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Evolution of Vertebrate Vision by Means of Whole Genome Duplications

Zebrafish as a Model for Gene Specialisation

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Abstract
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The signalling cascade of rods and cones use different but related protein components. Rods and cones, emerged in the common ancestor of vertebrates around 500 million years ago around when two whole genome duplications took place, named 1R and 2R. These generated a large number of additional genes that could evolve new or more specialised functions. A third event, 3R, occurred in the ancestor of teleost fish.

This thesis describes extensive phylogenetic and comparative synteny analyses of the *opsins, transducin* and phosphodiesterase (PDE6) of this cascade by including data from a wide selection of vertebrates. The expression of the zebrafish genes was also investigated. The results show that genes for these proteins duplicated in 1R and 2R as well as some in 3R.

Expression analyses of the zebrafish genes revealed additional specialisations for the 3R gene duplicates. The *transducin* beta subunit genes, *gnb1a* and *gnb1b*, show co-localisation in rods but are expressed at different levels. *Gnb3a* and *gnb3b* show different expression in the adult retina with low expression of *gnb3a* and expression of *gnb3b* in cones of the dorso-medial retina. The *transducin* gamma subunit genes *gngt2a* and *gngt2b* are expressed in the ventral and dorso-medial retina respectively. The both of PDE6 gamma subunit genes, *pde6ga* and *pde6gb* are both expressed in rods but *pde6ga* shows rhythmic changes of expression with low daytime levels. *Pde6ha* and *pde6hb* are expressed in cones however *pde6ha* show high daytime expression. All investigated *transducin* and PDE6 subunit genes, but *gnb1b*, were also expressed in the adult pineal complex or at some point during development.

These results provide compelling evidence that the 1R and 2R genome duplications facilitated the evolution of rods and cones by generating gene duplicates that could evolve distinct expression and function. This supports existence of colour vision before the origin of vertebrates, elaboration of this in the early vertebrate ancestor, along with origin of the black-and-white dim-light vision of rods. Furthermore, the different expression patterns observed in the zebrafish retina for teleost 3R duplicates demonstrate multiple additional specialisations.

Keywords: phylogenetics, evolution, vision, visual opsin, transducin, PDE6, genome duplications, subfunctionalisation

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”So long and thanks for all the fish”

-Douglas Adams

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Lagman, D**.*, Ocampo Daza, D.*, Widmark, J., Abalo, XM., Sundström, G., Larhammar, D. (2013) The vertebrate ancestral repertoire of visual opsins, transducin alpha subunits and oxytocin/vasopressin receptors was established by duplication of their shared genomic region in the two rounds of early vertebrate genome duplications. *BMC Evololutionary Biology*, 13:238.

II **Lagman, D.**, Sundström, G., Ocampo Daza, D., Abalo, XM., Larhammar, D. (2012) Expansion of transducin subunit gene families in early vertebrate tetraploidizations. *Genomics*, 100(4):203-11.

III **Lagman, D.**, Callado-Pérez, A., Franzén, IE., Larhammar, D*., Abalo, XM.* (2014) Transducin duplicates in the zebrafish retina and pineal complex: differential specialisation after the teleost tetraploidisation., *PloS One, Accepted.*

IV **Lagman, D.**, Larhammar, D.*, Abalo, XM.* (2014) Evolution and expression of phosphodiesterase 6 genes show vertebrate novelty in the control of photosensitivity. *Manuscript.*

*These authors contributed equally

Additional publications

In addition to the papers included in this thesis, the author has published the following papers.

i. Dahlbom, SJ., **Lagman, D.**, Lundstedt-Enkel, K., Sundström, LF., Winberg, S. (2011) Boldness predicts social status in zebrafish (*Danio rerio*). *PloS One.* 6(8):e23565.

ii. Larhammar, D., **Lagman, D.** (2014) Turtle ghrelin. *Nature Genetics* 46(6):524-5.

Contents

Abbreviations

1R	First round of whole genome duplication
2R	Second round of whole genome duplication
3R	Third round of whole genome duplication
dpf	days post fertilisation
cGMP	Cyclic guanosine monophosphate
G-protein	Guanine nucleotide-binding protein
GCL	Ganglion cell layer
GNAT	Guanine nucleotide-binding protein (G protein), alpha transducing activity polypeptide
GNB	Guanine nucleotide-binding protein (G protein), beta polypeptide
GNGT	Guanine nucleotide-binding protein (G protein), gamma transducing activity polypeptide
hpf	hours post fertilisation
INL	Inner nuclear layer
IPL	Inner plexiform layer
ISH	*in situ* hybridisation
LWS	Long wavelength sensitive *opsin*
NJ	Neighbour-joining
OFL	Optic fiber layer
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Photoreceptor outer segments
PDE6	Phosphodiesterase 6
PE	Pigmentary epithelium
PhyML	Phylogenetic maximum likelihood
RH1	*rhodopsin*
RH2	Green (medium wavelength) sensitive *opsin*
RT-qPCR	Reverse transcriptase quantitative PCR
SWS1	Short wavelength sensitive *opsin* 1
SWS2	Short wavelength sensitive *opsin* 2
WISH	Whole-mount *in situ* hybridisation

Introduction

Eyes provide a way for us to visualize our environment, to find food or mates and avoid being eaten. Since the early days of evolutionary theory the evolution of eyes has intrigued biologists. Charles Darwin admitted in *On the Origin of Species* that the different intricacies of the eye with a lens, cornea and the ability to perform optical corrections 'to be formed by natural selection seems […] absurd in the highest degree'. However, it is only absurd if one does not take into account the numerous gradations from a simple ancestral eye to the complex eyes seen for instance in contemporary vertebrates or cephalopods [1]. As Darwin stated later in the text, we can observe that eyes have evolved in many different lineages and are present in many different forms, from the simplest, a single light sensitive cell containing pigment, to the complex compound eyes of arthropods or the camera eyes seen in many animal groups. It seems as if eyes have evolved through steady gradations from a simple light sensitive cell that could only detect differences in illumination, into a complex system of cells being able to transmit information about movement, colour and illumination etc., to the brain for image processing [2].

