length of the body which, when combined with seawater create a slime that clogs the gills and effectively disarms predators (Fudge et al., 2005).

In comparison with lamprey, less is known about hagfish in part because they have been a historically challenging experimental model to work with. Most hagfish species cannot be reared in the lab, though recently one research group was successful in producing *Eptatretus burgeri* embryos (Ota and Kuratani, 2009). Most of the published developmental studies in hagfish originate from collections during the early 1800s by Bashford Dean (Ota and Kuratani,

2006). Since that time, attempted embryo collections have failed. Genetic information is also limited. The entire mitochondrial genome (Delarbre et al., 2002) has been sequenced and published, and a scaffold level draft genome of the Japanese hagfish has been recently uploaded to GenBank. Despite these limitations, hagfish still occupy an important position in efforts to understand vertebrate evolutionary history because they can complement the interpretations and work done in lamprey (Janvier, 2007).

Hagfish eyes are small, unpigmented and lack diagnostic jawed vertebrate features such as a duplex retina, lens, cornea and extraocular musculature (Locket and Jorgensen 1998). The eye-cup is relatively simple and masked by semi-transparent or non-transparent overlying epidermis, the transparency of the skin varies across species: 68% in *E. burgeri*, 61% in *Myxine glutinosa*, and 33% in *Paramyxine atami* (Fernholm and Holmberg, 1975). In the eyes of *M. glutinosa*, the eye is additionally covered by a layer of muscle, further obscuring the eye from light stimuli.

In the hagfish retina, only photoreceptors and retinal ganglion cells have been identified. The photoreceptors appear to comprise only one subtype, a short cylindrical rod-like structure in many *Eptatretus* species, and a small whorled structure in *M. glutinosa* (Holmberg, 1971a). Early work using bovine rhodopsin antibody staining showed immunoreactivity in photoreceptor outer segments and in the optic nerve (Vigh-Teichmann et al., 1984). However, the authors caution that this optic nerve immunoreactivity may have actually been staining for other opsin immunoreactive elements. One such cross reaction could be melanopsin, which is known to be expressed in hagfish retinal ganglion cells (Sun et al., 2014). Electrical responses of the eyes of *M. garmani* to flashes of low intensity light resemble electroretinograms (ERGs) of basal fish (elasmobranchs) (Kobayashi, 1964). Maximum saturation of the photoreceptors is reached at a

very low level of light, reminiscent of other deep water dwelling fish (Kobayashi, 1964). To further support this rod characterization, studies show the highest spectral sensitivity of the hagfish eye is to wavelengths between 500-520 nm, which fits the rhodopsin (rod associated opsin) photosensitivity of other vertebrates (Kobayashi, 1964; Lamb et al., 2016; Steven, 1955). Transcriptome analysis of *Eptatretus cirrhatus* has also identified that at least part of the phototransduction in hagfish is conserved with that of jawed vertebrate rods (RH1, PDE6G, GNAT1) (Lamb et al., 2016).

The cells of the hagfish retina are loosely organized into two nuclear layers, and lack distinct plexiform layers between these nuclei. The presence of two and not three nuclear layers has been interpreted as a lack of interneurons in the retina, though no studies have identified the presence or absence of interneurons (Locket and Jorgenson 1998, Holmberg 1970). A majority of optic nerve fibres in *M. glutinosa* project to an area considering homologous to the vertebrate optic tectum, which is reduced in lamination (four strata versus seven in lamprey) and in the density of neurons (Iwahori et al., 1996; Jansen, 1930; Kennedy and Rubinson, 1977). In *E. burgeri,* the afferent projections from the optic nerve lead to the dorsal thalamus and pretectal areas of the brain (Kusunoki and Amemiya, 1983). Projection to the pretectum is indicative of a population of intrinsically photosensitive retinal ganglion cells which are involved in the pupillary reflex and in circadian rhythms (Hattar et al., 2002; Lucas et al., 2003).

Reaction rates to light stimuli have been examined in several species by behavioral responses to light. In hagfish, light mediated responses are limited and consist of two phases: (1) general local movements followed by (2) swimming (Newth and Ross, 1955; Steven, 1955). *Myxine* hagfish have been shown to have a limited response, directly comparable to blind vertebrates and non-vertebrate chordates (Kobayashi, 1964; Newth and Ross, 1955; Steven,

1955). At low intensities, behavioural reaction times increase with light intensity increases, and at higher intensities reaction time plateaus (Steven, 1955). This plateau effect at high intensities supports the findings of Kobayashi (1964), that showed a maximum saturation at high intensities, reminiscent of rod-dominated retinas of deep sea fishes. It was reported that in *M. glutinosa,* removal of the eyes produced no difference in the behavioural response to light (Newth and Ross, 1955). Instead, it was proposed that the major photoreceptive source was in the skin rather than the eyes. Several years later, removal of the eyes in *M. garmani* was shown to prolong behavioral latency but not eliminate it response to light (Kobayashi, 1964). The discrepancies between these two studies is likely a result of the differences in the biology between the two fish. Though *M. glutinosa* is in the same genus as *M. garmani* and so would be expected to be quite similar, its retina shows a greater degree of disorganization and lies beneath muscle (Holmberg, 1971a; Kobayashi, 1964).

Studies of behavioural responses to light in *E. stoutii* have not been conducted, though it appears that they generally follow patterns similar to those reported for other hagfish (Emily Dong, personal observations). Other Eptatretinae species, *E. burgeri* and *Paramyxine atami* do display a reaction to general illumination which is faster than that of *Myxine* by several seconds (Patzner, 1978). Eptatreninae species possess more developed retinas, these findings illustrate that functional differences exist when compared with Myxininae.

Evidence has shown that hagfish eyes are functionally photosensitive, though it remains unclear if they are capable of forming images. Instead of serving image forming vision, the primary function of the eyes in hagfish has been suggested to be a basic light/dark detector used to entrain circadian rhythms (Ooka-Souda et al., 1993a; Ooka-Soudaa et al., 1995). Disruption of the flow of retinal information to the circadian pacemaker by surgical lesions to the pretectal

area (shown to be the circadian pacemaker) alters the rhythm, further suggesting light entrainment by the eyes as a relevant zeitgeiber for circadian rhythm. The life history of many hagfishes leaves much to be understood, and it is not entirely clear if light stimuli is consistent over the course of an individual's life.

Much of the biology of the hagfish eye remains unknown. Within the retina, interneurons have not been satisfactorily characterized, and additional photoreceptor subclasses have not been identified. Though we know that the hagfish photoreceptors are capable of responding to light signals, the functional physiology of the eye has not been interrogated sufficiently to know how this information passes through the retina, or if the supporting epithelia can maintain the high metabolic needs of a functional neural retina. Most of all, the development of the hagfish eye remains a near complete mystery. Though early studies have given us an approximate understanding of the morphological characters, we have virtually no information about the molecular mechanisms and genetic controls of hagfish eye development (Gorbman, 1997; Stockard, 1906; Wicht and Northcutt, 1995). With advances in molecular techniques, the answering of these questions has never been so tangible.

1.5 Objectives and purpose of study

Hagfish represent one of two living jawless fish lineages, which makes the study of their eye biology extremely pertinent in exploring vertebrate eye evolution. Building on the work of earlier studies, the objective of this project was to explore the structures, and molecular markers of the Pacific hagfish (*Eptatretus stouii)* eye. Among hagfish, Pacific hags are a choice model for eye studies because there is a substantial body of work that has already begun to characterize existing features. The eyes of *E. stoutii* are also less rudimentary than the eyes of *Myxine* species: possessing stacked photoreceptor lamellae, rather than whorled, and not having their eye

concealed by muscle (Fernholm and Holmberg, 1975; Jørgensen et al., 1998). Additionally, because hagfish are not easily reared in laboratory conditions, samples must be collected directly from the ocean. Pacific hagfish are the only known hagfish species inhabiting the waters surrounding the nearest marine research center: Bamfield Marine Sciences Centre, at Bamfield, BC, Canada.

The apparent "primitive" structure of the eye, in combination with the hagfish's basal phylogenetic position, tempts an attractive hypothesis that the hagfish eye might represent the basal condition of the vertebrate eye. However, substantial evidence in the form of phylogenetic confirmation of cyclostome monophyly, and the identification of vertebrate features in recently produced lab-reared hagfish embryos, suggests these features are instead a result of secondary loss or degeneration. In balance, we hypothesized that the hagfish eye possessed a greater number of conserved vertebrate eye characters than previously identified. The identification of these features is anticipated to confirm whether the eye of extant species evolved from functional, vision forming eye which has regressed over evolutionary time.

The first aim was to characterize photoreceptor morphology and gene expression to better understand the characteristics of photoreceptors in hagfish. In other vertebrate models, photoreceptor identity can be determined by molecular markers and ultrastructure of the outer segment. A majority of previous studies of the hagfish photoreceptors were completed before the use of modern gene and protein detection assays were commonplace, and so we endeavored to complement these studies by exploring the molecular characteristics of these cells. A homolog of vertebrate rod opsin (RH1 or rhodopsin) was expected to be found in hagfish photoreceptors as they are in other vertebrates. Additionally, if all photoreceptors express this

key rod identifier, more informed comparisons can be made with the photoreceptors of other vertebrates.

The second aim was to explore the retinal epithelium for identifiers of function and conservation with RPE in other vertebrates. The RPE is a necessary retinal tissue that supports the high demands of the neighboring photoreceptors. A photoreceptors capacity to maintain continuous phototransduction necessitates a functional RPE, and so in an ancestrally functional eye, we expected the hagfish to possess (at the very least) relics of a functional RPE such as retinoid cycling enzymes and pigment.

The third aim was to identify other cell types present in the inner nuclear layer of the retina, which is stratified into two approximate layers. Cells types within the inner layer had not yet been identified with any certainty. We hypothesized that interneurons would be present within this layer, as seen in other vertebrates, determined by cell specific gene expression.

Without hagfish, half of the information about jawless fish is missing. This is why it is so crucial to study this underutilized model. The results generated from this study will lend support for the monophyly of hagfish and lamprey and bring the overall characters of this lineage into greater view.

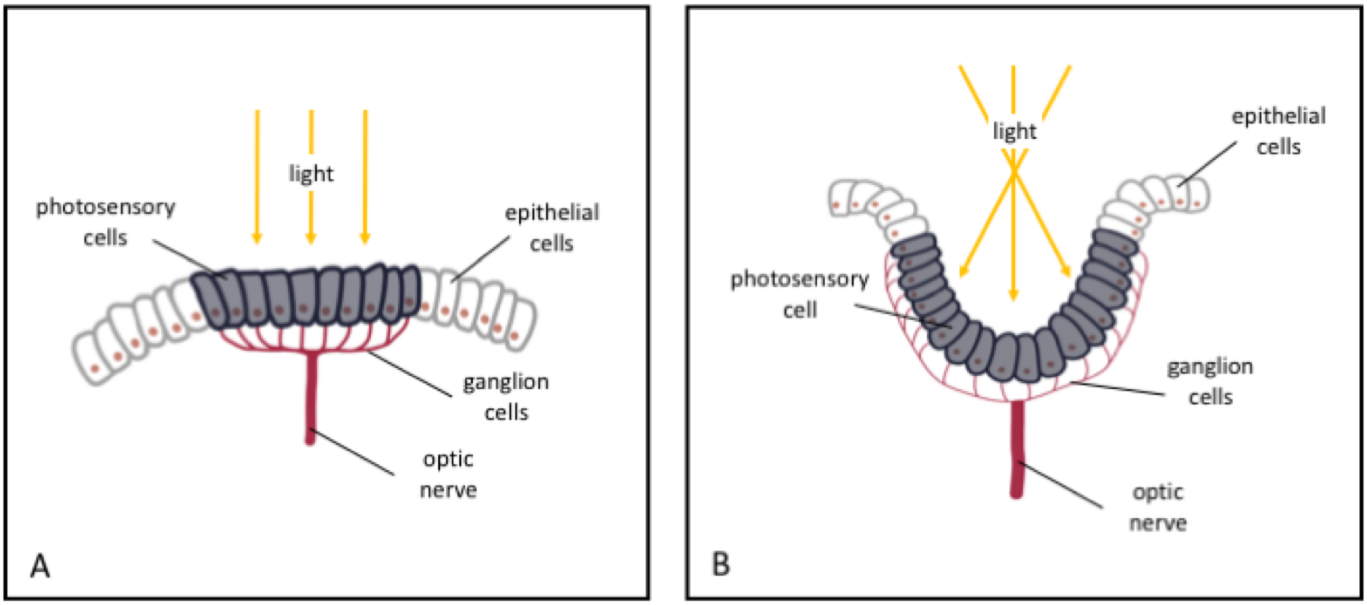

Figure 1.1. A photoreceptive field with a two dimensional cup-shape allows for directional sensing.

(A) In an eye patch type photoreceptive organ, the photoreceptive cells are arranged on the same horizontal plane. Any light that is received can only be detected in one dimension. These organs are useful in detecting shadow from light, but are unable to provide sufficient information for the brain to form an image. (B) Cup shaped eyes offer the advantage of being able to detect the directionality of incoming light. This allows for the perception of depth. From Dong & Allison (2017).

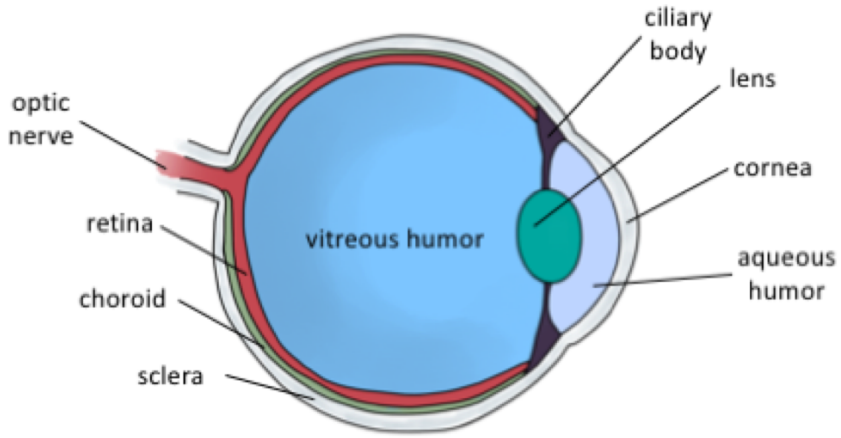

Figure 1.2. Anatomy of a generalized vertebrate camera-style eye

Vertebrate eyes are one of two camera-style eyes found in metazoans. These eyes are capable of forming high quality images by the coordination of shared features labelled here. Labelled features are highly conserved across vertebrates. From Dong & Allison (2017)

2 CONSERVED VERTEBRATE FEATURES IN THE RETINA OF PACIFIC HAGFISH (*EPTATRETUS STOUTII*)

2.1 Introduction

The vertebrate camera-style eye is a complex and intricate organ that differs from the light sensing organs of other chordates by its capacity to form images. Image formation is possible by the cooperation of a highly organized array of tissues that, in the canonical vertebrate eye, includes a lens, extra-ocular musculature, and a neural retina supported by a pigmented retinal epithelium. Light enters the eye and interacts with light sensitive photoreceptor cells, initiating a cascade of information through a three-layered strata of interconnected neurons. Photoreceptors, the cells that transduce this light signal, have a specialized structure called an outer segment, consisting of membranous discs studded with light sensitive proteins (opsins). Photons strike the opsin and transduce the light signal into an electrochemical signal that is relayed from photoreceptors to bipolar cells and on to retinal ganglion cells (RGCs). RGC axons come together to form the optic nerve, and transport visual input to the brain for further processing. Before this visual signal reaches the optic nerve, it is exposed to modulation by a complicated set of interactions from interneurons (bipolar, amacrine and horizontal cells), and input from adjacent photoreceptors. Background noise caused by stray photons is reduced by absorption by the retinal pigment epithelium (RPE), a layer of pigmented cells at the back of the eye which, in addition to providing for the metabolic needs of the eye, also aids in reducing background noise in visual information by the absorption of stray photons. The homology of these features across vertebrates is supported by shared morphology, developmental processes and molecular markers (Chow and Lang, 2001).

Much is known about the biology of the eyes of extant vertebrates, but little is known

about how the eyes of the earliest diverging vertebrate lineages evolved into a complex camera-style eye. Thus, to investigate the evolutionary history of the camera-style eye we must study lineages that came first in the evolutionary history of vertebrates: lampreys and hagfish. Lampreys and hagfish are the two most ancient lineages among extant vertebrates. Both are jawless fish which diverged approximately 450 million years ago and according to the fossil record, extant species have maintained a largely similar body plan to their extinct relatives (Kuraku and Kuratani, 2009). The lamprey eye shares remarkably high conservation with that of jawed vertebrates, possessing a lens, extra-ocular musculature, and a retina comprised of the same striated subsets of cells—photoreceptors, interneurons and RGCs (Dickson and Graves, 1979). In contrast with lamprey, we know comparatively little about the cellular composition of hagfish eyes.

Two limitations have complicated the progress of hagfish eye research. There is only a scaffold level draft genome for a single hagfish species (*Eptatretus burgeri)* , and a near inability to rear animals in the lab, with one exception (*E. burgeri)* (Ota and Kuratani, 2009). However, as they represent one of only two extant jawless vertebrate taxa, understanding the eyes of hagfish is indispensable in understanding vertebrate eye evolution.