In Darwin's time it was not known how the light sensitivity of certain nerves in the eye came about, and he even wrote that it probably was as tough a question to answer as the origin of life itself [1]. Thanks to modern technologies such as electrophysiology, protein purification, DNA sequencing and immunohistochemistry, we now know a lot more about the mechanisms behind the light sensitivity of different photoreceptor cells. We also know about the differences between photoreceptor cells and eyes of different animal groups, and what they are specialised to do. Eyes have evolved several times in many lineages [3]. Calculations have shown that it might take as little as 400,000 generations for a complex camera-type eye to evolve from a simple light sensitive organ [4]. This thesis will focus on the vertebrate eye, in particular the phototransduction cascade of rods and cones and how it evolved in the vertebrate lineage.

Origin of the vertebrate eye

Most vertebrates have image-forming camera eyes. The only exceptions being the rudimentary eyes of hagfishes and the cave- or ground dwelling

vertebrates that have partly or completely lost their eyes. Vertebrate eyes contain a cornea, a lens, a vitreous humor, a retina and a pigmentary epithelium. The retina is the light sensitive organ, which, like the sensor of a camera, detects incoming photons. The retina is very similar in structure and function across vertebrate species, with two main types of photoreceptor cells: rods and cones. The closest relatives of vertebrates, the tunicates, lack vertebrate type eyes. They are members of a lineage whose ancestor split from a vertebrate ancestor during the early Ediacarian period (around 625 million years ago) [5]. However, some tunicates have a light-sensitive *ocellus* during their free-swimming larval stage, which express a type of *opsin* closely related to the vertebrate visual *opsins* [6]. The *ocelli* of tunicates seem to share a common origin with the pineal organ (medial eye) of vertebrates, a light sensitive structure involved in entrainment of the endogenous clock, as it is located in the dorsal forebrain and develops from the same region of the neural plate [6]. Kusakabe and Tsuda (2007) also mention the possibility of photoreceptive cells being present in an organ of the tunicate larvae called *stomodæum* and that the tunicate *stomodæa* are related to the lateral eyes of vertebrates, as they seem to have a common developmental origin [6].

Vertebrate groups

Vertebrates belong to the phylum Chordata together with tunicates and cephalochordates (Figure 1). They are bilaterian animals whose most prominent common feature is the notochord, which defines the dorsal anterio-posterior axis of the animal. Among living members of these three groups, only the vertebrates possess camera-type eyes. Vertebrates can be divided further into jawed (gnathostomes) and jawless (cyclostomes) vertebrates, the latter group including lampreys and hagfishes [7]. Gnathostomes and cyclostomes diverged during the Cambrian period [5]. It is around this stage in vertebrate evolution that the vertebrate eyes appear, as this type of eyes is present in both cyclostomes and gnathostomes. Hagfish eyes, which are much simpler than lamprey and gnathostome eyes, have been proposed to be closer to the ancestral vertebrate eye [8]. However, recent data shows that lampreys and hagfishes are monophyletic [7], which would require a high level of convergence between gnathostome and lamprey eyes. An alternative hypothesis is that the eyes of hagfishes are degenerated or represent a neothenic state [8]. Because they are the sister group of gnathostomes, the genomic sequences of cyclostomes are of much interest for the understanding of the evolution of vertebrates. Currently there are genome assemblies for two cyclostome species: the sea lamprey (*Petromyzon marinus*) [9], and the Arctic lamprey (*Lethenteron camtschaticum*) [10].

The common ancestor of cartilaginous fishes and bony fishes is the next node of the three of the now living vertebrate lineages. Cartilaginous fishes

consist of two subclasses: chimeras and elasmobranchs (including rays, skates and sharks). Thus, cartilaginous fishes provide an excellent out-group for studying the two classes of bony fishes: ray-finned and lobe-finned fish, actinopterygians and sarcopterygians respectively. There are currently two genome assemblies of cartilaginous fish one chimera, the elephant shark (*Callorhinchus milii*) [11,12] included in this thesis, and a skate, the little skate (*Leucoraja erinacea*) [13].

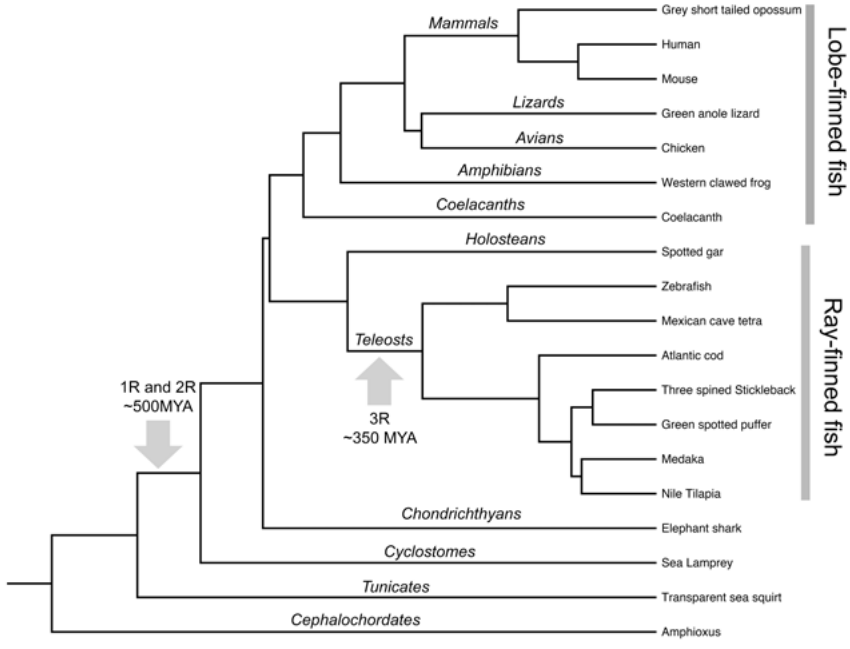

Figure 1. A species tree depicting the evolutionary relationship of the major vertebrate groups and invertebrate species of particular interest for this thesis. The branch lengths are approximate and all but the teleost branch lengths are from TimeTree.org [14]**.** Teleost branch lengths are based on Near *et al.* (2012:2013) [15,16]. Arrows indicate the 1R, 2R and 3R whole genome duplication events.