The eyes of extant hagfish lack a lens, ocular musculature, and possess an un-pigmented retina that strays from the typical jawed vertebrate three-layered organization (Lamb et al., 2007). The eye is obscured by overlying skin and in some species, like the Atlantic hagfish (*Myxine glutinosa*), it is also buried under muscle (Fernholm, 1974). Though a lens and ocular musculature are absent, photoreceptors are present, as are retinal ganglion cells. Little is known of the interneurons. Hagfish photoreceptors are simpler than that of other vertebrates, found either as a whorled structure (in *Myxine* genus), or shortened and non-tapered in *Eptatretus*

species. Rhodopsin (RH1) or rod opsin, is the only identified visual opsin in hagfish, and has been proposed to act through a phototransduction cascade shared with that of other vertebrates (Lamb et al., 2016). Studies of several genera of hagfish have shown that functionally, hagfish have a slow response to light. In two different hagfish species in which the eye is covered by skin, *Eptatretus burgeri* and *Paramyxine atami*, direct illumination produces a swimming response that is delayed by several seconds, and is not immediate (Patzner, 1978). In *M. glutinosa*, where the eye is buried beneath skin and muscle, response to light stimuli is reported to be comparable with animals that have limited vision or no proper eye, such as larval stages of *Ciona,* a genus of tunicates with a simple eye spot consisting of a few photoreceptors and pigment cells, and *Proteus*, blind cave salamanders which produce eyes that are normal during development that later regress significantly (Newth and Ross, 1955; Steven, 1955). These studies suggest that though the eye can perceive light signals, it may not have the image formation capacity of its sister group, lamprey.

Molecular and genetic analysis of a number of nuclear and mitochondrial genes and the identification of neural crest cells and vertebral elements have confirmed that hagfish and lamprey are sister taxa forming a monophyletic group that is equally related to jawed vertebrates (Delarbre et al., 2002; Heimberg et al., 2010; Kuraku et al., 1999; Kuratani et al., 2018; Ota et al., 2013; 2011; 2007; Stock and Whitt, 1992). Given the phylogenetic position hagfish occupy within Vertebrata, one interpretation of this relatively rudimentary eye is that hagfish are representative of a more ancient state which predates the eye of lamprey and jawed vertebrates (Lamb et al., 2007). A competing hypothesis suggests that the lack of numerous key vertebrate identifiers is the result of secondary losses over evolutionary time (Fernholm and Holmberg, 1975). We hypothesize that hagfish eyes have regressed secondarily by a partial loss

of typical vertebrate features, resulting in a character state that is rudimentary, but not ancient. By utilizing molecular genetic techniques, this study aims to explore these competing hypotheses by probing for remnants of derived eye characters which we believe may be present in the hagfish eye.

The availability of genetic information in hagfish is limited, though a draft genome has been produced for *Eptatretus burgeri*, the Japanese hagfish. In order to identify novel transcripts and search for molecular clues of derived features in the Pacific hagfish, we obtained a transcriptome by RNA-Seq from eye tissues of the Pacific hagfish (*Eptatretus stoutii*). From this transcriptome, we have identified a number of eye-related transcripts which, in other vertebrates, are specific to cell types and processes not previously shown in any hagfish species. Novel transcripts have been identified for interneuron markers, pigmentation genes, and key enzymes involved in the function of retinoid cycling in the RPE. Overall, these data suggests a higher degree of homology exists between the eyes of jawed vertebrates and hagfish than previously appreciated, and weakens the hypothesis of ancient hagfish eye origins.

2.2 Materials and Methods

2.2.1 Animal ethics and tissue collection

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

Pacific hagfish (*Eptatretus stoutii*) were collected (N=100) from Barkley Sound, Vancouver Island, BC Canada via a bottom-dwelling trap baited with hake. Hagfish were size selected on board the vessel (MV Alta) and brought ashore to Bamfield Marine Sciences Centre (BMSC) to be housed in aerated, outdoor holding tanks receiving flow-through seawater at 12°C.

Hagfish were held unfed for 1-3 days before tissue collection at BMSC. Eyes were obtained from animals euthanized by immersion in a lethal dose of fish anaesthetic MS222 (tricaine methanesulfonate) at 4g/L. Eye tissue was also harvested from fish obtained and used by Dr. Greg Goss and his lab members at the University of Alberta in Edmonton.

Eyes collected for RNA extraction were either (1) placed into RNAlater (Invitrogen, Cat. No. AM7020) at 4°C for 1-14 days before storage at -80°C; or (2) flash frozen in liquid nitrogen before storage at -80°C. Eyes used in transmission electron microscopy (TEM) were fixed in a solution of 2.5% glutaraldehyde/2% PFA/0.1M phosphate buffer. Eyes used for *in situ* hybridization or immunohistochemistry were fixed in 4% paraformaldehyde(PFA)/5% sucrose/0.1M phosphate buffer (referred to hereafter as 4% PFA).

2.2.2 Tissue preparation for cryosectioning

Following fixation in 4% PFA, tissue was cryopreserved by four sequential sucrose washes: 1 each in 12.5% sucrose/0.1M phosphate buffer and 20% sucrose/0.1M phosphate buffer, over-night in 30% sucrose/0.1M phosphate buffer, and then 1 hour in 1:1 ratio of 30% sucrose/0.1M phosphate buffer:OCT (VWR, Cat No. 25608-930). Following these washes, tissue was embedded inside a mold made by the severed end of a 1.5mL microcentrifuge tube attached to a glass slide with nail polish. Tissue blocks were stored at -80°C until used. Prior to sectioning, tissue blocks were brought to -20°C and then tissue was sectioned at 10µm at -20°C on a Leica CM1900 UV

cryostat and mounted onto SuperFrost Plus glass slides (Fisher Scientific, Cat No. 12-550-15). Sectioned slides were air dried at room temperature and stored at -80°C until use.

2.2.3 RNA extraction

RNA was extracted using TRIzol (Invitrogen, Oregon, Cat. No.15596026) chloroform extraction procedure. For every 50mg of tissue 750l of TRIzol reagent was used to homogenize with POLYTRON PT 1200 hand held homogenizer (Kinematica Inc.). A volume of 0.2mL of chloroform per 1mL TRIzol was added for phase separation, followed by centrifugation at 12 000xg for 15 min. The supernatant was removed and washed with 75% EtOH. RNA samples were quality checked on Agilent 2100 Bioanalyzer (Agilent RNA 6000 NanoChip). Samples used for downstream experiments had RNA integrity numbers (RIN) of 7 or higher.

2.2.4 Riboprobe production and in situ hybridization

In situ hybridization was performed based on previously described protocol (Barthel and Raymond, 2000). Riboprobes were made using primers designed against transcripts identified by homology searching **(Table 1**). Primers were designed with a T7 RNA polymerase promoter site (TAATACGACTCACTATAGGG), and used to amplify template from cDNA (**Table 2).** cDNA was generated using qScript cDNA Supermix (Quanta Biosciences, Cat. No. 95048-025) as per the manufacturer's instructions. PCR products were first identified by band size run, through 1% agarose gel by electrophoresis and then confirmed by DNA sequencing. Reverse transcription of PCR products with either DIG or FLR labelled nucleotides by T7 occurred at 37^{o}C overnight. RNA product was precipitated by ethanol at -80^{o}C and final DIG- or FLR-labelled riboprobe quality was assessed by RNA gel electrophoresis on a 1% gel and final concentration was determined by Nanodrop spectrophotometry (GE Healthcare, Cat. No. 28 9244-02).

For *in situ* hybridization, sections were brought to room temperature after storage at -80°C and then fixed for 10 min in 4% PFA fixative at room temperature followed by proteinase K digestion for 5 minutes, and a second fixation in 4% PFA for 10 minutes at room temperature. Sections were incubated with 0.3% acetic anhydride and 0.1M triethanolamine for 10 min at, then dehydrated by an ethanol series (50%, 75%, 90%, 100%) and air dried at room temperature. Hybridization occurred at 65-74°C (probe dependent, **Table 2**) for 12-20hrs**.** Probe temperatures were determined for each probe individually to improve specificity. Sections were then washed at 65-72°C in 2xSSC 5 min, 0.2xSSC + 0.1% Tween-20 for 20 min, 0.2xSSC + 0.1% Tween-20 2x20 min.

After blocking for at least 60 minutes at room temperature using 10% normal goat serum in phosphate buffer saline, pH 7.4 (PBS) with 1% Tween-20, sections were incubated in a 1:1000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics, Cat. No. 11207733910) then stained with nitro blue tetrazolium (Roche Diagnostics, Cat. No. 11383213001) and 5-bromo4-chloro-3-indolyl phosphate toludinium (Roche Diagnostics, Cat. No 11383221001). Images were taken on a Zeiss Axio Compound Light Microscope with Optronics MacroFire Digital Camera.

For fluorescent *in situ hybridization,* tissue was incubated with POD (horseradish peroxidase) conjugated anti-DIG or anti-FLR secondary antibodies (1:100 dilution, Roche Diagnostics, Germany, Cat. No. 11093274910, 11426346910) at 4 ^{o}C overnight. The following day, tissue was incubated in 1:100 tyramide conjugated to AlexaFluor 488, or 555 (Thermo Fisher Scientific, Cat. No. B40953, B40955). Following development with POD conjugated antibody POD was deactivated by incubation in 3% H_2O_2 in PBSTw for 30 minutes at room temperature. Slides were coverslipped with 70% glycerol and sealed with nail polish for imaging.

2.2.5 Immunohistochemistry

Frozen cryosectioned tissue was brought to room temperature, and fixed with 4% PFA at room temperature for 10 minutes, then rinsed with PBSTw. Primary antibodies (Table 3) were applied to tissue and incubated overnight at 4°C and then rinsed with PBS/0.1%Tween 3x 15 mins. Following that, secondary antibody was applied and incubated overnight at 4°C and then rinsed off with PBS/0.1%Tween 3x 15 mins. Secondary antibody used was either AlexaFluor-647 anti-mouse-IgG (Invitrogen, Cat. No. A31571) or AlexaFluor-488 anti-mouse-IgG (Invitrogen, Cat. No. A21202). Secondary antibodies were applied at a dilution of 1:1000 prepared in PBS/0.1% Tween. Sections were then coverslipped with 70% glycerol and sealed with nail polish. To assure specificity, negative controls were performed where no primary antibody was applied to the tissue, only secondary antibody.

2.2.6 Hematoxylin and eosin staining

Tissues were fixed in 4% PFA and embedded in paraffin wax. Fifteen μm sections were mounted onto glass slides. Wax was removed by rinses with toluene. Hematoxylin Gill III (Surgipath, Leica) was applied to the tissue for 2 minutes, followed by 15-minute rinse in distilled water and 2 minutes in 70% ethanol. Eosin (Surgipath, Leica) was applied for 30 seconds, followed by 100% ethanol rinses and 2x 2min toluene rinses. Slides were coverslipped with DPX (Thermo Fisher Scientific).

2.2.7 Transmission electron microscopy

Tissue preparation and thin sectioning for TEM was performed at the University of Alberta Advanced Microscopy Facility with the help of Arlene Oatway.

After fixation, tissues were stained with 1% osmium tetraoxide/0.1M phosphate buffer at room temperature. Eyes were then embedded in Spurrr's resin and sectioned at 70-90nm thickness. Sections were further treated with uranyl acetate and lead citrate at room temperature. Micrographs were taken with a Philips-FEI Transmission Electron Microscope (Morgagni 268, operating at 80kV, Hillsboro, Oregon) with Gatan Orius CCD Camera.

2.2.8 RNAseq

2.2.8.1 Transcript isolation and sequencing

Enucleated eyes were isolated from surrounding muscle in the head. Extraneous tissue was trimmed away. Entire eyes (retina, and vitreous surrounded by a substantial network of collagen and fat) were pooled into a single homogenate and sent for further tissue processing and sequencing procedures at the BGI (Beijing Genomics Institute) at the Children's Hospital of Philadelphia (CHOP) Genome Center. Entire brains (cut at the brain stem) were pooled, homogenized and sent to BGI@CHOP as well.

2.2.8.2 Methods carried out at the BGI@CHOP Genome Center.

Total RNA was extracted with TRIzol (Invitrogen, Oregon, Cat. No.15596026), and quality checked using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). cDNA was synthesized from samples with RIN values of greater than 7. Fragments were sequenced using Illumina HiSeq 4000.

Low quality, adaptor polluted reads, and reads composed of more than 10% unknown bases were filtered by BGI internal software and eliminated from further analysis. Trinity software (v2.0.6) (Grabherr et al., 2011) was used to perform de novo assembly with clean reads. Next, TGicl software (v2.0.6) was used to cluster transcripts and form unigenes (Pertea et al.,

2003)

BLAST (v2.2.23) was used to annotate Unigenes by aligning sequences to NT, NR, COG, KEGG and SwissProt databases. Blast2GO (v2.5.0) was used to obtain GO (gene ontology) annotation, and InterProScan5 (v5.11-51.0) was used to attain InterPro annotation (Altschul et al., 1997; Conesa et al., 2005; Jones et al., 2014). The segment of Unigene that best mapped to functional databases (in order of priority: NR, SwissProt, KEGG, COG) was selected as its coding sequence (CDS). CDS for Unigenes that could not be aligned to any of these databases was predicted by ESTScan (v3.0.2), with Blast-predicted CDS as a model (Christian Iseli, 2002). To determine gene expression, clean reads were mapped to Unigenes using Bowtie2 (v2.2.5), and FPKM gene expression levels were calculated with RSEM (v1.2.12) (Langmead and Salzberg, 2012; Li and Dewey, 2011).

2.2.9 Homology searching, transcript alignment and tree making

Following *de novo* transcriptome assembly, transcript annotation with 7 functional databases was performed (NR, NT, GO, COG, KEGG, Swissprot, Interpro). To confirm annotations of transcripts of interest, known protein coding sequences from a representative cross section of vertebrates (including zebrafish, mouse, chicken, and frog) were used to search the hagfish eye transcriptome using BLAST 2.6.0 (Altschul et al., 1997) and HMMER 3.1b1 (Eddy, 2011). The top three highly matched sequences were retained, and aligned to homologs using MAFFT under default settings (Pearson, 2014). For genes that are part of large and closely related protein families, transcript identity was further confirmed by phylogenetic analysis conducted using IQ-TREE (Nguyen et al., 2015). Branch support was determined using ultrafast bootstrap (1000 bootstrap replicates) (Hoang et al., 2018; Nguyen et al., 2015). The top candidate was used in subsequent analyses.

2.2.10 RACE-PCR of Hagfish Maf

Hagfish Long *Mafs* were identified by homology searching methods described above. RNA-sequencing does not always guarantee full length transcripts, and in the case of our putative hagfish Long-*Maf* we wanted to determine whether we were missing partial sequence information. Rapid amplification of cDNA ends (RACE) PCR was performed in order to obtain missing sequence information using SMARTer® RACE 5'/3' Kit (Clontech, Cat. No. 634858). RNA was isolated from fresh eye tissue as described above. Phylogenetic analysis using the RACE-PCR determined sequence was performed using IQTree as previously described. Modelfinder identified the best fitting model (Dayhoff+F+I+G4), that was chosen according to BIC.

2.2.11 Cell density comparisons

Using micrographs of DAPI (4',6-diamidino-2-phenylindole) stained nuclei in zebrafish and hagfish, total nuclei per 10μm at three points across the retina were counted (n=4). The data fell into a normal distribution with no outliers. Student's t-test was performed, which found nuclear densities to be significantly different between hagfish and zebrafish retina ($p=0.012$).

2.2.12 Microscopy

Fluorescent micrographs were taken using an LSM 700 inverted confocal microscope in combination with Zeiss Axio Observer.Z1, and captured using ZEN 2010 software (version 6.0, Carl Zeiss AG, Oberkochen). Images were compiled and constructed in PowerPoint (version 1710, Microsoft Office 365). Brightness of images was increased in ImageJ (Schneider et al., 2012).

2.3 Results

2.3.1 The Pacific hagfish retina is regionalized

Pacific hagfish (*E. stoutii*) eyes are found beneath a layer of translucent skin (**Fig. 2.1A).** Each eye is imbedded in the surrounding craniofacial muscle, and is small, non-pigmented, and lacks extra-ocular musculature (**Fig. 2.1B,C**). No lens is evident, though the vitreous of the eye is protein-dense, evident by the high degree of hematoxylin staining (**Fig. 2.1D**). As in lamprey, no intraocular musculature can be found within the eye.

In cross section, the retina of most vertebrates (exemplified here by zebrafish) is stratified into three distinct nuclear layers (**Fig. 2.2A**), divided by two synaptic layers (**Fig 2.2B-C**). As previously described in the hagfish, the retina of *E. stoutii* is comprised of two approximate cellular layers: the outer layer, and the inner layer (**Fig. 2.2D-F).** While the precise identities of cells comprising each layer are still under investigation, photoreceptors are known to occupy the outer layer, while retinal ganglion cells are known to occupy the inner layer. In hagfish, a third interneuron-housing inner nuclear layer equivalent is missing (**Fig. 2.2A**). Layers of synapses between cells in the hagfish retina do not cleanly define layers of nuclei (**Fig. 2.2E-F**). Instead, it appears that the cells of the inner layer, and synapses between them, exist in and around the axons and cell bodies of their neighbors. A synaptic vesicle marker, SV2 (synaptic vesicle protein 2) stains all throughout the inner layer, with greatest staining localized to the innermost region (**Fig. 2.2F**), which also corresponds to the highest density of cell projections (**Fig. 2.2E**). Hagfish retinas have a lower cell density than zebrafish, which may be contribute to the comparative differences in organization (**Fig. 2.3**). A more detailed investigation of cell identities within the inner layer is described later on in the text.

In jawed vertebrates, a population of proliferating undifferentiated cells exists at the outer edges of the retina, known as the ciliary marginal zone (CMZ) (**Fig. 2.4**) (Fischer and Reh, 2000; Johns, 1977; Kubota et al., 2002; Wetts et al., 1989). In hagfish, the cells at the ciliary marginal zone lack photoreceptor outer segments, and have irregularly shaped nuclei that are distinct from other retinal cells further along the retina (**Fig. 2.4A**). A gradient of cell morphologies can be seen as you move closer to the center of the retina, with increasing resemblance to fully differentiated cells of the rest of the retina (**Fig. 2.4A'-A''**). Molecular studies have revealed that cells in the vertebrate CMZ have characteristic gene expression profiles. *PAX6* is one such marker of undifferentiated retinal cells in the CMZ (Marquardt et al., 2001). We identified a homolog of *PAX6* in hagfish that shares strong conservation with other vertebrate homologs (**Fig. 2.5**). *PAX6* expression is most concentrated at the tip of the hagfish marginal zone (**Fig. 2.4B-C'**), suggesting that not only do these cells appear undifferentiated by morphology, but that they also express transcripts known to be related to the maintenance of a multipotent cell state (Marquardt et al., 2001).

2.3.2 Photoreceptor morphology is rod-like

To further determine whether the Pacific hagfish retinal photoreceptors were rod or cone-like, we assessed morphological features. By morphology, previous studies have shown that across different species, hagfish possess one identifiable class of photoreceptor in their outer nuclear layer, and *E. stoutii* are no different (**Fig. 2.6A**). The membranes of the hagfish outer segment display the rod-like characteristic of having stacked lamellae organized into discrete discs, evident by their lack of continuity with the surrounding membrane of the outer segment (**Fig. 2.6B**). This outer membrane appears to wrap continuously around the outer segment, and can

be seen on both edges of the outer segment in cross section of the photoreceptor (**Fig. 2.6C**). No cone-like morphological features were observed.

2.3.3 Molecular features of E. stoutii photoreceptors

Next, we determined whether these photoreceptors, which morphologically appear rod-like, express rod markers. Rhodopsin (RH1) is the only visual opsin known to be present in the hagfish eye, though it remained undetermined whether or not it is the only visual opsin expressed (Lamb et al., 2016). We identified one (highly expressed) visual ciliary opsin: *RH1* (**Table 1, Fig. 2.7**). Additional homology searching using HMMER (Eddy, 2011) and BLASTP 2.6.0 (Altschul et al., 1997) did not reveal any additional visual opsins. In comparison with other vertebrates, *E. stoutii RH1* shares high sequence similarity, and is clustered with other vertebrate rhodopsins with high certainty (**Fig. 2.7**).

In situ hybridization of hagfish *RH1* using RNA probes designed from transcriptome sequences shows highly specific staining in the photoreceptors, closely associated with the nuclei (**Fig. 2.8A-B**). *RH1* is expressed in all photoreceptors, making the presence of other ciliary photoreceptor types unlikely. Additionally, the photoreceptor outer segments are readily immunolabeled by ZPR-3 (zebrafish rod labelling polyclonal antibody) and 1D4 (monoclonal antibody raised against bovine rhodopsin) (**Table 3, Fig. 2.8C-D'**). Immunoreactivity was not detected in hagfish eye tissue with any other robust non-rod photoreceptor antibodies (**Table 3).**

2.3.4 Markers of vertebrate interneurons are expressed in the inner nuclear layer of the retina

Apart from photoreceptors and retinal ganglion cells, cell identities within the hagfish retina are inadequately characterized. A lack of identified interneurons within the hagfish retina has been

compared to the two layered morphology of pineal organ (Lamb et al., 2007). Comparisons to the pineal support the ancient eye hypothesis. Thus, finding interneurons which are absent in the pineal, is key in rejecting this hypothesis. The inner layer of the hagfish retina is a diverse mix of cells, which do not appear to be homogenous in shape or size (**Fig 2.9**). We hypothesized that additional cell types aside from retinal ganglion cells populate this region. RNA-sequencing returned a number of putative interneuron markers (**Table 1**). Using these transcripts, RNA probes were designed and used for *in situ* hybridization (**Table 2)**. *PKC-*α (**Fig. 2.10C**), a marker of rod-bipolar cells is shown to be expressed in the inner nuclear layer (Greferath et al., 1990), along with *PAX6* (**Fig. 2.10A**) (marker of amacrine cells and RGCs) and *calbindin* (**Fig. 2.10D**) (marker of horizontal cells) (Marquardt et al., 2001).

RGCs have previously been examined in the hagfish retina. In jawed vertebrates, the majority of retinal ganglion cells are strictly concerned with the trafficking of photoreceptor derived visual information to the brain. However, there is a subset of RGCs that are also intrinsically photosensitive (ipRGCs), due to the presence of melanopsin, another light sensing opsin not expressed in the visual photoreceptors of the retina (Hattar et al., 2002). We have found *melanopsin* expressed within this inner nuclear layer (**Fig. 2.10B**).

2.3.5 E. stoutii possess a retinal (non)pigment epithelium

In jawed vertebrates, the retina is supported by the RPE, a monostratified epithelium found between the photoreceptor outer segments and the choroid (a network of vasculature that supports the outer retina). This tissue is a vital partner with the neural retina, and is unique to vertebrates. At the outer edge of the hagfish retina, beyond the photoreceptors, there is a single row of cells has been unsatisfactorily characterized. Previous studies have shown that these cells possess microvilli on their basal surface and are closely associated with nearby photoreceptor

outer segments (Fernholm and Holmberg, 1975; Holmberg, 1971). We have found substantial evidence showing that these cells in hagfish are a homolog of the jawed vertebrate RPE.

Melanin is a pigment found in the RPE of vertebrates and is key to sustaining healthy function in the eye and absorbing stray photons. An immediately striking feature of the hagfish eye is its apparent lack of pigment (**Fig. 2.1C**). Thus, we have termed this non-pigmented RPE equivalent the "Retinal (non)Pigment Epithelium" or RnPE. Curiously, though the eye is unpigmented, we have found that pigment related genes are expressed in the eye, including *tyrosinase* (necessary for melanin synthesis) and *PMEL* (necessary for stable melanin deposition in the melanosome) (**Table 1, Fig 2.11**).

In addition to shielding the retina from stray photons, the RPE serves a highly necessary role in retinoid cycling – specifically, the production of 11-cis retinal, a chromophore associated with visual opsins that is required for visual function. Without the renewal of this chromophore by the RPE, phototransduction cannot be sustained. Retinoid cycling is achieved by the enzymatic action of lecithin-retinol acyltransferase (LRAT), 11-*cis*-retinol dehydrogenase 5 (RDH5) and *RPE65*, supported by cellular retinaldehyde-binding protein (CRALBP) (Batten et al., 2004; Moiseyev et al., 2003; Strauss, 2005). The transport proteins cellular retinol binding protein (*CRBP*) and interphotoreceptor retinoid-binding protein (IRBP) are also involved in the transport of highly hyrdophobic retinoids between the RPE and photoreceptors (Strauss, 2005). Analysis of our transcriptome revealed that homologs of *RPE65, LRAT, RDH5, CRALBP* and *CRBP* are expressed in the hagfish eye (**Table 1, Fig. 2.12**). *IRBP* could not be identified. Further, we found that the expression of *RPE65* is extremely specific and highly expressed within all cells of the RnPE (**Fig. 2.13A**).

Another major function of the RPE is to maintain the integrity of the photoreceptor outer segments by phagocytosing shed photoreceptor discs. This process renews the photoreceptors and in part prevents oxidative stress. As previously seen, we have found evidence of phagosomes in the RPE which contain pieces of photoreceptor outer segments (**Fig. 2.13B**) (Holmberg, 1971).

2.3.6 Hagfish photoreceptors express an NRL homolog

NRL (neural retina-specific leucine zipper), a Long-MAF belonging to the MAF family of transcription factors is of particular importance in the vertebrate eye because of its role in rod photoreceptor development (Kim et al., 2016). Transcriptome analysis revealed a long Maf transcript expressed in the eye of Pacific hagfish (**Fig. 2.14A-B**). RACE-PCR was used to identify a more complete coding sequence of the RNA-sequenced Maf transcript (**Table 2)**. The sequence that was identified using RACE-PCR possesses both the BZIP binding domain and N-terminal region domain which is characteristic of other vertebrate long MAFs (Friedman et al. 2004). The hagfish *Maf* homolog identified here clusters among other vertebrate long *Maf*s, and most closely with *NRL* and *MafB* (**Fig. 2.14A**). Hagfish *Maf* is expressed specifically in the photoreceptors (**Fig. 2.14C**).

2.4 Discussion

Vertebrate eyes share a remarkable degree of morphological, developmental and functional conservation. The unity among vertebrate eyes has been confounded by the discrepancy between the typical vertebrate style eye of lamprey and the more rudimentary eye of hagfish. The hypothesis that hagfish eyes are an ancient intermediate between chordates and lamprey implies that the derived vertebrate features seen in lamprey were not present in the common cyclostome ancestor, and thus not in the shared vertebrate ancestor, meaning that the lamprey

would have had to acquire these jawed vertebrate-like features independently. The strongly supported monophyly of cyclostomes, and our data greatly weakens this hypothesis (Delarbre et al., 2002; Heimberg et al., 2010; Kuraku et al., 1999; Kuratani et al., 2018; Ota et al., 2011; 2007; Stock and Whitt, 1992; Takezaki et al., 2003; Yu et al., 2008). Here, we have identified previously obscured vertebrate features present in the hagfish retina, providing evidence in support of the idea that hagfish eye features have secondarily degenerated over evolutionary time.

2.4.1 The rudimentary appearance of the eye is not necessarily ancient

Increasing evidence supports the idea that many of the features not seen in hagfish are a result of degeneration (Fernholm and Holmberg, 1975; Gabbott et al., 2016; Ota et al., 2013; 2011; 2007). We, and others, have hypothesized that some of the rudimentary features of the hagfish eye are also due to degeneration (Fernholm and Holmberg, 1975). Agnathans diverged over 450 million years ago (Kuraku et al. 2006), and in that time, *E. stoutii* have adapted to a life at depths of 80m or deeper, burrowing and relying largely on non-visual sensory input (Jørgensen et al., 1998; Theisen, 1973). Many cave dwelling, burrowing, deep sea, and nocturnal animals exhibit eye reduction and loss as a result of limited visual stimuli which is hypothesized to have reduced the selection pressure on visual system (Collin and Partridge, 1996; Emerling, 2018; Munk, 1965; Partha et al., 2017). Relaxed selection for high acuity vision in these fish is a likely factor in the evolutionary degeneration of the hagfish eye. We have revealed several key pieces of evidence in support of the degeneration hypothesis.

2.4.2 Interneurons

In the three-layered retina of jawed vertebrates, the outermost layer is comprised of photoreceptors, the middle layer of interneurons (horizontal, bipolar, and amacrine cells), and

the innermost layer, of retinal ganglion cells. In order for image formation to be possible there are a number of highly complex relationships among photoreceptors, and between photoreceptors and interneurons. Hagfish lack these three distinct cellular layers, but seem to retain the cell type diversity as seen in other vertebrates. We have produced the first evidence that hagfish possess interneurons. The presence of *calbindin*, *PAX6* and *PKC-*α labelling within the inner nuclear layer of the retina suggests that, like lamprey, hagfish possesses a number of previously unidentified presumptive interneurons (**Fig. 2.10**). The presence of these markers suggests that the basic cell composition within the retina has been retained, and though it remains untested whether or not the same retinal network persists. The presence of these additional cell types certainly implies that it is or was possible for vision to occur.

2.4.3 Photoreceptors and the RnPE

E. stoutii possess a single visual photoreceptor which appears to be rod-like. The entire outer segment, though much shorter than rods of other vertebrates, has a cylindrical rod-like shape and does not taper (**Fig. 2.6A**). Confirming what has been observed in previous studies of *E. stoutii* and other hagfish species, we have found membrane surrounding the majority of the outer segment. The inner membranes within the outer segment are organized into discs and do not appear to be continuous with the outer membrane, and this is a defining feature of rods (not cones) (Holmberg, 1971a). We have shown that these morphologically rod-like photoreceptors all express rhodopsin transcripts, further confirming them as rod-like (**Fig 2.8**). Robust outer segment immunoreactivity by 1D4 (bovine rhodopsin) and ZPR-3 (zebrafish rod-labelling) antibodies suggests that these rhodopsin transcripts are translated and trafficked to the outer membranes, where they are likely responsible for eliciting the known photosensitive response in photoreceptors (**Fig. 2.8**). Our work elevates and confirms studies identifying the hagfish

photoreceptors as rods by their rod-typical spectral sensitivity, and electrophysiological response to low light stimuli (Kobayashi, 1964; Steven, 1955). Additionally, these findings do not match those of a recent study that identified LWS and SWS opsin immunoreactivity in Pacific hagfish photoreceptors (Zeiss et al., 2011). We did not identify any other photoreceptor subtypes, nor did we find immunoreactivity indicative of the presence of any LWS or SWS cones (**Table 3)**

Photoreceptors can be defined by their morphology, but are also defined by molecular markers. Vertebrates express any one of five visual opsins in their photoreceptors. Rods express rhodopsin (*RH1*) and cones express a number of opsins which cover the visual light spectrum (*RH2, LWS, SWS1, SWS2*). Each is tuned to a particular wavelength allowing for high acuity daylight vision in the case of cones, and dim light in the case of rods. We know from sequence analysis that rhodopsin is the most recently evolved visual opsin, arising from a duplication of RH2 (Okano et al., 1992; Trezise and Collin, 2005). The presence of *RH1* expressing photoreceptors alone in hagfish tells us that they have secondarily lost the capacity to express the other visual opsins, and that their retina may have once more closely resembled lamprey, in which some species which possess all five visual opsins (*RH1, RH2, LWS, SWS1, SWS2*) (Collin et al., 2003). The cell density in the neural retina of hagfish is significantly smaller than in the zebrafish retina (**Fig. 2.3**), which could be as a result of the loss of corresponding connectivity and network that supports cones (Masland, 2001). Future work will strive to identify lingering cone networks, if any exist. Gene expression identified by RNAseq can only tell us about transcribed genes, and so genomic information will be necessary to interrogate the full extent of opsin gene loss.

The presence of a *Maf* transcription factor which possesses the characteristic BZIP and N-terminal long-MAF domains (Friedman et al. 2004) that is localized to the photoreceptor nuclei

implies a shared rod photoreceptor identity programme which has been present in vertebrate retinas since before agnathans diverged (**Fig. 2.14**). In mammals, *NRL* is necessary for rod vs. cone identity, in *NRL* mutant animals, rods fail to develop (Kim et al., 2016; Mears et al., 2001). In zebrafish, *nrl* seems to be sufficient to drive a rod-like fate (Oel et al. unpublished). In chicken, no *nrl* homolog has been identified. Instead, chickens possess a *MAFA* that is expressed in rods and is sufficient to drive the expression of rhodopsin (Kim et al., 2016; Ochi et al., 2004). Expression of this long Maf transcription factor in hagfish photoreceptors suggests that the hagfish not only share mature cell markers with other vertebrates, but also developmental gene networks.

While the presence of rods confirms the propensity for the retina to receive and transduce light signals, our identification of the presence of a retinal pigment epithelium indicates that the proper function of those photoreceptors can be maintained. The RPE provides a number of services for the photoreceptors including retinoid cycling, nutrient and metabolite exchange between vasculature and the retina, and phagocytosis of sloughed photoreceptor outer segments. These two tissues exist in a highly intertwined relationship, which when disrupted, can result in serious malfunction and disease (Strauss, 2005). The presence of ciliary opsin expressing photoreceptors in conjunction with an epithelial cell layer capable of retinoid cycling is derived in terms of chordate eye evolution, and exclusive to vertebrates. This partnership is absent in early diverging chordates such as *tunicates,* where visual cycle components are expressed in the photoreceptor cells (Tsuda et al., 2003). The shared presence of retinoid cycling components within a separate epithelial tissue in hagfish, lamprey and gnathostomes suggests that this partnership predates the divergence of jawed from jawless vertebrates.

IRBP is a carrier protein that transports all-trans retinol from photoreceptors to RPE and was the only retinoid cycling constituent not found in our transcriptome. In mice, a loss of IRBP slows the recovery of photosensitivity after photobleaching (Palczewski et al., 1999; Ripps et al., 2000). This suggests that IRBP may not be necessary for retinoid cycling, though it does facilitate a faster recovery in photoreceptor sensitivity. Interestingly, $IRBP^{-/-}$ mice have shortened photoreceptors (Ripps et al., 2000), a feature also seen in hagfish. Absence of IRBP expression in the eye transcriptome reflects only the transcripts that were captured, and do not necessarily mean that the gene is missing from the genome.

A loss of pigment is another common correlate with deep sea or dim light living. In hagfish, the majority of the body is highly pigmented, and loss is exclusive to the RPE (**Fig. 2.1A**). In most vertebrates, the RPE is pigmented by the presence of melanin. In hagfish, though the eye is not pigmented, we have shown that it does express pigment related transcripts including *PMEL* (required for melanin deposition) and tyrosinase (necessary for melanin synthesis) (Julien et al., 2007; Oetting and King, 1999). Hagfish skin is richly pigmented, implying that these fish have not entirely lost the capacity for pigment development. It remains unclear if these transcripts are functional in the eyes of modern day hagfish. However, limited information about fossil hagfish tells us that the eyes of at least one extinct hagfish species possessed pigment (Gabbott et al., 2016). In addition to its own degeneration, the loss of melanin from the eye is likely to have been a contributing factor in the degeneration of other eye tissues. In models of albinism, eye function is seriously impacted in connection with an absence of melanin (Schraermeyer et al., 2006).Photoreceptors are particularly prone to bleaching and oxidative stress in the absence of melanin which may explain the short and sparse photoreceptors of hagfish.

It is possible that the loss of melanin has had an effect across the eye, beyond the retinal epithelium. In human albinism, lack of pigment in the eye is associated with mis-routing of the optic nerve from eyes to the brain (Creel et al., 2009). In hagfish the optic nerve projections are composed of fewer nerve fibres than in other vertebrates (Kusunoki and Amemiya, 1983; Ooka-Soudaa et al., 1995). It remains unclear how or why pigmentation is absent only in the retina and in the epidermis overlying the eye. Further studies of pigmentation development in the eyes embryos and in very young hagfish may provide answers to these questions.

2.4.4 Ciliary marginal zone

Toward the aperture of the eye, the retina thins and is populated by a collection of multipotent progenitor cells. This area is termed the "ciliary marginal zone" (CMZ). The cells in the CMZ are capable of proliferating and adding to the fully formed retina post-development (Fischer and Reh, 2000; Hollyfield, 1971; Johns, 1977; Kubota et al., 2002). Hagfish eyes possess a population of cells at their marginal zone which, in the case of the photoreceptors lack the outer segments of fully differentiated cells, and express Pax6, a marker of progenitor cells (Marquardt et al., 2001) (**Fig. 2.4**). Hagfish eyes increase with the size (=age) of the fish (**Fig. 2.15**), and so it is likely that this CMZ is active throughout maturation to provide new photoreceptors. As we do not have access to embryos, this region of differentiation could be key in understanding developmental processes within the retina. Expression and timing of key genetic signals may be present in this region that could recapitulate development and modulation of cell types within the embryonic hagfish retina.