The two bony fish classes contain most of the living species of vertebrates. Lobe-finned fishes is a class that includes lungfishes, coelacanths and tetrapods, the four-legged land vertebrates: mammals, birds, non-avian reptiles and amphibians. Lungfishes are the closest relatives to tetrapods, however there is no genome sequence from any lungfish species yet. Therefore the genome of the coelacanth (*Latimeria chalumnae*) provides an good out-group to tetrapod genomes in comparative genomic analyses [17]. The earliest node among tetrapods is the node separating amphibians and amniotes. There is currently one amphibian genome assembly available from the Western clawed frog (*Xenopus (Silurana) tropicalis*) [18]. Among the amniotes, there are currently numerous genome assemblies available, most of

which are of mammals. The major groups of living amniotes are mammals as well as birds and non-avian reptiles (lizards, turtles, snakes and crocodilians). There are currently a number of reptile and bird genomes available. The primary ones used in this thesis are the green anole lizard (*Anolis carolinensis*) [19] and the chicken (*Gallus gallus*) [20]. Among mammals the earliest node is the split between monotremes, the egg laying mammals platypus (*Ornithorhynchus anatinus*) [21] and echidnas, and therians, which includes marsupials and placental mammals. Among therians the genome assemblies of the grey short tailed opossum (*Monodelphis domestica*) [22], human (*Homo sapiens*) [23] and mouse (*Mus musculus*) [24] were used extensively in the analyses included in this thesis.

The ray-finned fishes is a class that includes over 30,000 species living in almost every body of water on this planet. The great majority of ray-finned fishes belong to the group of teleost fishes. This hugely diverse group is of great interest because of their multitude and diverse set of specialisations. Although there are many species in this class, the ray-finned fishes are underrepresented when it comes to sequenced genomes. The genome of a non-teleost ray-finned fish spotted gar (*Lepisosteus oculatus*), a holostean, has been sequenced. This species provides well needed out-group when studying teleost genomes [25]. A representative selection of teleost genomes were also investigated in this thesis; zebrafish (*Danio rerio*) [26], Mexican cave tetra (*Astyanax mexicanus*), Atlantic cod (*Gadus morhua*) [27], Nile tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*) [28], three-spined stickleback (*Gasterosteus aculeatus*) and green spotted pufferfish (*Tetraodon nigroviridis*) [29]. In some cases more species were included in order to improve the phylogenetic analyses. See papers for further details.

Vertebrate whole genome duplications

In his book *Evolution by Gene Duplication* published in 1970, Susumu Ohno proposed that gene duplications would be required to provide new genetic raw material for rapid evolution to occur [30]. Based on chromosome numbers or DNA content of the nucleus of various vertebrate species, he suggested that the genome of the vertebrate ancestor had duplicated two or even three times. The evidence for tetraploidisations, or whole genome duplications, was later shown by comparing the genomic locations of genes and their paralogs (genes separated by a duplication event within a species) between human and mouse [31]. Following this, many studies of gene families and their genomic regions have provided more evidence and suggested the time periods for two rounds of tetraploidisation in the vertebrate ancestor, called 1R and 2R for the first and second rounds of duplication. Among these gene families are the developmentally important *HOX* gene clusters [32,33], the neuropeptide Y system [34–36], the opioid system [37,38], the

insulin-like growth factor binding proteins [39], the voltage gated Na^+ channels [40], the somatostatin receptors [41], the kisspeptin receptors [42] and the endothelin system [43]. More global analyses of conserved synteny between vertebrate genomes also provides extensive evidence for the 1R and 2R events (Figure 1) [44–46]. Subsequently, in the common ancestor of all teleost fish, a third round of whole genome duplication, called 3R, occurred [29]. Additional support for 3R has been shown by other studies investigating the genomes of ray finned fish lineages diverging close before, spotted gar (*Lepisosteus oculatus*) [25], or close after, European and Japanese eels (*Anguilla anguilla* and *Anguilla japonica*, respectively) [47,48]. In some vertebrate lineages independent more recent whole genome duplications have taken place, such as the salmonid lineage [49], some carps [50] and *Xenopus* frogs [51–53].

There are two primary ways for whole genome duplications to occur namely allopolyploidy or autopolyploidy. Allopolyploidy occures when two diploid gametes (which may arise accidentally when cell division has failed) from two different species fuse and form a tetraploid zygote. Autopolyploidy occurs when the genome of a zygote has been replicated, but then fails to divide [54].

Genomic rearrangements such as fusions and fissions of chromosomes are common after whole genome duplication events. This jumbles the duplicated chromosomes and makes analyses of conserved synteny, co-localisation of genes in the same chromosomal region in several species, more difficult. Often the duplicated chromosomes will be fused, making it appear as though they were duplicated through segmental duplications of chromosomal regions. This is likely due to recombination between the duplicated chromosomes and the process of re-diploidisation, a reversion to a diploid state from the post duplication tetraploid state. However, analyses of various genomes have helped delineate these events and show that chromosomal fusions and rearrangements were common after vertebrate whole genome duplications [25,28,41,45,55,56].

It has been long debated whether cyclostomes diverged from the gnathostome lineage between 1R and 2R or after 2R. Recent evidence from both whole genome [9] and individual gene families [57] strengthen the hypothesis that cyclostomes diverged after 2R. This is further complicated by data from the Arctic lamprey (*Lethenteron camtschaticum*) genome, which suggests that lampreys, just as teleost fish, have experienced an independent third whole genome duplication event [10].