2.4.5 ipRGCs

The role of the hagfish eye in the overall sensory capacity of the hagfish is minor, rather than forming images, and it is likely a light-sensitive organ used to entrain circadian rhythm (Ooka-Souda et al., 1993b). Studies have shown that hagfish will react to light by locomotor response, but with a significant latency, and that the removal of the eyes in the hagfish (*M. garmani*) does not entirely eliminate, but lessens the behavioural response to light (Kobayashi, 1964; Ooka-Souda et al., 1993b; 2000; Ooka-Soudaa et al., 1995; Patzner, 1978; Steven, 1955)..

Consistent with findings in the Japanese hagfish (*Eptatretus burgeri),* we have found melanopsin-positive retinal ganglion cells within the retina of *E. stoutti,* which are known as intrinsically photosensitive retinal ganglion cells (ipRGCs) (Sun et al., 2014). Intrinsically photosensitive retinal ganglion cells allow for the entrainment and maintenance of circadian rhythm and pupillary response in jawed vertebrates. Melanopsin, an opsin which (in the eye) is only expressed in retinal ganglion cells, mediates this retinal ganglion cell response to light (Hattar et al., 2002). Because they live in such a poorly lit environment, there may have been a redundancy between the capabilities of ipRGCs and the canonical photoreceptors under these conditions, causing positive selection on photoreceptors for vision to relax. This effect has been seen in other vertebrates such as blind mole rats, which also have a highly degenerated eye and possess more ipRGCs compared with non-fossorial mammals (Esquiva et al., 2016). Microbats, which are nocturnal, also have more melanopsin positive RGCs (Jeong et al., 2018).

2.4.6 Limitations and conclusions

One limitation to our interpretations here is that the properties of the adult hagfish may not represent those of embryonic hagfish. As we do not yet have access to embryos of this species, we cannot know how genetic developmental networks differ during development of the

individual. Other species of hagfish may give us greater insight into the evolutionary history of degeneration of this lineage. For example, the Atlantic hagfish which belongs to a different genus (*Myxine*), is in some ways even more rudimentary than that of the *E. stoutii* (Fernholm and Holmberg, 1975). Additionally, the limited nature of available fossil hagfish limits our understanding of extinct lineage eye morphology, particularly in relation to the morphologies of the living hagfishes.

The results of this study reiterate the high potential for function within vertebrate retinas and strengthen the argument for a common origin of vertebrate eyes from a single ancestor that predated the divergence of the agnathans from the gnathostomes. The rudimentary appearance of the hagfish retina has masked many newly identified vertebrate features now identified including interneurons, photoreceptors, and an RnPE that contains machinery necessary for function. A greater understanding of hagfish eye biology can supplement the findings in lamprey and better inform interpretations of the eye in the last common ancestor of extant agnathans and jawless vertebrates. In light of our confirmation of the presence of interneurons and a retinal (non)pigment epithelium, the eyes of hagfish appear less far removed from that of other vertebrates.

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awarded to EMD.

Table 1. Markers of retinal cell types found in hagfish

Marker	Associated retinal cell type	FPKM
PKC-α	Rod bipolar cells	12.03
PAX6	Amacrine, RGCs	12.9
Calbindin	Horizontal, RGCs	2.69
SNCG	RGCs	1623
Melanopsin	Intrinsically photosensitive RGCs	4.5
PMEL	RPE	12.03
Tyrosinase	RPE	5.61
LRAT	RPE	9.14
RPE65	RPE	525.43
RDH5	RPE	6.35
CRALBP	RPE	102.67
CRBP	RPE	116.64
RH1	Rod photoreceptors	366.9
Maf	Photoreceptors	27.89

Table 2. Primers used to produce riboprobes for in situ hybridization designed against E. stoutii transcripts, and hybridization temperatures for each probe, and primer used for RACE-PCR and the resulting sequence.

Probe	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon size (bp)	Hybridization temperature °C
Rhodopsin	GAGAACTCTGTTCCTACCACCATC	TAATACGACTCACTATAGGGCACCGTCATCTTCCTCTTCAGTAC	1387	65
Maf	CTCAAGTTCGAAGTGAAGAAGGAGCC	TAATACGACTCACTATAGGGGCCAGTTTCTCGTACTTCTCCTTG	875	65
Pax6	GATTCTGGGCCGATACTATGAGAC	TAATACGACTCACTATAGGGGATGGTTGAGGGATAGGTTGGTAC	744	74
PKC-a	GAGGGAGAGTACTACAATGTTCCC	TAATACGACTCACTATAGGGGTCTCTTATATCATGCTCCCCCTC	930	74
Calbindin	CGCTGAAGACCTTCTCAAGATTTGGG	TAATACGACTCACTATAGGGGATAGAGCTGGCCATTATCGACC	701	74
Melanopsin	ATGGCGTCTCGCCAAGATA	TAATACGACTCACTATAGGGGAGCAATCCCTCCCTCATCG	1342	67
RPE65	CTACCTACTGCATACTGGTGACAC	TAATACGACTCACTATAGGGCATGAAATCCTCACGGTTGTGTCC	816	65
MAF RACE-PCR primer	GATTACGCCAAGCTTCTGTTGCTGCTGCTGATGCCCTTGCT			
Hagfish Maf RACE-PCR fragment	AVVSTQSTWEELPFRVRV*LNARAAFASPTEFPERVRRGERASALRDR*EGTHAWPCSALLCRDEGCPGGVVYA LPRRLKPLLNEYDDPSVTRWKSVQAGHRWSQQPPALPELDGRQMRSQVTAADLAVHNNLPTSPLAMEYVNDF DLLKFEVKKEPLEALAAGRLCSRVAGSLSSTPMSTPCSSVPSSPSFSAPSPSDPKTHLEELYWMGGYQQLNPEALH LSPEDAVDALISSQHAAQLHNFDGYRGPGHHHHHHHHQPGPGQGGPPPGQQHYPVLGPHDELSAACPQPPMQ SAHHHPHHHPHHHHQPQQQQQQHHHHHPQQGHQQQQQQSQHHEHNQHSQSHSDDRFSDDQLVSMSVR ELNRQLRGCGKDEVIRLKQKRRTLKNRGYAQSCRYKRVQQKHVLETEKVDLIRQVEQLRQEIARVGRERDAYKEKY EKLAGGSLAAVVAAAAHGGYLKGGSCAPETPKSPEFYL			

Table 3. Antibodies used for immunohistochemical labelling

Antibody	ID/name	Host	Dilution	Source	Tissue labelled in hagfish
Bovine rhodopsin (1D4)	ab5417	Mouse	1:100	Abcam, MA	Rods
Synaptic Vesicle Protein 2	AB_2315387	Mouse	1:50	DSHB	Plexiform layers
ZPR-3	ZPR-3	Mouse	1:100	ZIRC	Rods
Protein Kinase C	P5704/Ab1-prkc	Mouse	1:75	Sigma-Aldrich, ON	Bipolar Cells
ZPR-1	ZPR-1	Mouse	1:100	ZIRC	None (robustly label cones in zebrafish)
Zebrafish blue opsin	Ab1-opn1sw2	Rabbit	1:100	D. Hyde, Notre Dame	None (robustly label cones in zebrafish)

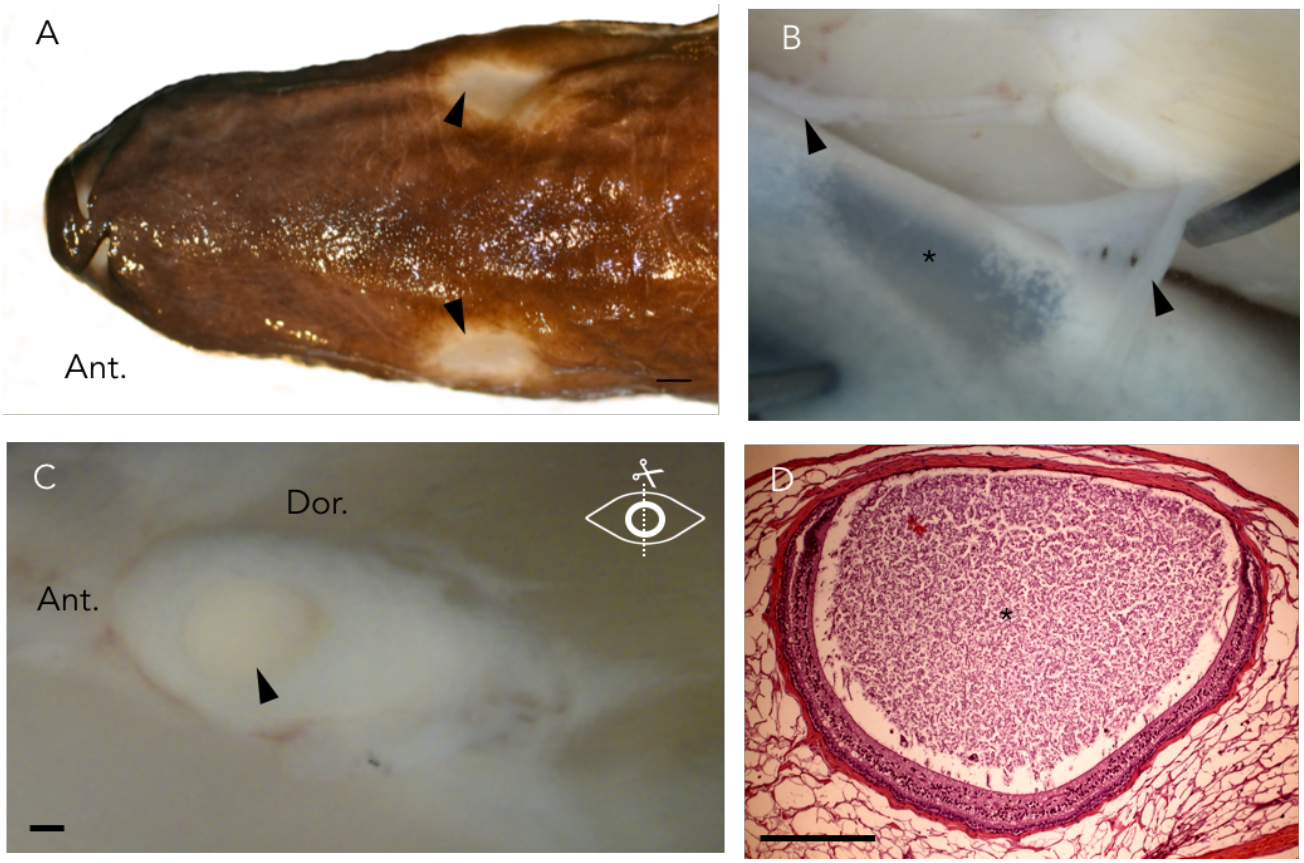

A
Ant.
B
*
C
Dor.
Ant.
D
*

Figure 2.1. Anatomy of the hagfish eye.

(A) Dorsal view of the Pacific hagfish head. Arrows indicate the translucent windows of epidermis overlying the eyes. (B) The non-pigmented eye is revealed by dissecting away the overlying epidermis, which is loosely attached to the head by two ligamentous attachments. Arrows indicate the ligaments securing the overlying epidermis to the head. Asterisk indicates the translucent window, through which light passes to reach the retina. (C) External view of the hagfish eye, arrow indicates the aperture, or opening through which light enters the eye. The eye is embedded in the surrounding musculature, and does not possess extraocular muscles. (D) H&E stained hagfish eye cross section. The muscle and collagen tissues in which the eye is embedded can be seen around the periphery of the eye. The eye lacks lens and intra-ocular musculature, though it does possess a protein rich vitreous, stained by hematoxylin and indicated by an asterisk. Scale bars are 0.25mm.

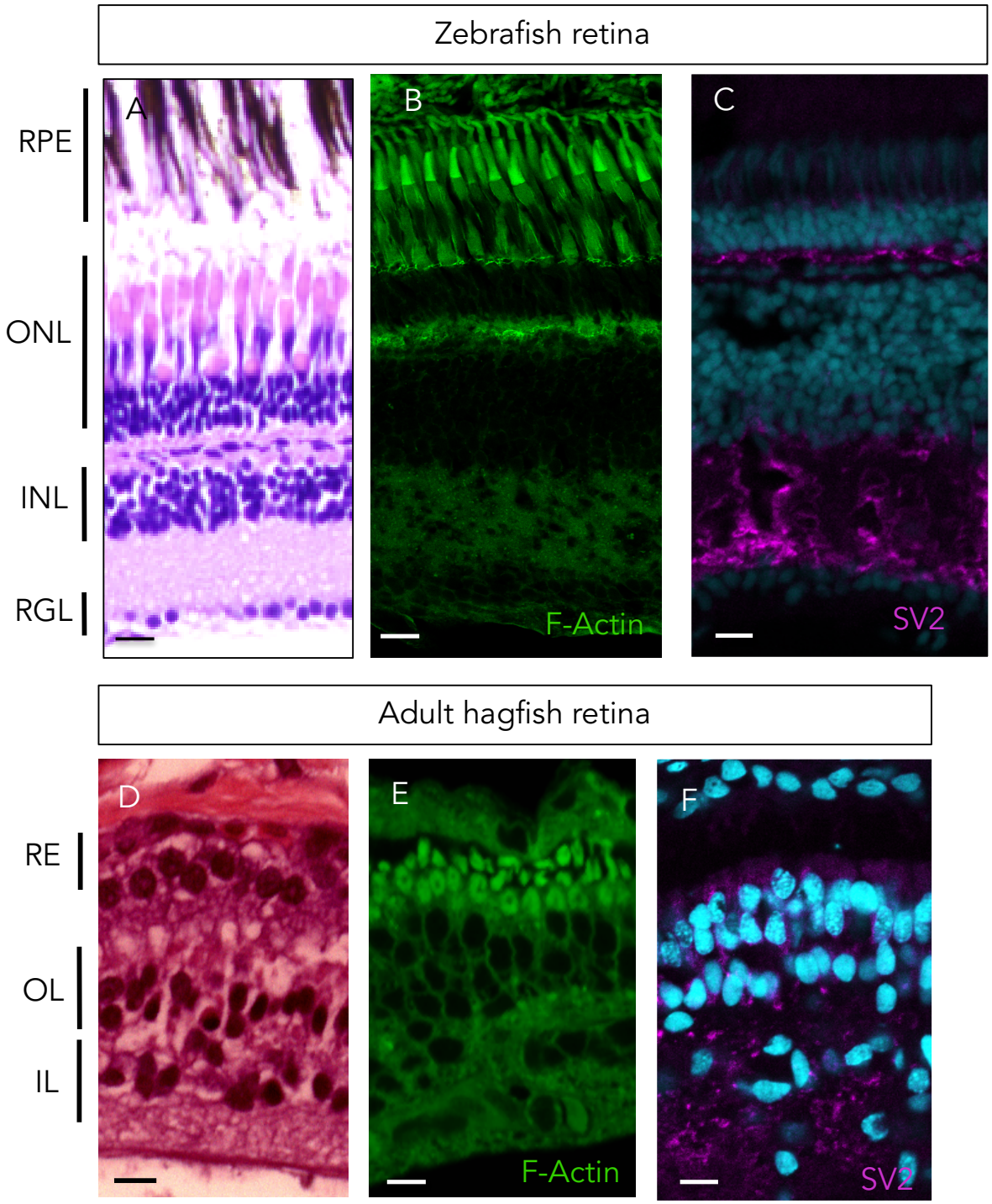

Zebrafish retina
RPE
ONL
INL
RGL
A
B
F-Actin
C
SV2
Adult hagfish retina
RE
OL
IL
D
E
F-Actin
F
SV2

Figure 2.2. Retinal lamination of the hagfish retina consists of two nuclear layers.

(A-C) Zebrafish retinas are laminated in the typical vertebrate fashion, having three nuclear layers alternating with two plexiform layers. (D-F) The neural retina of the hagfish is organized into two approximate layers, the outer layer (OL) and the inner layer (IL). (E) Phalloidin (F-Actin, green) staining illustrates the lack of distinct synaptic layers, which, in zebrafish (B), distinguishes nuclear layers and corresponding cell types within the IL. (D) H&E staining of hagfish retina displays the scarcity of nuclei within the neural retina, particularly within the IL. (F) Immunofluorescence of monoclonal antibody specific to the synaptic vesicle protein SV2 (magenta). This further clarifies the lack of distinct synaptic layers, as staining is shown to be diffuse throughout the retina, and lacks any discernable lamination within the inner layer. Nuclei are stained with DAPI and pseudo-coloured cyan. Scale bars are 10μm.

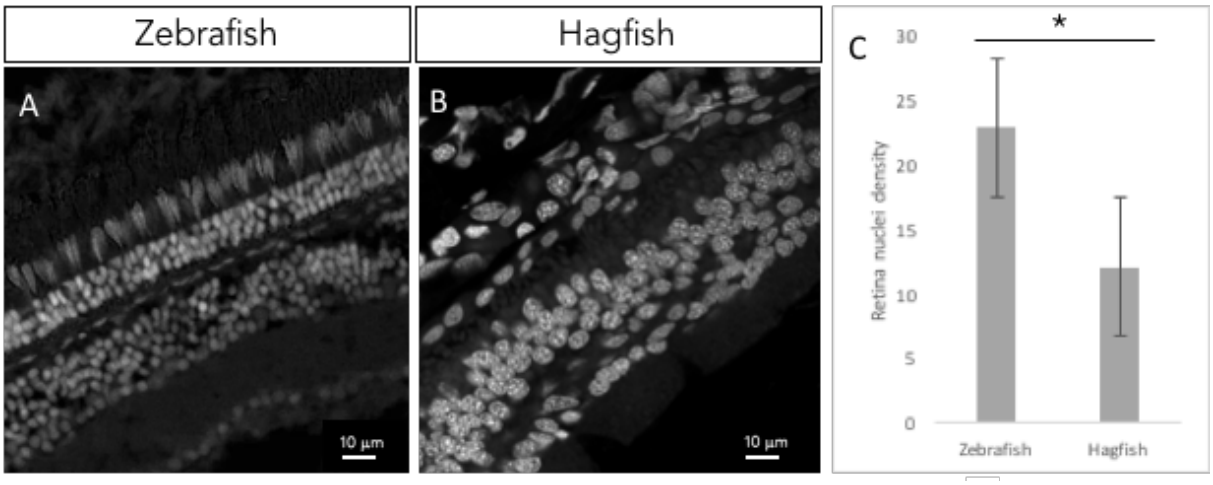


Figure 2.3 Retinal cell densities of two vertebrates: zebrafish and hagfish.

(A) Zebrafish have greater cell densities in their retinas when compared with (B) hagfish. (C) The cell densities (total nuclei per 10µm identified by DAPI staining) are significantly different ($p=0.012$, $n=4$). Scale bars are 10µm.

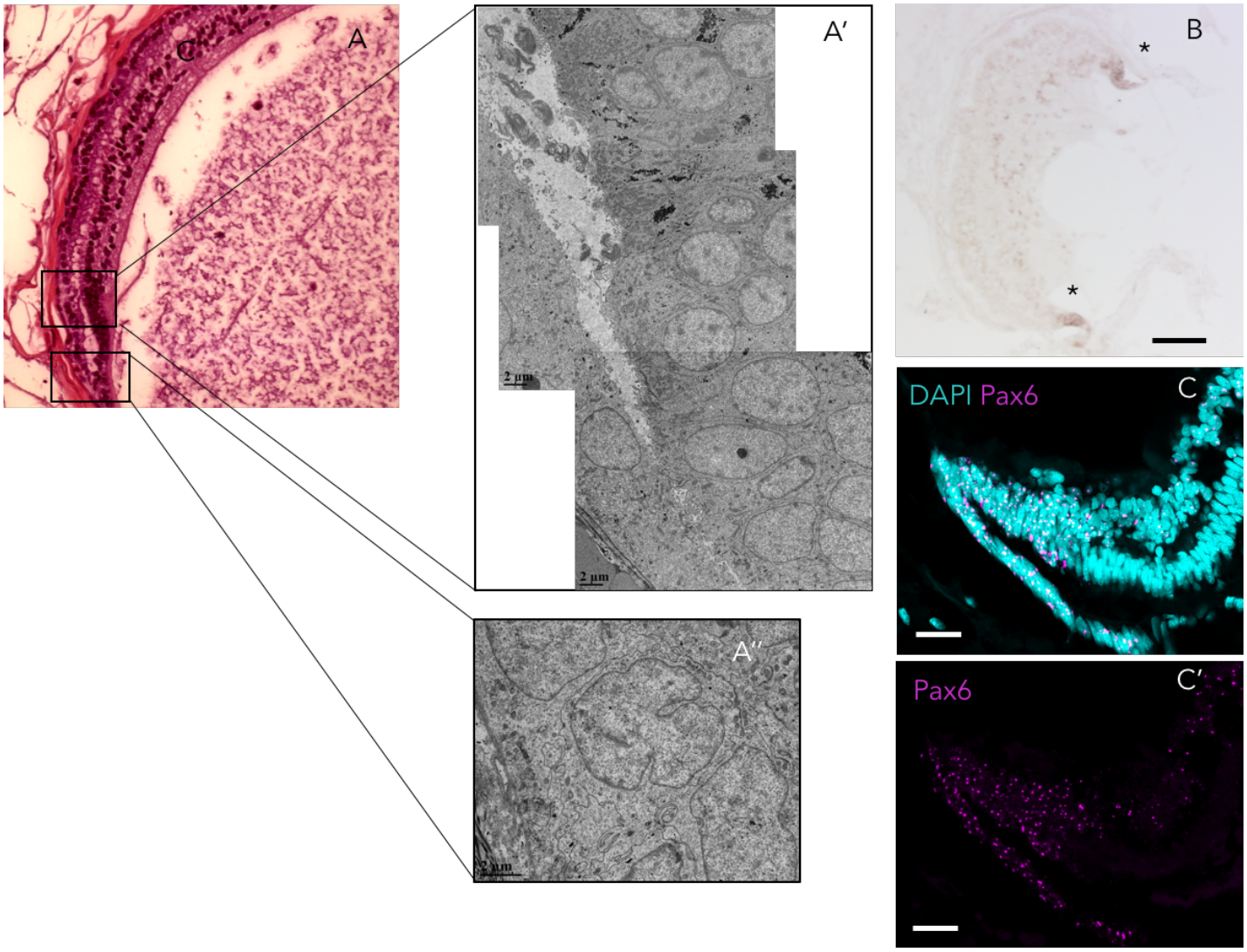
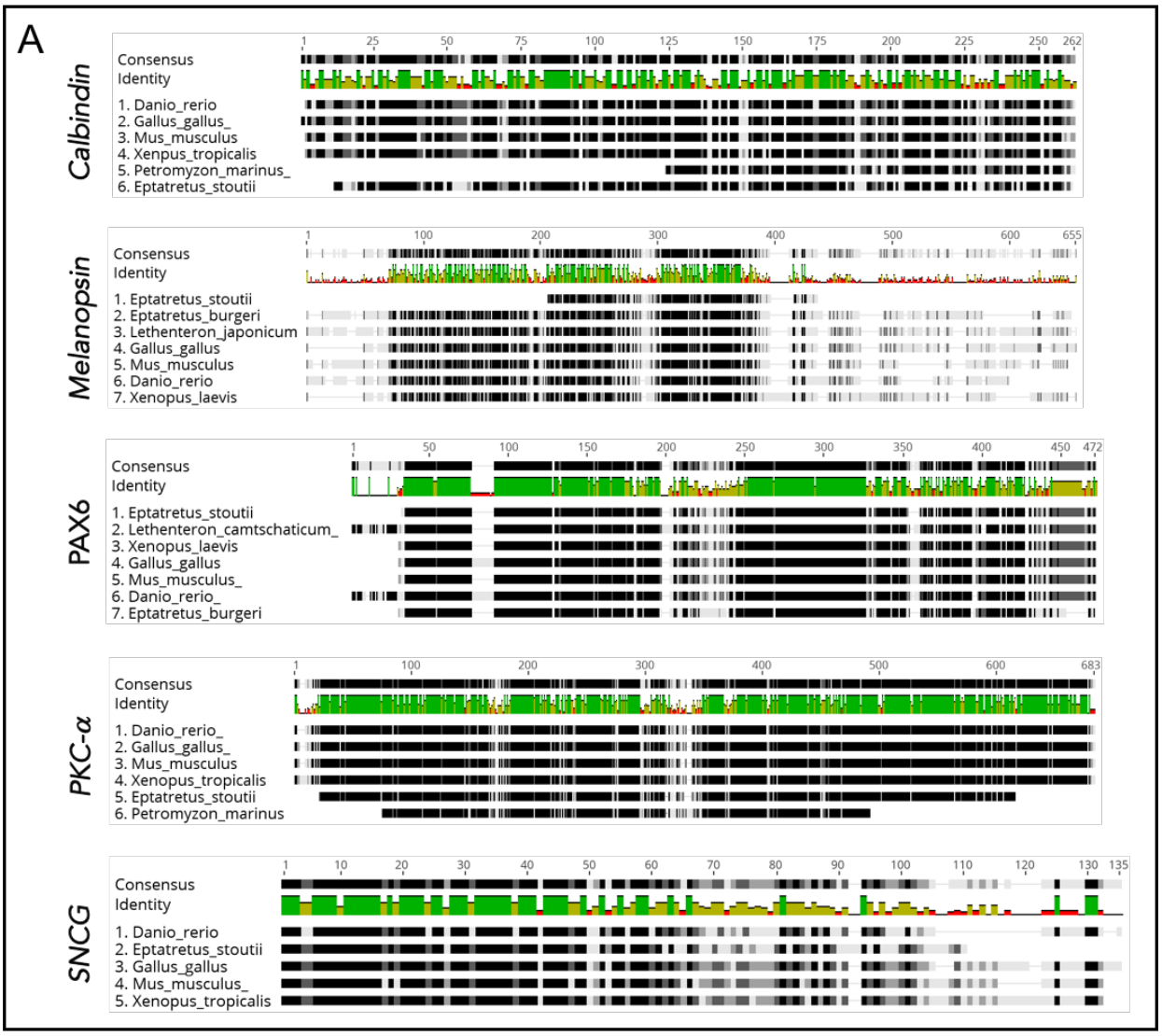

A
A′
A″
B
C
C′
DAPI Pax6
Pax6
2 µm
2 µm
2 µm

Figure 2.4 A CMZ-like tissue is identified at the margin of the retina.

(A) H&E stain of the peripheral hagfish retina. (A'-A''). Differences in lamination can be observed across the length of the retina; the cells located more peripherally appear less organized, while those more centrally located are seen within distinct photoreceptor outer segments, and begin to laminate into two approximate layers. (B) Pax6 transcript is shown to be localized at the outer edges of the retina by *in situ* hybridization. Transcript is also localized to some putative mature amacrine cells. This staining corresponds with the immature morphology of peripheral cells. (C) *In situ* hybridization staining of Pax6 (magenta) can be seen all throughout the width of the marginal retina, but is limited to occasional cells within the inner retina (Pax6 is also a mature amacrine cell marker). Nuclei are stained with DAPI (cyan), scale bars are 100μm.

A



B

Gene name	Avg % identity	Compare with
Calbindin	58%	Lamprey: 65%
Melanopsin	57%	Lamprey: 47%
PAX6	77%	Lamprey: 77%
PKC-α	80%	Lamprey: 79%
SNCG	58%	Zebrafish: 52%

Figure 2.5 Cell markers for vertebrate interneurons and RGCs are expressed in the hagfish eye.

(A) MAFFT amino acid alignments of predicted protein coding sequences of hagfish transcripts with homologs from a representative group of vertebrates. (B) Sequence identity of hagfish transcripts with other vertebrates is reported as an average of individual sequence similarities with each aligned homolog from another vertebrate. For context, the average % identity of Lamprey is reported when annotated gene sequences are available, when unavailable Zebrafish is reported.

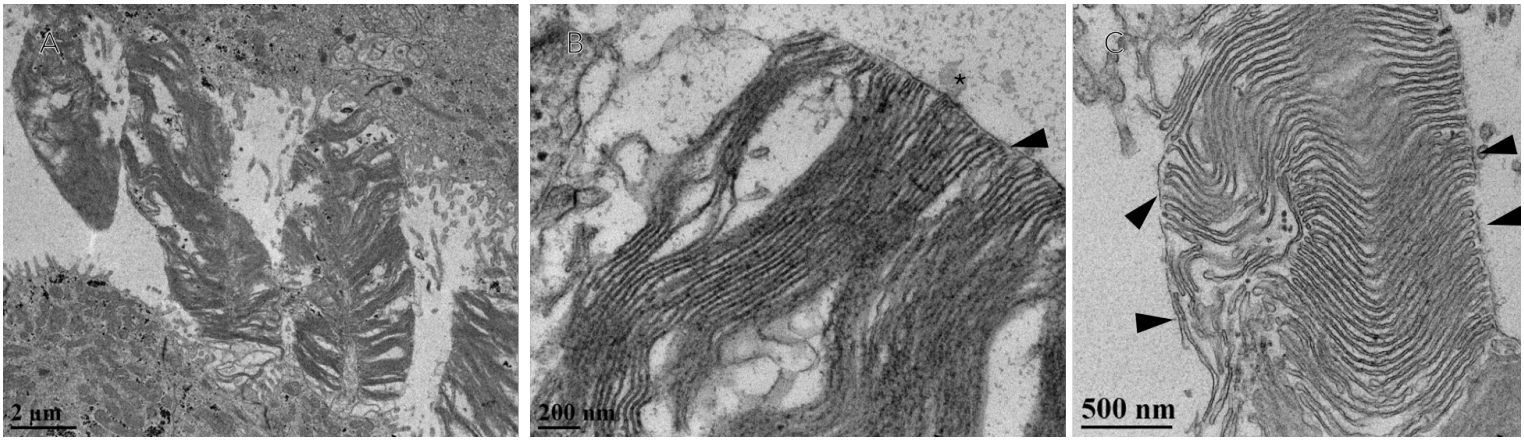
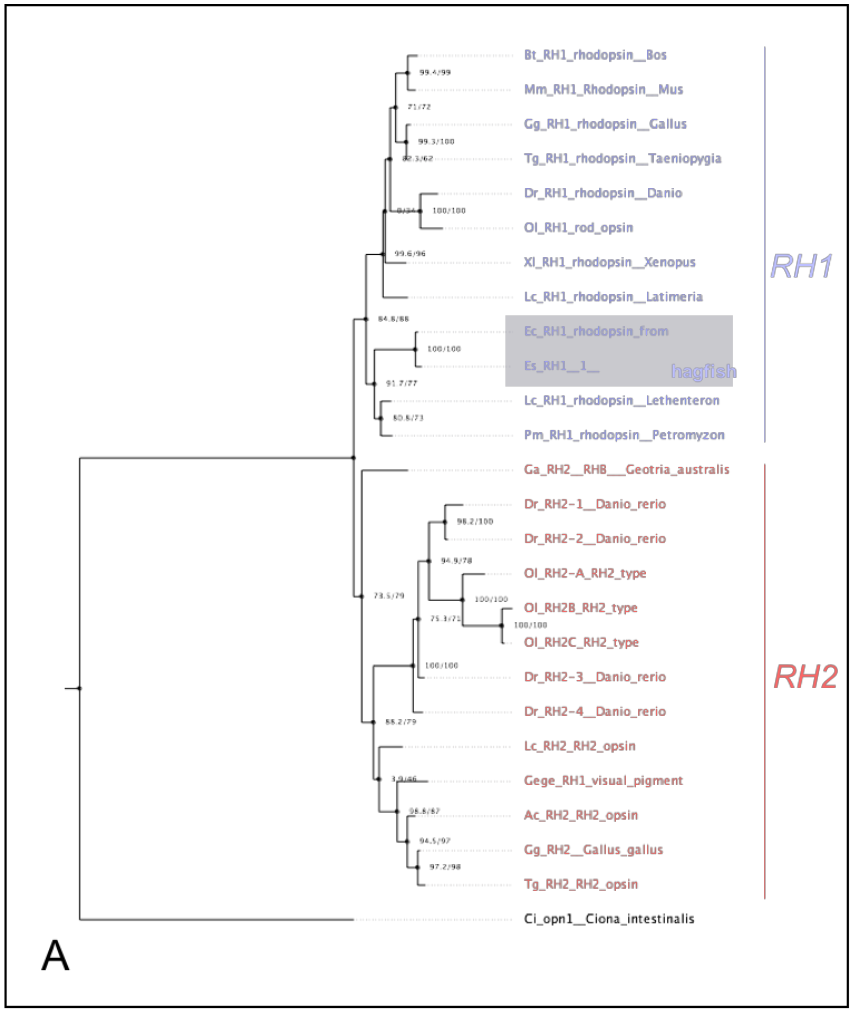
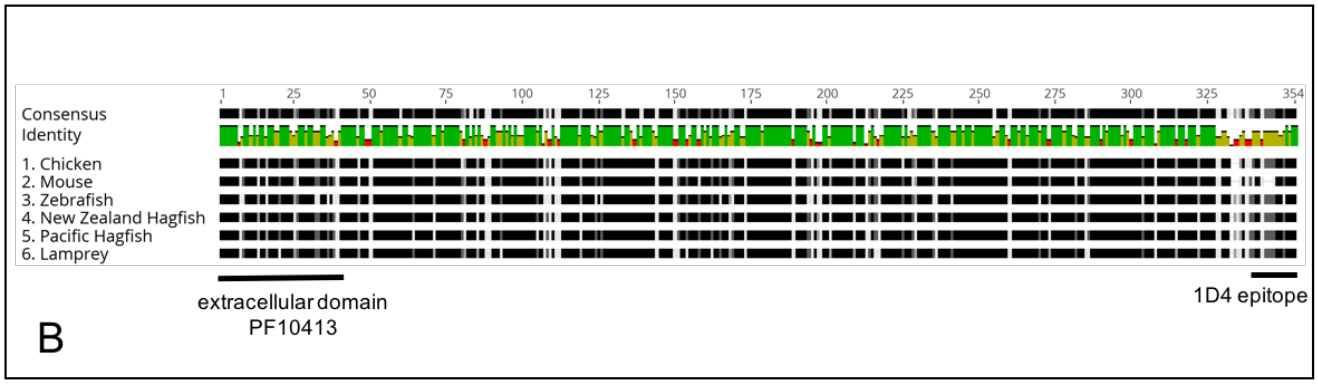


Figure 2.6 Hagfish photoreceptor cell ultrastructure is rod-like.

Transmission electron microscopy of the outer segments of adult hagfish photoreceptors. (A) Hagfish outer segments are loosely packed, and are in close association with the overlying retinal epithelia. (B-C) Increased magnification of the photoreceptor outer segments, shows an outer membrane (asterisk) surrounding the photoreceptor outer segment discs (arrowheads). Outer segment membranes appear to be arranged into discs as seen in rod photoreceptors, due to their hairpin morphology and lack of continuity with the outer membrane.





	Chicken	Mouse	Zebrafish	New Zeala...	Pacific Ha...	Lamprey
Chicken		86.040%	79.379%	79.603%	78.754%	81.020%
Mouse	86.040%		77.119%	73.654%	72.805%	77.904%
Zebrafish	79.379%	77.119%		72.881%	72.316%	75.141%
New Zealand Hagfish	79.603%	73.654%	72.881%		96.317%	81.870%
Pacific Hagfish	78.754%	72.805%	72.316%	96.317%		80.737%
Lamprey	81.020%	77.904%	75.141%	81.870%	80.737%	

C

Figure 2.7 Hagfish rhodopsin is a vertebrate rhodopsin.