Gene specialisation

When genes are duplicated, a common fate for additional gene copies is redundancy and loss through pseudogenisation (nonfunctionalisation) due to

relaxed selection pressure. The half-life of gene duplicates in animals has been calculated to approximately four million years [58]. Most of the genes that are retained after a genome duplication event are genes involved in signal transduction due to the expansion of all components of the cascade [54]. In some instances, when selection is favourable, one of the duplicated genes partition the original function (subfunctionalisation) or gain completely new functions (neofunctionalisation) [59].

Subfunctionalisation has been observed after 3R or other types of duplications in zebrafish (*Danio rerio*): the *arr3a* and *arr3b* genes that are expressed in different subtypes of cones in the retina [60]; the green opsin duplicates, *rh2-1, rh2-2, rh2-3* and *rh2-4*, which have different spectral sensitivities [61] and are expressed in different parts of the retina [62]; the fatty acid-binding protein 1 duplicates, *fabp1b.1* and *fabp1b.2*, [63]; and the *pax6a* and *pax6b* genes important for the development of the eyes and brain [64].

Neofunctionalisation, when gene duplicates gain new functions, has also been observed. The 3R generated *scn4aa* (a voltage-gated Na^+ channel alpha subunit) gene in teleost fishes has independently been selected for use in myogenic electric organs in two different lineages [65]. Another example of neofunctionalisation is from another Na^+ channel alpha subunit gene, *SCN7A*, which emerged in therian mammals through local duplication and subsequently lost its function as a sodium channel and evolved a Na^+ sensor function [66].

Subfunctionalisation but also neofunctionalisation can in some cases be attributed to the differential loss or gain of *cis*-regulatory elements, respectively. This results in differential expression of the gene duplicates. Gains and losses of regulatory elements has been shown for the *pax6a* and *pax6b* genes [64] and the genes involved in egg spots on the anal fins of Halpochromine cichlids, *fhl2a* and *fhl2b* [67].

The vertebrate phototransduction cascade

The signalling cascade of rods and cones (Figure 2) starts with the activation of a light receptor protein, an *opsin*, by a photon. The activated *opsin* in turn activates *transducin,* the heterotrimeric G protein of the phototransduction cascade. *Transducin* dissociates into the activated alpha subunit and a heterodimer of the beta and gamma subunits. The activated alpha subunit activates phosphodiesterase 6, PDE6, a heterotetramer of two catalytic and two inhibitory subunits, by removing the inhibitory gamma subunits. This starts the hydrolysis of cGMP to GMP by PDE6, which lowers the intracellular cGMP levels. This results in the closure of cyclic nucleotide gated channels (CNGs) and the hyperpolarisation of the cell. Rods and cones use related but different protein components in this cascade. For example, there are five

ancestral vertebrate *opsin* types: one *rhodopsin* gene expressed in rods and four colour *opsin* genes expressed in cones.

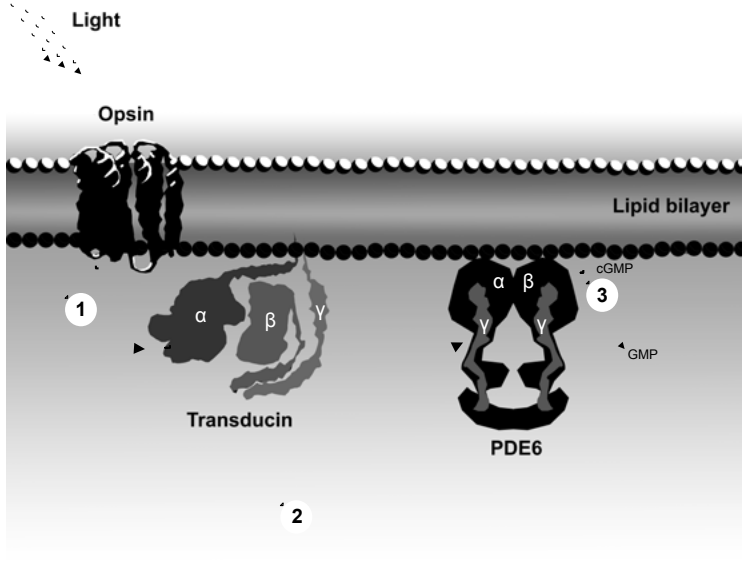

Figure 2. The first steps of the vertebrate phototransduction cascade. (1) Light activates the *opsin*, which in turn activates *transducin*. (2) The activated *transducin* dissociates and the activated alpha subunit removes the inhibitory gamma subunits of PDE6. (3) This results in the activation of PDE6, which hydrolyses cGMP into GMP.

The visual opsins

The visual *opsins* are members of a large group of *opsin* G protein coupled receptors. Their function and evolutionary history have been studied extensively since their initial discovery. For vertebrate visual *opsins*, the identification and phylogenetic analyses of five visual *opsin* subtypes in chicken (*Gallus gallus*); red sensitive LWS, violet/ultraviolet sensitive SWS1, blue sensitive SWS2, the *rhodopsin* RH1 used in dim-light vision, and green sensitive RH2, showed that the green sensitive *opsin* is most closely related to *rhodopsin*. Thus, showing for the first time that dim-light vision is a newer phenomenon than colour vision [68]. Subsequently, these five visual *opsin* subtypes were identified in the pouched lamprey (*Geotria australis*) [69]. In phylogenetic analyses, these five lamprey sequences cluster within each of the five different vertebrate visual *opsin* classes [69,70]. It has been clear since the identification of all five visual opsin subtypes in a lamprey as well in gnathostomes that the gene duplications that gave rise to the five visual *opsin* subtypes must have taken place before that split. However, the exact

pattern of duplication generating these genes, and if the early vertebrate whole genome duplications were involved, has been unclear. Partly due to a lack of large-scale analyses of conserved synteny. The topologies of many sequence-based visual *opsin* trees suggest a serial duplication pattern that does not conform to duplications in 1R and 2R. Conversely, early synteny analyses of the human genome suggested that the visual *opsins* resulted from an ancestral gene pair that gave rise to two subfamilies after duplications in 1R and 2R [71,72]. These scenarios are summarised in Figure 3. One of the visual *opsin* subfamilies has only one surviving member (LWS) while the other subfamily has four surviving members (SWS1, SWS2, RH1 and RH2) [71,72].