(A) Maximum likelihood tree using protein sequences, including predicted Pacific hagfish rhodopsin, highlighted in blue. (B) Protein alignment of *RH1* across vertebrates, hagfish are highly conserved with other vertebrate *RH1*. The 1D4 antibody epitope is at the C-terminus. (C) Distance matrix (% identity) for protein alignment in B. Node strength is indicated by SH-aLRT support (%) / ultrafast bootstrap support (%).

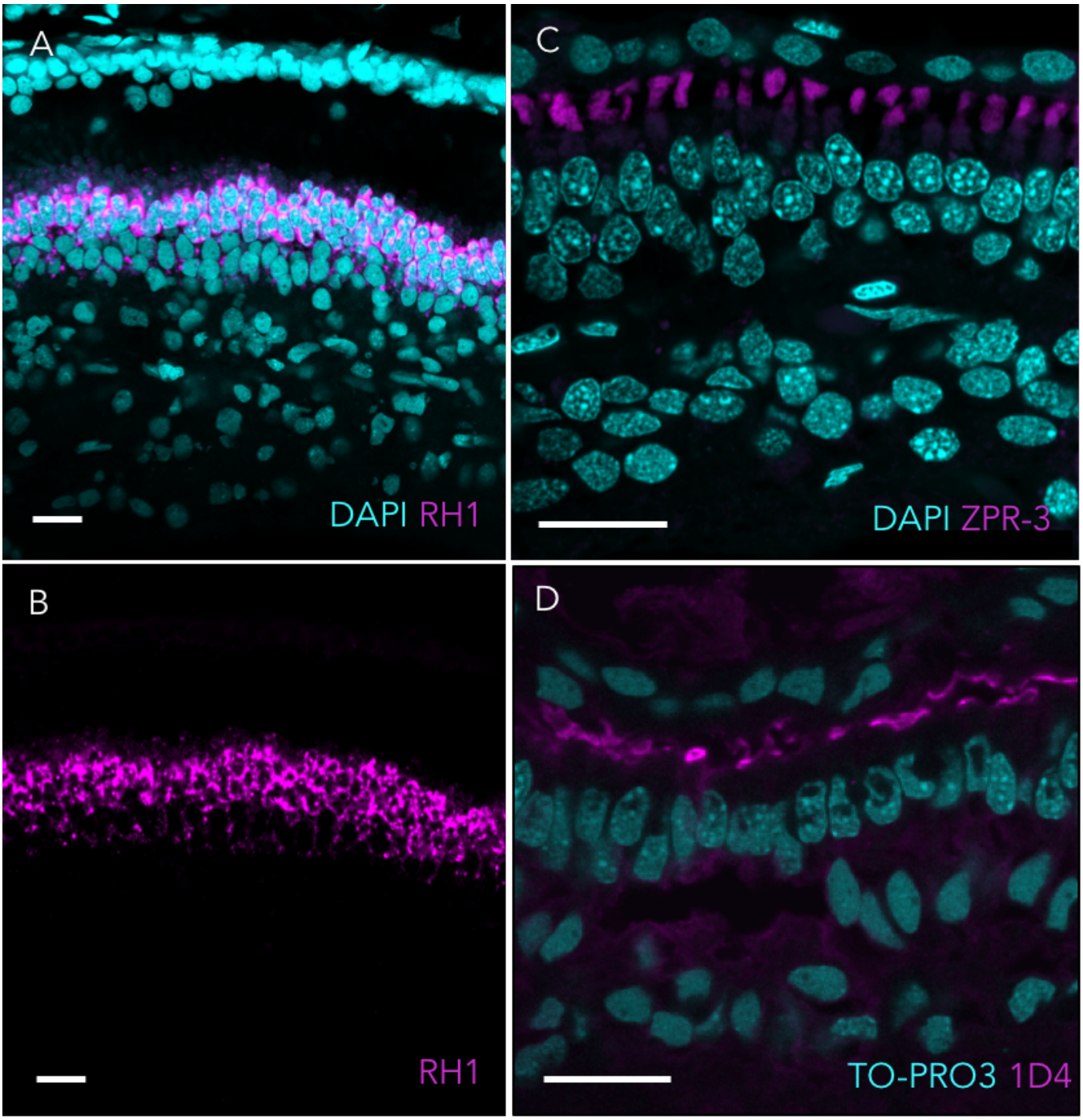
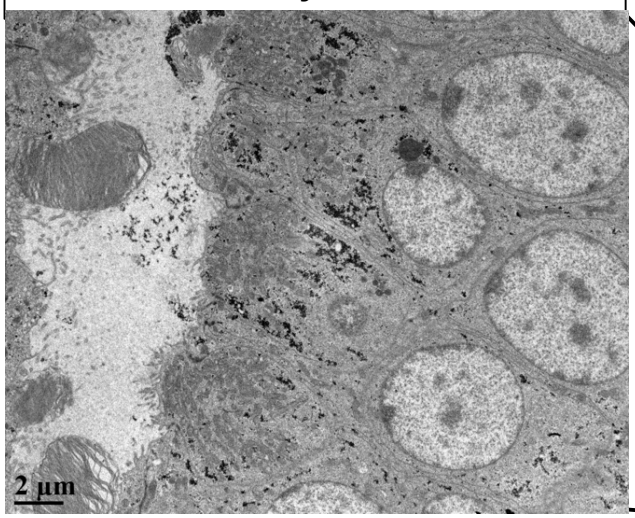

A
DAPI RH1
C
DAPI ZPR-3
B
RH1
D
TO-PRO3 1D4

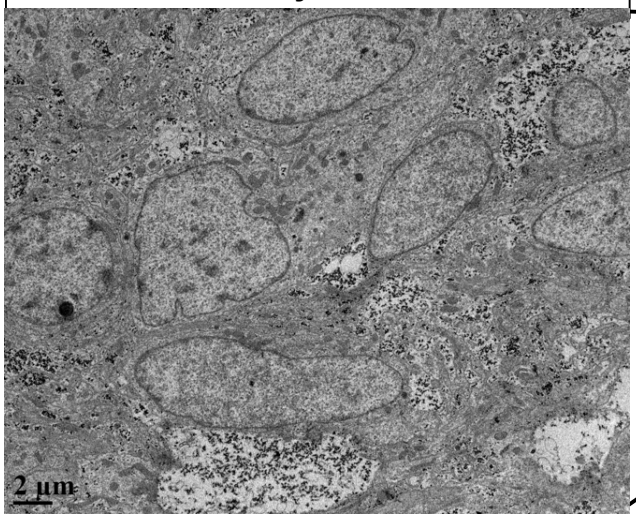
Figure 2.8 Single photoreceptor cell type expresses rhodopsin and other rod markers.

(A-B) *In situ* hybridization of RH1, or hagfish rhodopsin (magenta), labels all of the photoreceptors. Rhodopsin expression is localized to the outer layer of the retina, and no obvious non-rhodopsin expressing photoreceptors are seen. Overlap of rhodopsin and DAPI nuclear stain (cyan) is seen across all photoreceptors. (C) Antibody staining with ZPR-3 (polyclonal antibody, zebrafish rod outer segment marker) and DAPI nuclear stain (cyan). ZPR-3 is immunoreactive with the photoreceptor outer segments. (D) Antibody staining with 1D4 (monoclonal antibody designed against bovine rhodopsin) and TO-PRO3 nuclear stain (cyan). As in (C), 1D4 antibody labels the photoreceptor outer segments. All scale bars are 20μm.

Outer layer nuclei



Inner layer nuclei



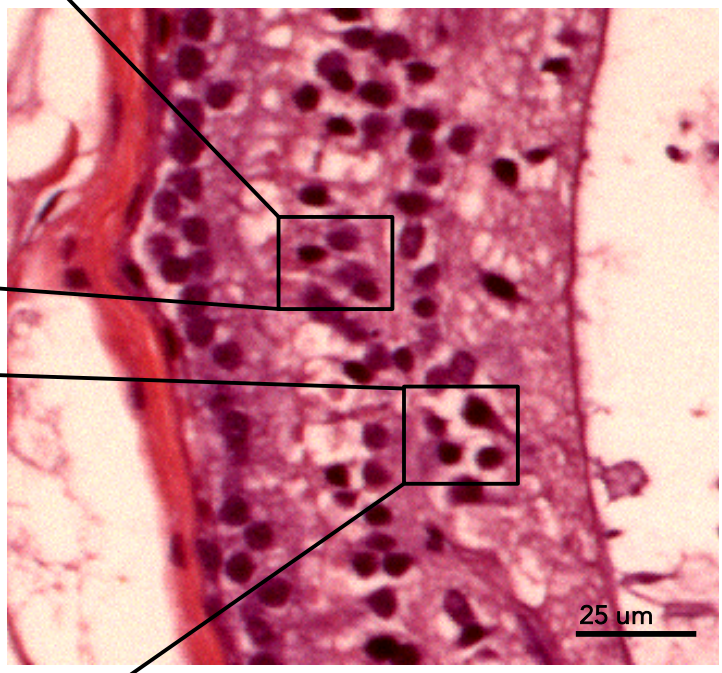


Figure 2.9 Nuclei in the inner layer are a non-homogenous group of cells.

Transmission electron microscopy of hagfish retinal nuclei. (A) Photoreceptor nuclei from the outer nuclear layer appear to be relatively regularly shaped and sized. (B) Inner nuclear layer nuclei are different in shape and size. This is an indication that the cells within the inner layer are not a homogenous population.

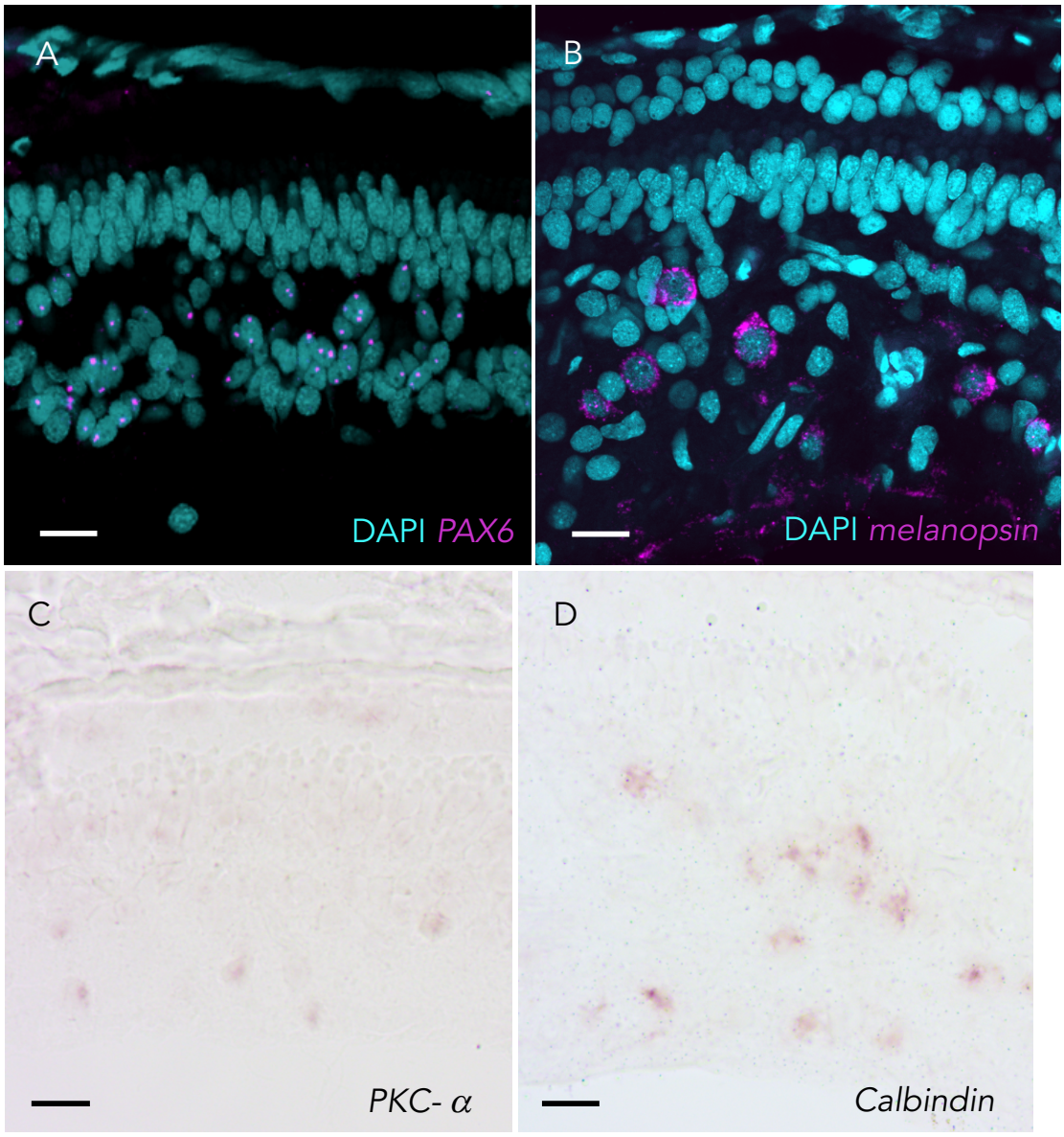
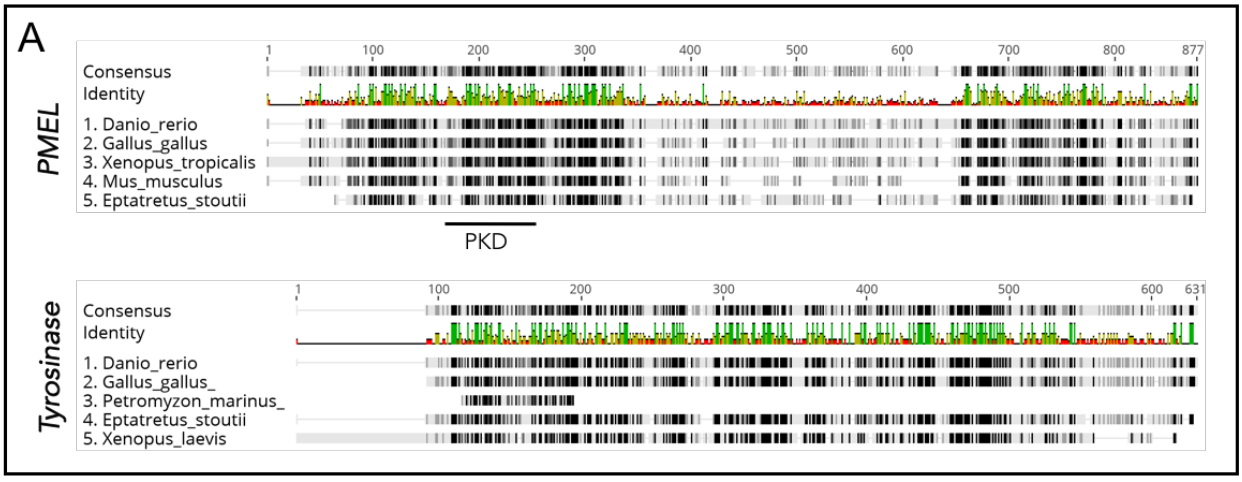

A
DAPI PAX6
B
DAPI melanopsin
C
PKC- α
D
Calbindin

Figure 2.10 Cells within the inner retinal layer express markers of classic retinal interneuron types.

In situ hybridization of hagfish retinal cells. (A) *Pax6*, a marker of amacrine and retinal ganglion cells. (B) melanopsin, a marker of intrinsically photosensitive retinal ganglion cells. DAPI nuclear stain is in cyan. (C) Protein kinase C alpha, a highly conserved marker of rod bipolar interneurons within the retina. (D) Calbindin, a marker of horizontal cells and retinal ganglion cells. Scale bars are 20μm.



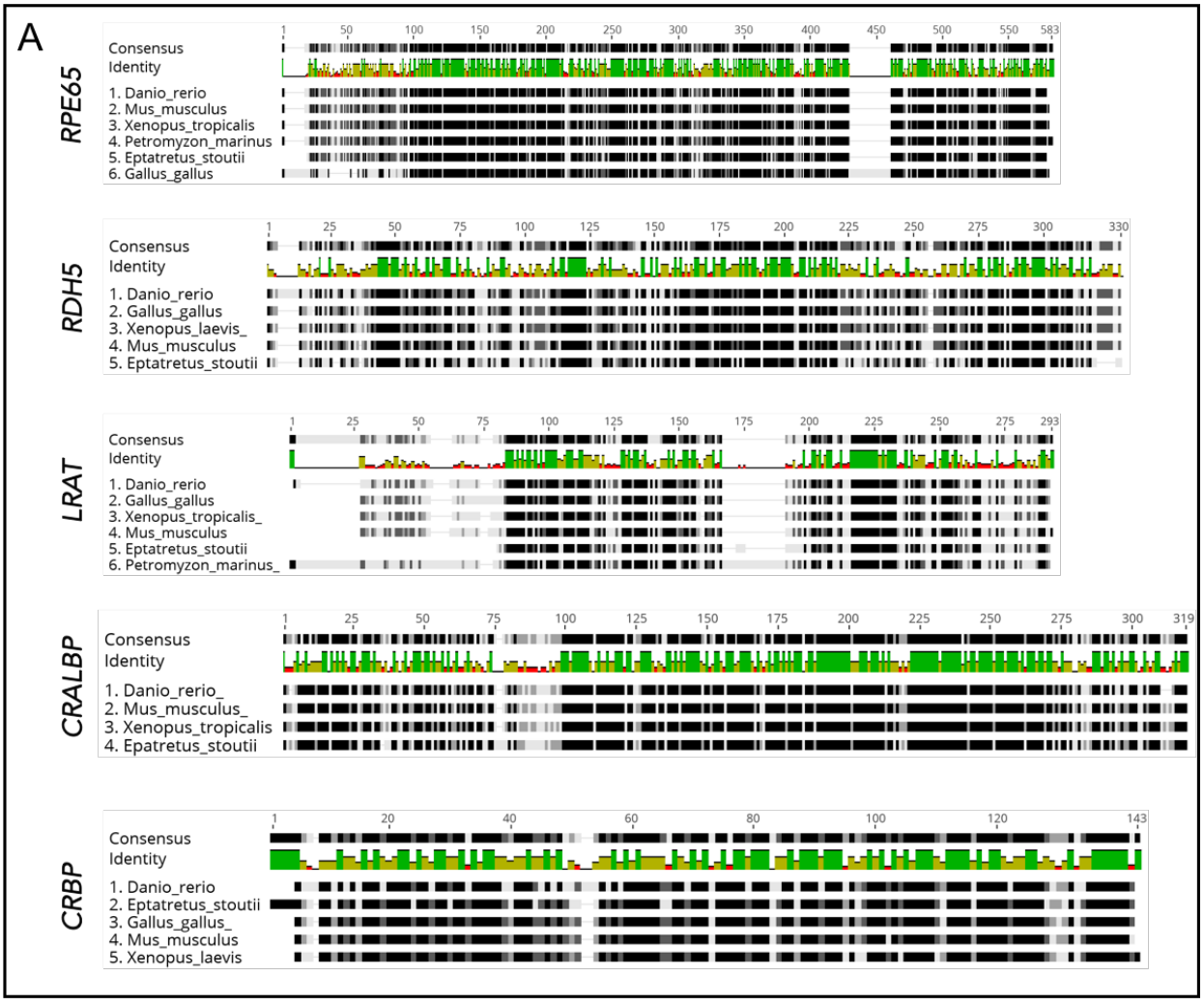
B

Gene name	Avg % identity	Compare with
PMEL	27.7% overall 48% at the PKD	Zebrafish: 32% Zebrafish PKD: 56%
Tyrosinase	41.5%	Lamprey: 47.7%

Figure 2.11 Two melanin-pigment related transcripts (*PMEL* and *tyrosinase)* are expressed in the hagfish retina.

(A) MAFFT amino acid alignments of predicted protein coding sequences of hagfish transcripts with homologs from a representative group of vertebrates. (B) Sequence identity of hagfish transcripts and other vertebrate is reported as an average so similarity with each aligned homolog from another vertebrate. For context, the average % identity of Lamprey is reported when annotated gene sequences are available, when unavailable Zebrafish is reported. For *PMEL,* conservation was further examined at the highly conserved PKD (polycystic kidney disease domain) as overall sequence identity was low.

A



B

Gene name	Avg % identity	Compare with
RPE65	67%	Lamprey: 69%
LRAT	51%	Lamprey: 42%
RDH5	67%	Zebrafish: 69%
CRALBP	62%	Lamprey: 66%
CRBP	63%	Zebrafish: 59%

Figure 2.12 Transcripts of enzymes and transport proteins involved in vertebrate retinoid cycling are expressed in the hagfish retina.

(A) MAFFT amino acid alignments of predicted protein coding sequences of hagfish transcripts with homologs from a representative group of vertebrates. (B) Sequence identity of hagfish transcripts and other vertebrate is reported as an average so similarity with each aligned homolog from another vertebrate. For context, the average % identity of Lamprey is reported when annotated gene sequences are available, when unavailable Zebrafish is reported.

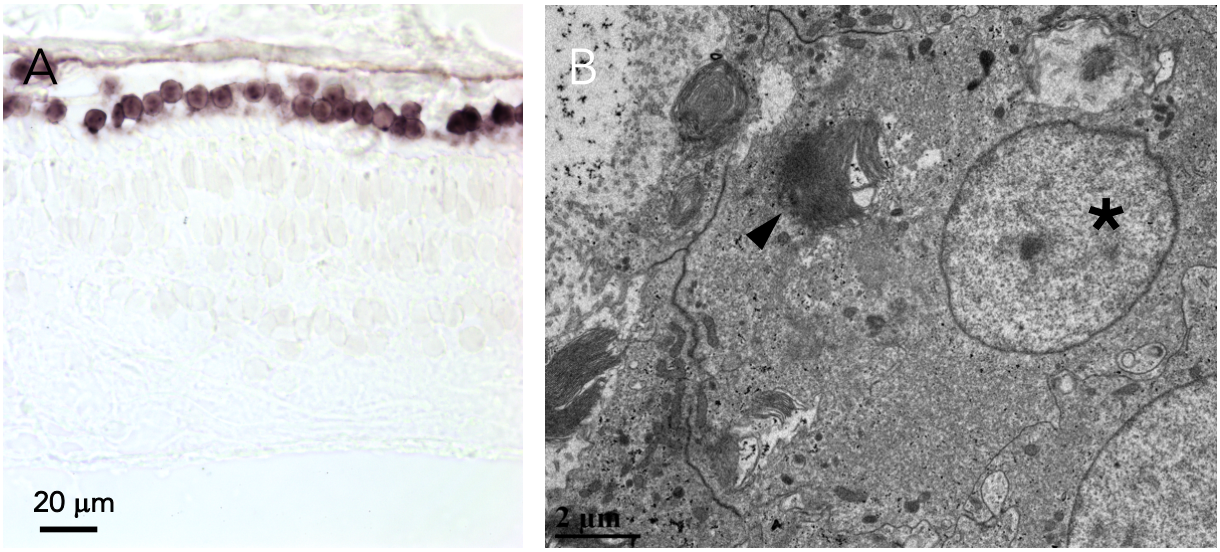


Figure 2.13. The hagfish RnPE possesses conserved features of vertebrate RPE that are essential for phototransduction. (A) The retinal pigment epithelium is responsible for retinoid cycling, in part by the action of RPE65, a crucial isomerase. *RPE65* transcript can be seen in the hagfish retinal epithelium by *in situ* hybridization. (B) Another function of the vertebrate RPE is to phagocytose expired portions of the outer segments of the photoreceptors. Here we see a portion of an outer segment which has been engulfed by a phagosome (arrowhead) into the RnPE. Asterisk denotes retinal epithelium nucleus.

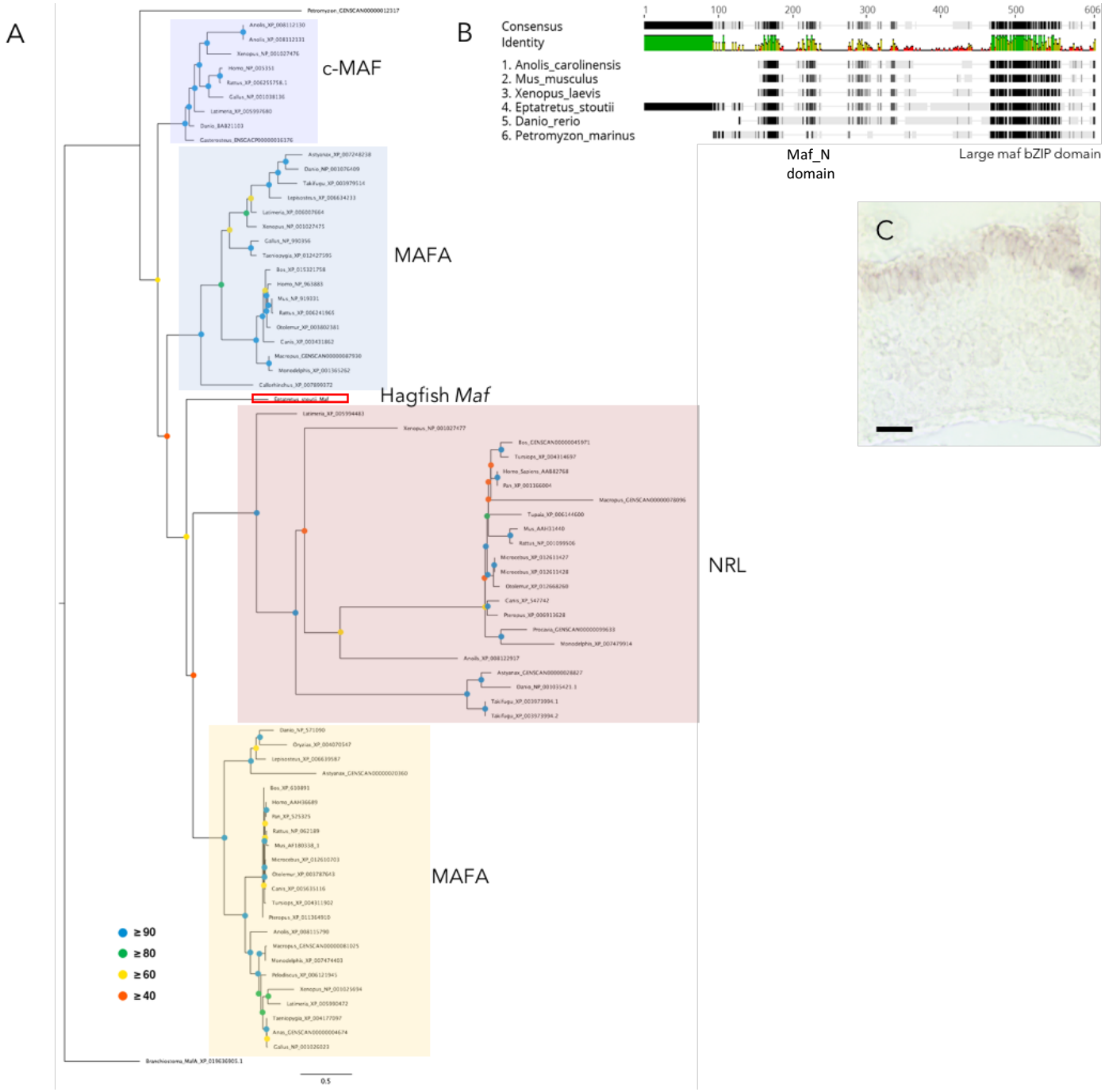

A
c-MAF
MAFA
Hagfish Maf
NRL
MAFA
≥ 90
≥ 80
≥ 60
≥ 40
0.5
B
Consensus
Identity
1. Anolis_carolinensis
2. Mus_musculus
3. Xenopus_laevis
4. Eptatretus_stoutii
5. Danio_rerio
6. Petromyzon_marinus
1
100
200
300
400
500
606
Maf_N domain
Large maf bZIP domain
C

Figure 2.14. **Hagfish photoreceptors express an *NRL* homolog.**

(A) Maximum-likelihood phylogenetic tree of vertebrate long mafs (c-*MAF, MAFA, MAFB,* and *NRL*). Hagfish *Maf* is more closely related to vertebrate *NRL* than is the singular lamprey *Maf*. Nodes are supported by ultrafast bootstrap values indicated in the legend as %. (B) Comparison of NRL amino acid sequences within vertebrates shows high conservation across the large maf bZIP domain (pfam03131) and Maf_N domain (pfam08383). (C) *In situ* hybridization of hagfish *Maf* in hagfish retinal sections reveals specific staining within the photoreceptors. Scale bar is 20μm.

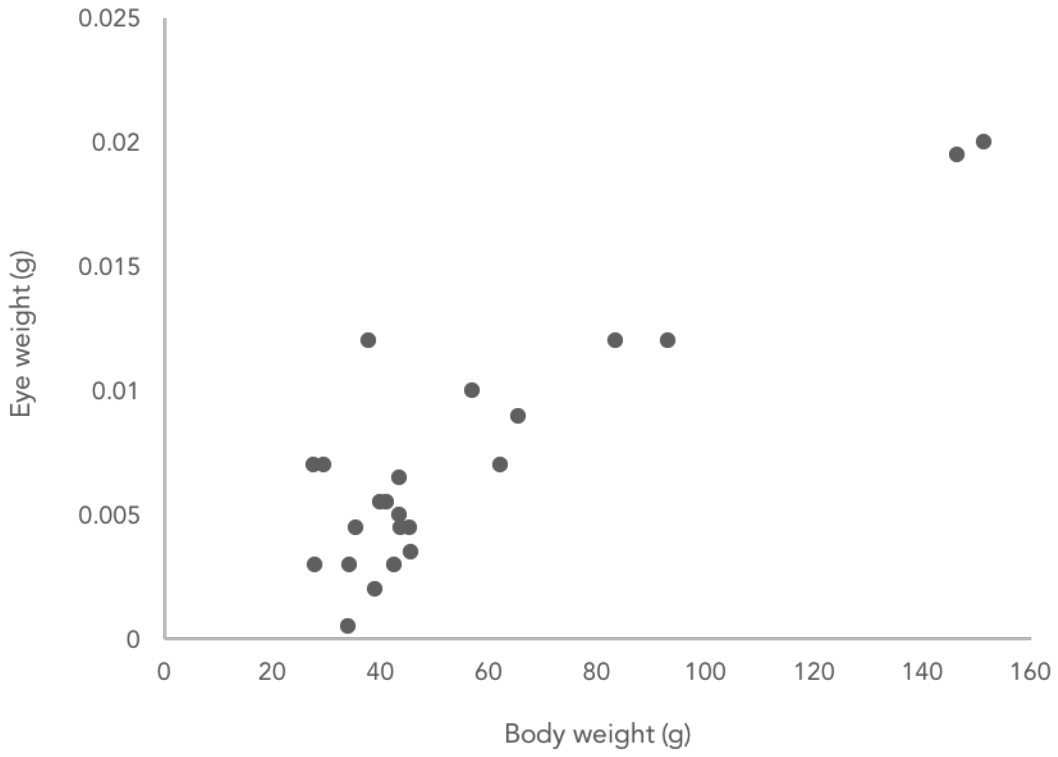


Figure 2.15 The size of the eyes of hagfish increases with the size of the body.

As hagfish increase in weight, their eyes correspondingly increase in weight. This growth in eye size over the life time of hagfish implies that changes continue to occur in the eye beyond embryonic development.

3 DISCUSSION

Our identification of novel vertebrate characters in the eyes of *E. stoutii* has opened the door to a number of future directions. This chapter will explore some of the questions that remain untested in the hagfish eye, and will propose experiments to address them. The focus here will be on elucidating the means by which hypothesized pigmentation, retinal lamination, and lens loss have occurred in these fascinating fish.

3.1 Pigment loss in the RnPE of hagfish

3.1.1 Pigment is required for healthy maintenance of the eye

In even the simplest of eyes, a common trend is shared across metazoans: where there is a photoreceptive eye-like organ, there is pigment. Not all light that enters the eye is absorbed by the photoreceptors—some travels beyond the photoreceptors to the back of the eye where it can cause damage to the delicate vasculature of the choroid, RPE, and reflect back to the photoreceptors (Creel et al., 2009; G. Jeffery, 1997; Peters et al., 2006). Eye associated pigments offer a number of supportive roles in the eye by maintaining longevity and improving visual function. The role of pigment in these tissues is largely to protect the eye from light damage by absorption of stray photons across the visible and UV light spectrum, and scavenging of reactive oxygen species (Meredith and Sarna, 2006; Peters et al., 2006).

Melanin is the primary pigment in vertebrate eyes and is found in the iris, RPE, and choroid. Pigment accumulation occurs within specialized cells called melanocytes that are derived from two embryonic sources and are thus controlled by differing developmental processes. RPE melanocytes are derived from neuroepithelium, while iris and choroid melanocytes originate from neural crest cells. Regardless of developmental origin, melanocyte biosynthesis of

eumelanin (the primary melanin constituent within the eye) requires an input of tyrosine or dopa amino acids as substrate, from which the final product is derived by a series of enzymatic steps by the action of tyrosinase in fish, and with the added use of *Tyrp2*, and *Tyrp1* in mammals (Camp and Lardelli, 2001; Wakamatsu and Ito, 2002).

Melanin improves the directional sensing of photoreceptors by eliminating background light reflection from the back of the eye. It should be noted that in some nocturnal, or primarily dim-light dwelling animals, the reflection of stray photons is instead harnessed to enhance visual sensitivity by the tapetum lucidum, a biological reflector tissue typically associated with the choroid. Also referred to as "eye-shine," the tapetum lucidum increases the amount of light reaching the photoreceptors in low-light conditions by its capacity to reflect and not absorb light. However, in bright light, the tapetum lucidum is thought to negatively affect visual acuity (Ollivier et al., 2004).

3.1.2 Pigment in fossil hagfish eyes

Extant species of hagfish lack any obvious signs of pigment in their eyes, an uncommon feature among vertebrates. To further interrogate the history of this loss, we can look to the fossil record. Mass spectrometry of the head of a fossil hagfish revealed that unlike extant hagfish, melanin-bearing eye spots exist at the anterior of the head (Gabbott et al., 2016). Melanin in the eyes of extinct hagfish suggests that some hagfish speces at one time possessed a melanin pigmented visual system. Our reported findings of tyrosinase in the extant hagfish retina alongside the findings of melanin pigment in fossil hagfish indicate that melanin could have regressed by secondary loss. However, we do not know how this loss may have occurred. Because we have limited genomic and developmental information about hagfish, understanding

the different means by which other animals lose eye pigment will aid interpretations and explorations in hagfish.

In the natural world, it is rare to find pigment loss that is localized solely to the eye. The deep sea fish *Scopelarchus michaelsarsi* is an example of the rare situation where the eye is depigmented but pigment in the body occurs. This deep sea fish has an unpigmented RPE, as seen in hagfish, though the mechanism is unknown (Collin and Partridge, 1996). Pigment loss in animals tends to be global and is rarely exclusive to the eye. In cave fish (*Astyanax)* and cave salamanders (*Proteus*) pigment loss occurs across the whole body, and not just in the eye (Durand, 1976; Protas and W. R. Jeffery, 2012). In human cases of ocular albinism where pigment loss is greatest, hypopigmentation still occurs across the body (Shen et al. 2001).

There are several animal models of RPE-specific pigment loss. One gene known to be involved in eye-specific pigment loss is melanogenesis-associated transcription factor (*MITF*). Studies of *MITF* have shown that certain isoforms are highly prevalent and localized in the melanocytes of the RPE (Bharti et al., 2008; Takeda et al., 2002). *MITF* mouse mutants have a small eye without pigment in the RPE (Nakayama et al., 1998). *Xenopus laevis* adult *mitf*-deficient mutants lack melanin in their RPE (Fukuzawa, 2018). No other gene has yet been implicated in ocular-specific pigment loss, and so it remains a good candidate for interrogation in the pigment loss of the hagfish eye.