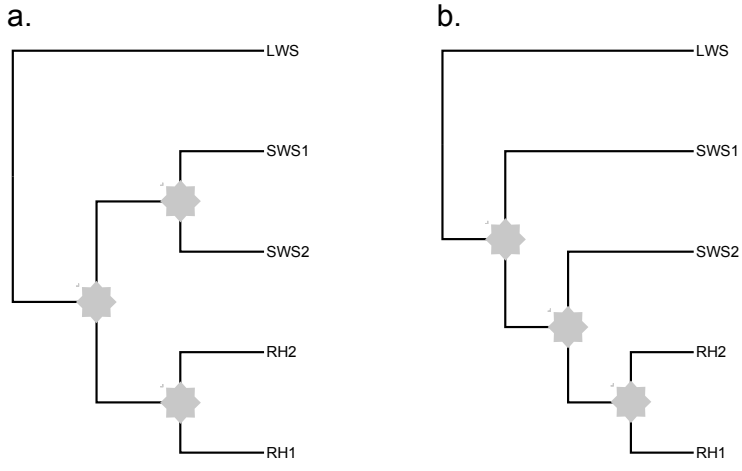

Figure 3. Summarised phylogenies of the vertebrate visual *opsins*. a) The expected topology of the visual *opsins* if the duplications follow a 1R and 2R scenario with a double bifurcating topology, 1R results in two genes that later are duplicated in 2R. b) The observed topology of the visual *opsins* in many phylogenetic analyses that instead suggest a step-wise duplication pattern rather than a 1R and 2R scenario. Stars represent duplication events.

In Paper I we performed comparative synteny analyses in combination with phylogenetic analyses of neighbouring gene families between several vertebrate species. These analyses, in contrast to previous analyses [71,72], included several ray-finned fish species for example the holostean spotted gar (*Lepisosteus* oculatus) and the teleost zebrafish (*Danio rerio*).

Transducin

Rods and cones express different but related *transducin* α, β and γ subunits. Rods express Gαt1, Gβ1, Gγt1 while cones express Gαt2, Gβ3, Gγt2 [73,74]. These proteins are encoded by the *GNAT1, GNB1* and *GNGT1* genes for

rods and *GNAT2, GNB3* and *GNGT2* genes for cones [72]. The Gα proteins are encoded by a gene family of 16 members in the human genome [75]. Among these members, the Gαt and Gαi proteins are closely related and the corresponding genes are often located close together on the same chromosome. They therefore have been proposed to constitute an ancestral pair that originated in a local duplication. These gene pairs are located in the same chromosomal regions as the visual *opsins* in the human genome, suggesting that the ancestral Gαt and Gαi gene pair later duplicated in 1R and 2R giving rise to the genes of modern vertebrates [71,72,76].

The Gβ proteins are encoded by genes forming a family of five genes in humans: *GNB1, GNB2, GNB3, GNB4* and *GNB5*, where the Gβ5 protein encoded by the *GNB5* gene is the most divergent at the amino acid level [77]. The five genes are located on different chromosomes and the *GNB1, GNB2, GNB3* and *GNB4* group, which include the rod and cone specific genes, were suggested to have originated in 1R and 2R. Although the analysis of the phylogenetic trees of this family is complicated, partly because of high sequence conservation, the chromosomal regions provide evidence for this hypothesis [71,72].

There are 13 genes encoding Gγ proteins in the human genome [78] two of which are expressed in rods and cones. The genes encoding the rod and cone specific Gγ proteins are closely related and share high similarity at amino acid level [77]. Analyses of the human genome led to the conclusion that they are located in the chromosomal regions housing the *Hox* genes and thus probably duplicated in a similar fashion, through 1R and 2R [71,72].

In Paper II we expanded on previous analyses of the *transducin* subunit gene families by investigating a wider selection of species and performing comparative synteny analyses. An extensive analysis of the paralogon housing the *GNAT1, GNAT2* and *GNAT3* genes is presented in Paper I.

Phosphodiesterase 6

Rods express the PDE6α and PDE6β catalytic subunits (encoded by the *PDE6A* and *PDE6B* genes) as well as the *PDE6G* inhibitory PDE6γ subunit genes. Cones express the PDE6α′ catalytic subunit (encoded by the *PDE6C* gene) and the *PDE6H* inhibitory PDE6γ subunit genes [79]. The human genome contains 21 genes encoding catalytic PDE subunits divided into 11 subfamilies: PDE1 through PDE11 [80]. The PDE6 genes form a subfamily of genes that previous analyses have shown are most closely related to the PDE5 and PDE11 genes [80–82]. The inhibitory PDE6γ subunits do not seem to be present in invertebrate genomes [81]. Previous analyses suggested that the *PDE6A, PDE6B* and *PDE6C* genes originated in 1R and 2R, as they are located in the same chromosomal regions as the neuropeptide Y receptor genes [72], which have been analysed before [35]. Sequence-based phylogenies of the PDE6 genes also support this scenario [71,81].

Larhammar *et al.* (2009) reported that the phylogeny and the chromosomal locations of the *PDE6G* and *PDE6H* genes on the same human and dog chromosomes as two of the *Hox* gene clusters indicated that these two genes also have their origin in 1R and 2R [72]. In Paper IV, we expand these analyses by including more species in the phylogenetic and comparative synteny analyses. We also investigated the expression of all zebrafish-specific PDE6 subunit genes in order to determine if there have been gene specialisations associated with the gene duplications.

The vertebrate retina

The overall structure of the retina is common to all vertebrates, except hagfish, (Figure 4). From outer to inner, the retina is composed of the pigmentary epithelium (RPE), the photoreceptor cell layer (PR), the outer limiting membrane (OLM), the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL), the ganglion cell layer (GCL) and the optic fibre layer (OFL). There is a minor divergence from this organisation in the lampeys, where retinal ganglion cells are located in the INL and the IPL [83].

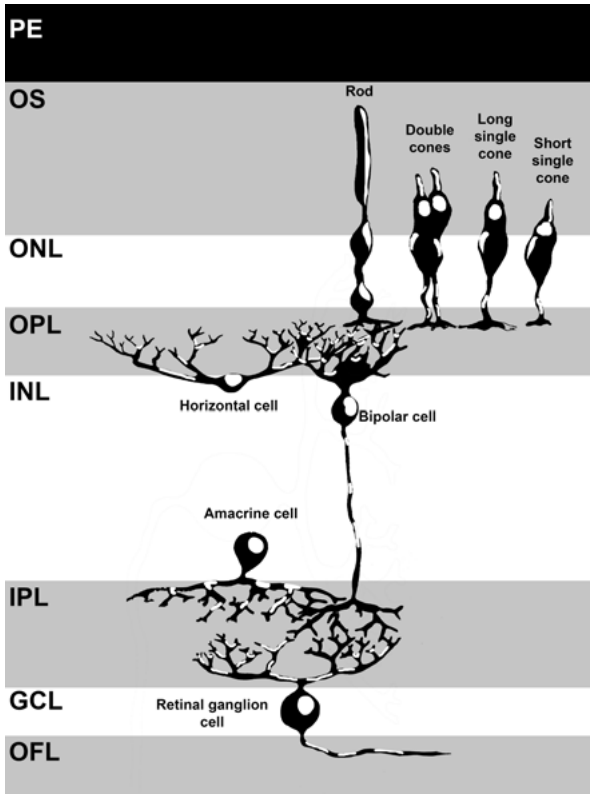

Figure 4. Schematic figure of the vertebrate retina with the connections of a rod cell. The rod sends signals that are modulated by horizontal cells to the bipolar cells. These bipolar cells send the signal further to retinal ganglion cells; in this instance amacrine cells modulate the signal. Finally the retinal ganglion cell send the signal to the brain through the optic nerve.

With some exceptions gnathostomes have five classes of visual photoreceptor cells; one rod type used for scotopic, low light vision and four cone types used for photopic vision, bright light color vision [2]. The pouched lamprey (*Geotria australis*) also has five photoreceptor cell types and five classes of visual *opsins* [2,69,84], while other lamprey species have less, probably due to secondary losses. The lineage of northern hemisphere lampreys have only two cell types, one cone-like and one rod-like (see [84] and references therein).

Rods and cones send signals modulated by horizontal cells to bipolar cells in the OPL. Rod photoreceptors connect to one type of bipolar cells and cones to another. Cone bipolar cells can be of the ON or OFF type, which depolarize in response to light and darkness, respectively, and rod bipolar

cells are always the ON type. The bipolar cells have synapses to retinal ganglion cells in the IPL. This input is modulated by amacrine cells. For a review of this see reference [85]. The axons of the retinal ganglion cells project to the brain through the optic nerve [83]. A subset of the retinal ganglion cells expresses *melanopsin* (encoded by the *OPN4* gene). These photosensitive ganglion cells are involved in the entrainment of the circadian clock pacemaker in the suprachiasmatic nucleus (SCN) in the hypothalamus through the retino-hypothalamic tract [86].

The zebrafish retina

Retinal structure and cell types

The zebrafish (*Danio rerio*) retina has a single rod photoreceptor type and four cone subtypes that express visual *opsins* with different spectral sensitivities, LWS (red), RH2 (green), SWS1 (UV) and SWS2 (blue) [61,87–90]. The different cones in the zebrafish retina have very different morphology; the SWS1 expressing cones are short and called short single cones (SS), the SWS2 expressing cones are longer and called long single cones (LS), the RH2 expressing cones and LWS expressing cones are the longest and fused into pairs of one red cone (the long cell) and one green cone (the short cell) and are called double cones (DC) [91,92]. The photoreceptors in the zebrafish retina are arranged into a mosaic made of alternating rows of different photoreceptor cells. One row contains SS and LS cones and the adjacent rows are of DC cones [91]. Robinson *et al.* (1993) showed that the SS cones in the zebrafish retina are sensitive to UV light. However, the gene they cloned and demonstrated to be expressed in SS cones was later shown to be *rhodopsin*. It was also shown to be expressed in rods rather than in SS cones [89,93]. Later, Raymond *et al.* (1996) showed that goldfish (*Carassius auratus*) UV *opsin* probes hybridized to the SS cones, demonstrating that these cones express SWS1 type *opsins* [89].

Even though there are four major classes of cones in zebrafish each expressing one type of visual *opsin* the genome contains 11 genes for visual *opsins* [61,94,95]. Some of these have a differential spatial expression in the retina [62] and one, the *errlo* gene encoding *exo-rhodopsin*, is expressed outside the retina in the pineal gland [96]. Three of the zebrafish visual *opsin* genes belong to the *rhodopsin* subtype: two *rhodopsin* (*rho*) genes, *rh1* and *rh1-2*, and *errlo.* While the *rh1* and *rh1-2* genes are both are both intron-less [94], *errlo* has a classical *rhodopsin* four-intron structure. This, together with phylogenetic analyses, suggests that *errlo* is the true ortholog of non-actinopterygian *rhodopsin* and that the actinopterygian intron-less *rho* is the result of a retrotransposition event in the ray-finned fish lineage [97]. Differential expression of visual *opsins* has also been observed in other species. For example in the retinas of the four eyed fish (*Anableps anableps*) and the

onesided livebearer (*Jenynsia onca*) possibly accounting for different specialisations of the different parts of the retina [98]. Different temporal expression has been observed for *rho* genes in the European eel (*Anguilla anguilla*), which changes expression between two different genes between different life stages, literally exchanging the spectral sensitivity of their rods [99].

In addition to its numerous types of cones, the zebrafish has four subtypes of horizontal cells: H1, H2, H3 and rod horizontal cells. They mostly connect to specific subtypes of photoreceptor cells: H1 to LWS, RH2 and SWS2-expressing cones; H2 to RH2, SWS2 and SWS1-expressing cones; H3 to SWS2 and SWS1-expressing cones; and rod horizontal cells to rods [100]. There are also 18 bipolar cell subtypes in the zebrafish retina, nine of which comprise 96% of the total bipolar population and none connecting exclusively to rods [101].