3.1.3 A role for MITF, and functional activity assays for tyrosinase in E. stoutii

As *MITF* plays a major role in vertebrate eye pigment loss, future studies should examine *MITF* expression in the developing eye of Pacific hagfish. Aside from our identification of pigment-related *PMEL* and tyrosinase transcripts, the expression of other pigment related genes in hagfish eyes is virtually unknown. A more intensive look into pigment associated genes and their

expression patterns will be informative in terms of understanding how hagfish have lost their eye pigment.

The next logical step following our identification of tyrosinase expression in the hagfish retina is to determine the endogenous activity of this enzyme. First, by confirming that the appropriate substrates (dopa or tyrosine) are present by the Millon histochemical reaction for identification of tyrosine (Humason, 1972) and the dopa reaction (Laidlaw, 1932). In the absence of the necessary precursors, melanin synthesis will not occur.

Second, we must identify the presence and activity of tyrosinase protein, as our study only identified the transcript. Fluorescent probes have been designed to assay the activity of endogenous tyrosinase. The interaction between the probe, and a functional tyrosinase produces a fluorescent signal that corresponds with the level of enzyme activity, with higher levels of fluorescent signal being produced as a result of greater tyrosinase activity (Wu et al., 2017). This technique has been used on cells to assay endogenous tyrosinase activity, but not yet on whole tissues. In theory, as long as tissues are freshly acquired, this technique has the potential to identify presence or absence of tyrosinase enzymatic activity in hagfish eye tissue. As an assurance that a lack of signal is not a result of assay failure, tissues with known pigment in the hagfish (i.e. skin) and pigmented eyes of zebrafish would be examined in parallel.

Melanin synthesis is highly conserved enzymatic process across eukaryotes. We could take advantage of this deep conservation by performing an entirely different test of the activity of hagfish tyrosinase by expressing it in bacteria. Several strains of *E. coli* have been engineered for the mass biotechnological production of melanin. Significant genetic engineering has produced optimal production by the introduction and alteration of existing tyrosinase enzymes from different organisms. By cloning hagfish tyrosinase, and introducing it into *E. coli* by previously

established plasmid expression schemes it would be possible to assay whether or not the hagfish tyosinase is functional (Sabido et al., 2013; G. Wang et al., 2000). In the case that it is non-functional, this approach could also allow for the modeling of evolutionary changes to the tyrosinase. By altering key residues in comparison with other fish tyrosinases, we can determine which changes may have contributed to a loss of pigment in extant hagfish. Positive controls to ensure the system was working would include expression of at least two tyrosinases known to be functional cloned from lamprey and zebrafish.

3.2 Hagfish lens evolution

The lens is a highly conserved feature of the vertebrate camera-style eye. It provides focusing power (in addition to the cornea) by the use of elongated fiber cells containing high concentrations of crystallin proteins (Bloemendal, 1977). The crystallins that compose the vertebrate lens belong to two superfamilies: α-crystallin and $\beta\gamma$-crystallin (Slingsby et al., 2013). α-crystalllins are heat shock proteins that participate ubiquitously in a number of cellular processes, whereas $\beta\gamma$-crystallins are mostly eye-specific proteins expressed in very few tissues outside the lens (Andley, 2007). In vertebrates, the lens develops from ectodermal epithelium overlying the optic cup. The contact between these two tissue signals the formation of lens precursor cells which form the lens placode.

Adult *E. stoutii* bear no signs of possessing a lens. Instead, they possess a protein-rich vitreous of which the composition remains unknown. Despite the absence of lens in adults, the formation of the lens placode has been identified in *E. stoutii* hagfish embryos (Stockard, 1906; Wicht and Northcutt, 1995). A projection of cells extends from the inner layer in the direction of the optic cup very early in development which disappears before the fish hatch. It has not yet

been determined how the embryonic hagfish lens disappears, or whether or not the persisting adult vitreous expresses lens specific proteins such as crystallins.

Lens absence in the eyes of other vertebrates that have undergone eye regression seems to follow a pattern of normal developmental formation and then subsequent loss. In the Somalian cave fish *Phreatichthys andruzzii,* ocular degeneration involves not only the loss of the lens, but loss of the entire eye. In adulthood, no eye is detectable, despite having normal development during early stages (Berti et al., 2001). In *Proteus anguinus*, a blind salamander, a normal eye develops and persists in the larval stage, but soon afterward begins to degenerate, with the lens forming lytic vacuoles and disappearing entirely with the rest of the eye (Durand, 1976). In the naked mole rate (*Heterocephalus glaber)* where a functional, albeit reduced, retina persists, the lens is present but abnormal. Organization of the lens cells is displaced in these mammals, disrupting the refraction of light passing through the lens to the retina (Nikitina et al., 2004). While the cave fish and blind salamander both lack a lens entirely in adulthood, they also lack a retina. The hagfish is thus fairly unique among described regressed eyes because it maintains an eye-like shape, and a rudimentary retina, but entirely lacks a lens.

There have been no studies to extensively examine the presence of a lens in the fossil hagfish, though a small clay deposit is noted in each eye. In other eyed vertebrate fossils found in the same Mazon Creek formation, similar deposits are seen and are proposed to fill in the space where a lens would have been (Bardack, 1991). In living hagfish, there is one species in which a lens has been reported but not confirmed, *M. garmani* the Japanese hagfish (Kobayashi, 1964). The presence of this lens in this singular extant species would imply that hagfish are capable of developing a lens. It is possible that this lens has arisen convergently with that of lamprey and gnathostomes, though like many other eye features it is more likely to have been lost in other

hagfish groups secondarily. The presence of lens placodes in *Epatatretus* species also supports this view that some key intervention occurs during early development to prevent the lens from developing.

3.2.1 Studies to reveal the nature of lenses in hagfish

No molecular studies have been done to further identify what is described as a "colorless transparent lens" in *M. garmani* (Kobayashi, 1964), and mention of this lens does not appear anywhere else in the literature. Because so little is known about this or any hagfish lens, I propose several experiments in order to investigate the intriguing loss seen in all but one hagfish species.

The transcriptome that we produced from *E. stoutii* was sequenced from whole eye tissue. Thus, we have an account of gene expression not only in the retina, but in the entire eye. Homologs of α- and $\beta\gamma$-crystallins will be identified using homology searching methods described in Chapter 2. Upon identification of any crystallin homologs, endogenous gene expression will be determined in *E. stoutii* using *in situ* hybridization. Crystallin expression originates from within the lens placode during development in other vertebrates (Cvekl and Ashery-Padan, 2014), and so it is possible that there may be no trace of crystallins within the vitreous of adult fish. As embryos are unavailable for this species, an alternative would be to collect young hagfish in which lens placode derived proteins may still be present.

Examination of *M. garmani* will be absolutely key in understanding the evolutionary loss of the hagfish lens. Further confirmation of its presence and molecular make-up is required before any further interpretations can be made. We have had success in designing RNA probes for *in situ* hybridization in *E. stoutii* using the sequence information from another hagfish species, *E. cirrhatus.* Thus, it may be possible to use *E. stoutii* primer sequences to produce *M. garmani*

RNA probes. If no crystallins are identified in the *E. stoutii* transcriptome, degenerate primers targeted at conserved regions can be designed and used to clone *M. garmani* crystallins. Capture of these fish at different life stages, particularly if embryos can be obtained, will be necessary in elucidating whether this lens is retained for the life of the fish, or undergoes any further regression.

3.3 Lamination in the hagfish retina

One of the key differences between the hagfish retina and retinas of other vertebrates is the lack of three nuclear strata. It remains unclear how this two-layered organization regressed, whether in ocular development or by regression post-development. We have identified the first evidence that interneurons (the inhabitants of the third nuclear layer that hagfish have been reported to lack) are present in the retina of hagfish. We have proposed that the odd lamination of the hagfish retina is likely due to differences in organization rather than an outright loss of this entire layer of cells.

3.3.1 Comparisons between hagfish and larval lamprey

Adult lamprey retinas are laminated in the three-layered fashion of a typical vertebrate; however, the eyes of larval lamprey (ammocoetes) are not. Larval lampreys are largely burrowing, spending a majority of their time at the sandy or muddy bottoms of streams. Thus, the visual system is not utilized like it is in the adults. As in hagfish, the eyes of the ammocoete are obscured by skin, and their response to light has a several second latency (Newth and Ross, 1955). Ammocoete retinas are primarily composed of undifferentiated cells, associated with a pigment epithelial layer. In *P. marinus,* only within a 60μm radius of the optic nerve head can

proper adult-like lamination be found (Dickson and Collard, 1979). During metamorphosis, lamprey continue the developmental growth of the retina, producing a full mature retina complete with photoreceptors, interneurons, and ganglion cells (Abalo et al., 2008; Rubinson, 1990; Villar-cheda et al., 2005).

Some comparisons have naturally been drawn between ammocoete larvae and hagfish, with one prevailing theory proposing that hagfish represent a paedomorphic form that has lost its adult stage, and that indirect development of lamprey represents the ancestral condition (Lamb et al., 2007). However, in the case of larval lampreys, the absence of proper lamination is due to halted development of cells within the retina during larval stages, rather than a failure to form layers altogether. Ammocoete retinas still possess the capacity for forming retinal laminae, albeit in a delayed fashion. Hagfish retina cells are differentiated, but have reduced lamination, suggesting that a retention of an ancestral larval state may not be the most plausible cause of altered lamination.

3.3.2 Lamination defects in jawed vertebrates

In other vertebrates, lamination of the retina develops as undifferentiated cells migrate and generate different cell-types in response to intricate time and space dependent signals (Livesey and Cepko, 2001). This process is immensely complex, and relies on a number of different genetic cues involved in cell adhesion and axon guidance. In the early retina, cells are differentiated from a population of retinal progenitor cells (RPCs) that span the width of the retina by processes that extend on either side. Spatial identity is achieved by the detachment and migration of RPCs into the correct position across the width of the retina. Following this, neural processes extend from the cells and make a series of complicated connections with varying connectivity partners. Exactly how lamination is produced in the retina is still under

investigation, though several studies have identified vital signals involved. Numerous animal model mutants with phenotypes affecting the organization of these cells, but not the formation of retinal cell types, have been identified and characterized. Here I will briefly describe some of the known factors involved in producing proper retinal circuitry, and how we might use this to understand the hagfish retina.

3.3.2.1 Cell adhesion

Cellular adhesion mutants have demonstrated a need for proper cell adhesion in the generation of retinal lamination. It is thought that the disruption of cell junction complexes affects the polarity of dividing cells, thereby abnormally impacting their localization and perturbing retinal lamination.

Mutations in the cadherin-catenin complex result in retinal lamination defects. Cadherin-catenin contributes to the formation of adherens junctions, which are cell junctions that occur in epithelial and endothelial tissues. N-cadherin disruption has been shown to produce defects in cell localization and adhesive interactions in the retina (Masai et al., 2003). When β-catenin is removed in a tissue-specific manner from the developing mouse retina, cell proliferation and differentiation is unaffected, but these cells migrate to a disorganized arrangement and fail to separate into lamina (Fu et al., 2006). Similarly, in the N-cadherin *parachute* (*pac*) zebrafish mutants, developing neuroepithelial cells fail to localize to the correct nuclear layer. RGCs and amacrine cells are found intermingled in a poorly defined inner nuclear layer, and possess mis-branched and aberrant positioning further implying that N-cadherin involvement is necessary in both cell localization and in the neurite pathfinding in the developing retina (Masai et al., 2003).

The Par-3/Par-6/aPKC complex plays a critical role in the formation and maintenance of epithelial tight junctions and has been implicated in the laminar separation of developing neural

retina cells (Hirose et al., 2002; Horne-Badovinac et al., 2001). Conditional knockouts of *aPKC* in mouse produces a failure to form layers altogether, and underdeveloped photoreceptors with uncharacteristic neuronal connections (Koike et al., 2005). Loss of *Par-3* in zebrafish likewise results in a failure to produce three nuclear layers (Wei et al., 2004). Additionally, the *mosaic eyes (moe)* loci has been proposed to participate with members of the Crumbs family in forming tight junctions (Jensen and Westerfield, 2004). Zebrafish *moe* mutants have possess no apparent retinal layers, though all cell types are present (Jensen et al., 2001; Jensen and Westerfield, 2004). This combined evidence supports the view that genes involved in regulating cell polarity and adhesion processes are necessary in the formation of retinal lamina.

3.3.2.2 Axon guidance

In maintaining the overall connectivity and organization in the retina, neuronal projections are restricted to highly specific areas (such as the retina plexiform layers) so that they can be available to cell dendrites with which they need to connect. The developmental cues by which this circuitry is established are largely unknown, though factors involved in axon and dendrite pathfinding may be involved in plexiform layer determination. Class 5 semaphorins and their PlexA1 and PlexA3 receptors provide repulsive axon cues and act during retinogenesis. In the absence of *Sema5A* and *Sema5B*-regulated guidance in mouse, amacrine and retinal ganglion cells extend irregular processes (Matsuoka et al., 2011). It is proposed that these irregular processes arise due to a lack of distinct molecular boundaries within the retina, allowing for amacrine cell neurite projection into the outer plexiform layer, rather than towards retinal ganglion cells. In another example, Sidekick proteins (Sdks), show sufficiency to encode laminar specificity by ensuring that neurites of interneurons and RGCs extend to the correct area within the plexiform layer. By ectopically expressing Sdks in normally Sdk-negative cells, the authors

found that Sdk was sufficient to produce re-routing of processes to where Sdk-positive cells are found (Yamagata et al., 2002). These cues act as attractant cues for the inner plexiform layer, to attract neuronal connections. Other attractant cues may exist within the retina, and it is likely together that the numerous cell types within the retina are able to make connections with the right partners.

Within the retina there is a large number of Müller glial cells surrounding and supporting the neurons. These cells span the entire width of the retina and are arranged among interneurons, photoreceptors and ganglion cells, and show an approximate spatial pattern with greater spatial density of cell bodies toward the inner layer (Willbold and Layer, 1998). This scaffold-like organization of glial cells has been shown to act as a foundational guide for migration of newly forming neurons in the developing central nervous system (Hatten, 1990). It has been suggested that the polar nature of Müller cells, and their lateral organization within the retina act as a landmark by which neuronal cells are arranged within the retinal layers. Müller cells derived from chicken retinas have been shown to be sufficient to produce lamination of retinal cells *in vitro* (Willbold et al., 2000). However, developmental glial guidance of neurites has not yet been shown, and in many models Müller glia develop after many of the other retina cell types.

Rather than effectors of a developmental organization, Müller cells more likely play a role in maintaining organization of adult retinas. It has been demonstrated that Müller cells are valuable in maintaining retinal organization in multiple planes. In zebrafish, ablation of Müller glial cells releases tensile force in parallel to the retinal margin, implicating it in the development of cone mosaic organization, and proper placement and spacing of cells within the retina

(Nagashima et al., 2017). Additionally, in adult zebrafish, retinoschisis (ripping between the retinal layers) occurs in response to the ablation of Müller glia (MacDonald et al., 2015).

3.3.3 Future directions: Investigate genes implicated in retinal lamination in hagfish

Retinal connectivity is highly specified and precisely regulated. In some cases, the lack of a single molecule is enough to relocate an entire class of cells to an abnormal location in the retina. For example, the loss of *Lim1* in the mouse retina is sufficient to mis-localize horizontal cells into an overlapping domain with amacrine cells (Poché et al., 2007). Thus, it is reasonable to hypothesize that the layering found within the hagfish retina may be the result of a few, or even a single, aberrant developmental cues.

In order to understand why the dendrites and cell bodies of interneurons and RGCs are housed in a single retinal inner layer, I propose a study of factors within the CMZ of the adult hagfish. Ideally, these studies would be performed on embryonic hagfish, however, attaining hagfish embryos is extremely difficult. Instead, we can make use of the newly described CMZ of hagfish, in which we have proposed that new retinal cells develop and contribute to the expanding mature retina.

Examination of cell adhesion-related gene expression by *in situ* hybridization in the peripheral retina and transcriptome analysis will focus on previously identified factors in lamination mutants described earlier in this chapter, including the cell adhesion Par-3/Par-6/aPKC, and catenin-cadherin complexes. Similarly, the tight junction-related gene *moe* suggests that RPE-derived factors are required for retinal cell localization (Jensen et al., 2001). Given that hagfish possess an abnormal RPE, this presents valuable relationship to explore.

As the role of Müller glia appears vital in the maintenance of vertebrate retinal lamination, it would be natural to explore the presence of Müller glia in both the CMZ and central mature retina. Previous studies have identified what are termed as "supporting glial cells" in the hagfish retina on the basis of their morphology in TEM, but not by any molecular markers (Holmberg, 1970). Further identification of Müller cells could be achieved by antibody labelling: HNK-1, Cralbp or Dkk3 antibodies are all highly specific to Müller glia in vertebrates. Analysis of Müller glia specific transcripts within the hagfish transcriptome in comparison with those identified in other transcriptomes would also provide evidence for presence of Müller glia (Roesch et al., 2008).

3.4 Conclusion

In vertebrate and invertebrate groups alike, eyes have evolved in a colourful array. The wide variety of image forming eyes that exist in the animal kingdom speaks to the great importance that light signals have played in the evolution of animals. In vertebrates, the benefit of the eye is most markedly illustrated by its conservation across the entire subphylum—evolved in some early ancestor, and left nearly unchanged through such evolutionary novelties as the jaw and tetrapod limb. As we gain a greater knowledge of living examples of eyes, we can begin to draw more decisive conclusions and better understand the events in the evolutionary history of the eye. In doing so we draw ever closer to better knowing the eye that developed 500 million years ago and has been serving vertebrates nearly unchanged since then.

To complement what is known of lamprey eyes, it is integral that hagfish biology is studied, as they are a vital piece to the puzzle of chordate eye evolution. Despite the challenges that they pose as a model, including the lack of obtainable embryos and the limited availability of genomic information, they remain one of only two living agnathan lineages. Though unassuming

and hidden, the hagfish holds a wealth of evolutionary information accumulated over the 450my+ existence of this lineage. We have identified novel vertebrate features within the eye, and thereby have provided evidence to support hagfish's inclusion within Agnatha as a monophyletic clade, and the degenerate eye hypothesis. It is thus invaluable to study the intriguing assembly of gains and losses accumulated over millions of years in these fish.

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