Circadian changes in the retina

The day and night cycle of light and darkness affects photoreceptor cells of vertebrates to a large extent, since they are depolarized in darkness and hyperpolarized in light and thus consume more energy during darkness [102]. In order to adjust to the light-dark cycle most of the cell types present in the retina have an endogenous circadian clock, with some differences between vertebrate groups. This cyclic expression controls the rhythmicity of different processes through a transcription-translation-feedback-loop of clock genes, much like the central timekeeper of the SCN. The clock is entrained by light input from all types of photoreceptor cells, including rods, cones and intrinsically photosensitive retinal ganglion cells expressing *melanopsin*. These photoreceptors send input (rods and cones through bipolar cells) to dopaminergic amacrine cells in the IPL. The amacrine cells then propagate the signal to the horizontal and photoreceptor cells, providing feedback and light entrainment. Thus the clock of rods and cones seem to be entrained both indirectly by themselves, but also by indirect light input from retinal ganglion cells. The major output of the central timekeeper in the brain is the release of melatonin from the pineal gland during the dark period. In the retina, rods and cones are the major source of melatonin (for review see [103]). The circadian clocks of rods and cones are entrained by a light-dark cycle of dopamine and melatonin release. Since dopamine is released during the day [104], it relays the major light signal, while melatonin is released during the night and acts as a night signal, in turn modulating dopamine release [105].

Studies have shown temporal differences in the expression levels of genes involved in the phototransduction cascade. The expression of the red sensitive *opsin* (LWS) in chicken (*Gallus gallus*) has been shown to have a circadian rhythmicity with the highest levels of expression in the late afternoon [106]. Similarly, Li *et al.,* (2005) showed that the zebrafish (*Danio rerio*)

retina is most sensitive to red light in the late afternoon and the least sensitive in the early morning. When they compared the mRNA levels for LWS opsin during different time points of the day they observed high expression in the late afternoon and low expression in the early morning, probably regulated by dopamine [107]. Analysis of the expression variation of red, green and blue *opsins* in the cichlid *Astatotilapia burtoni* (peviously *Haplochromis burtoni*) showed a similar variation over 24h as the zebrafish red opsin [108]. Both of these studies indicate that light is a strong regulator of expression of the visual *opsin* genes in these species [107,108]. Measurements of *rhodopsin* expression in the same cichlid species as well as the cane toad (*Bufo marinus*) showed high, almost constant, expression during the day and reduced expression during the night [109]. It is worth noting that the RNA probe they used was made from bovine (*Bos* sp.) *rhodopsin*, which may have resulted in the detection of other *opsins*.

Other circadian changes can also be observed in the zebrafish retina. The inner segments of cones elongate and the inner segments of rods retract during the night (Figure 5) and the pigment granules of the PE disperse during the day and aggregate during the night [110,111]. These changes probably increase visual acuity during the daylight hours and reduce the bleaching of rod photoreceptors [111].

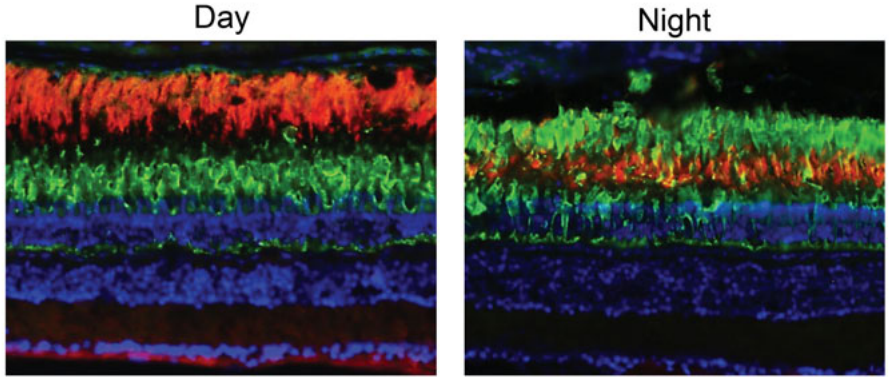

Figure 5. Structural differences between day and night in the retina of adult zebrafish, showing retinomotor movements of rods and cones. During the day the rod outer segments are located within the pigmentary epithelium due to an extension of their inner segments and they are retracted during the night. Cones, in this case double cones, extend their inner segments during night and retract them during the day. Rod OS are visualised using an anti-body against Gβ1 and a secondary antibody coupled with Alexa 555 (red). Double cones are visualised using an antibody against *zpr1* and a secondary antibody coupled with Alexa 488 (green). Nuclei are stained using DAPI (blue).

In addition to the above-mentioned differences between day and night in gene expression and cell shape, there are circadian changes in the structure of the outer segments (OS) of rods and cones. During the daylight hours when rods are extended they shed OS discs to the PE, while cones shed during the night when they are at their longest. These are the respective times when both cell types have most contact with the PE, which phagocytise the shed OS discs [112].

Disassembly, both complete and partial, of synaptic ribbons of photoreceptor cells and some bipolar cells has been observed in teleost fish during the night [113–116]. Opposite changes have been observed in the pineal of several vertebrates (for review of circadian changes of synaptic ribbons see [117]). These changes in the synaptic ribbons of the teleost retina might reduce the metabolic energy expenditure during night by reducing the efficiency of released neurotransmitter vesicles [115]. In addition to the disassembly of synaptic ribbons, Emran *et al.*, (2010) showed that the function of OS in zebrafish larvae is reduced at night and that retinal signalling in response to light is almost absent. This in combination with behavioural experiments, made them conclude that zebrafish larvae essentially shut their retinas down at night, thus saving energy [116]. Adult zebrafish have also been shown to have circadian changes in sensitivity, being most sensitive to visual stimuli in the late afternoon and the least sensitive in the early morning [118].

Aims

The overall aim of this thesis is to investigate gene families involved in the initiation of the phototransduction cascade in vertebrate rods and cones: their evolution in relation to vertebrate whole genome duplications as well as their expression profiles and possible sub-/neo-functionalisation in the zebrafish (*Danio rerio*) retina.

Specific aims of the different papers:

- Paper I. To investigate the evolution of the visual *opsins* and their chromosomal regions, which are shared with the α subunit genes of *transducin*.

- Paper II. To investigate the evolution of the *transducin* α, β and γ subunit gene families and their respective chromosomal regions.

- Paper III. To investigate the expression patterns and expression levels of the zebrafish *transducin* subunit genes.

- Paper IV. To investigate the evolution of phosphodiesterase 6 subunit gene families and their respective chromosomal regions, and the expression patterns and levels of the zebrafish PDE6 subunit genes.

Materials and methods

Bioinformatic analyses

Sequence identification and amino acid sequence alignments

Amino acid sequences of the *opsin*, *transducin* and phosphodiesterase 6 gene families were downloaded from the Ensembl genome browser for a representative set of vertebrates and invertebrates (see papers for details). Missing or incomplete sequences were sought in the genome assemblies using BLAST [119] searches. Sequences were aligned using Jalview 2.8 [120] and its ClustalW implementation (Paper I and II) or Seaview 4.5.3 [121] and ClustalO (Paper IV). Sequence stretches that were poorly aligned were manually inspected and if needed manually curated. Missing or faulty sequence stretches were curated manually by inspecting the respective genomic sequences in order to correct faulty or truncated gene predictions

Phylogenetic analyses

Amino acid sequence alignments of the included gene families were used to constructing phylogenetic trees with the neighbour-joining method (NJ) in ClustalX 2.1 [122] with standard settings and 1000 bootstrap iterations as well as the phylogenetic maximum likelihood (PhyML) method in PhyML 3.0 [123], using the web server (http://atgc.lirmm.fr/phyml/) or through Seaview 4.5.3. The most suitable amino acid substitution model for the PhyML analyses was determined through ProtTest 3.2 [124] based on each alignment.

Analysis of conserved synteny

In theory, genes that were co-localized in an ancestral chromosome region before whole genome duplications will form gene families with members co-localized across the duplicated chromosome regions, creating a pattern of conserved synteny. Thus, by analysing the phylogenies of these neighbouring gene families, it is possible to examine whether the chromosomal regions show signs of large-scale duplications in the time windows of 1R, 2R and/or 3R. In this way, the chromosomal regions of the visual *opsin*, *transducin* subunit and phosphodiesterase 6 (PDE6) subunit gene families were investi-

gated in several species. For each of these gene families, lists of genes located in the vicinity of each gene family member were collected from Ensembl.org and compared between each other. Gene families with members on at least two of the investigated chromosome regions were selected, and the amino acid sequences of their members were used to create alignments and phylogenetic trees as described above. The species chosen to select the neighbouring gene families varies between papers, based on the number of gene family members and their chromosomal locations. In Paper I and II the human and zebrafish (*Danio rerio*) genomes were used. In Paper II the chicken (*Gallus gallus*) genome was also used. In Paper IV only the human genome was used. Neighbouring gene families were defined according to their respective Ensembl protein family predictions.

One of the PDE6 subunit families, the gamma subunit genes, are located in the known paralogous chromosome regions of the *SSTR2, -3* and *-5* genes [41]. However, some of the PDE6γ genes did not conform to these chromosome regions or were located on small unmapped scaffolds. To confirm the orthology and paralogy relationships of these genes, regions 100kb upstream and downstream of the spotted gar PDE6γ genes were investigated. To do this, a list of all genes within these regions, as well as their predicted orthologs in a selection of vertebrate species, were retrieved from Ensembl.

Analyses of gene expression

In situ hybridisation

All experiments using animals were conducted according to regulations and were approved by Uppsala Ethical Committee on Animal Experiments (Uppsala djurförsöksetiska nämnd); permit numbers C33/10, C294/12 and C315/12.

Adult AB strain and transgenic Tg(*gnat2*:EGFP) zebrafish (*Danio rerio*) were kept in a 14/10 hour light/dark cycle (lights on at 08:00 and off at 22:00). Tg(*gnat2*:EGFP) zebrafish express EGFP in cones, driven by the cone specific *gnat2* promotor [125]. Fish were anesthetized using Tricaine (0.04% in system water) and immediately decapitated at different time points during the day. The heads were fixed in 4% paraformaldehyde in phosphate buffered saline for seven hours. Following fixation they were sectioned in 18-20μm thick transversal sections in a cryostat. To detect the expression of *transducin* and PDE6γ genes in the adult zebrafish retina, *in situ* hybridisation (ISH), using specific ribo-probes were performed according to Hauptmann and Gerster (2000) [126] with minor modifications.

The probes were designed to target the 3′ untranslated regions (3′UTR) of the genes, when possible. This was done to reduce cross hybridisation due to high sequence similarity in the coding regions of these genes. In addition to

experiments using adult zebrafish, embryo and larval AB strain zebrafish were collected during six consecutive days post fertilisation and used for whole mount *in situ* hybridisation (WISH) experiments.

Immunohistochemistry

In the ISH experiments performed on Tg(*gnat2*:EGFP) zebrafish (*Danio rerio*), the fluorescence in cones was enhanced using a mouse anti-GFP primary antibody (1:400) and an Alexa 488 coupled donkey anti-mouse secondary antibody (1:1000).

Immunohistochemical experiments were also performed to see the differences between day and night in the adult zebrafish retina. Rod outer segments were visualised using a primary rabbit anti-GNB1 antibody (1:500) and a secondary donkey anti-rabbit antibody coupled with Alexa 555 (1:1000). Double cones were visualised using a primary mouse anti-Zpr1 (1:400) and a secondary donkey anti-mouse antibody coupled with Alexa 488 (1:1000).

Quantitative polymerase chain reaction RT-qPCR

Zebrafish (*Danio rerio*) heads were collected at seven different time points during a 24-hour period (Zeitgeber time, ZT): 08:00 (ZT0), 12:00 (ZT4), 16:00 (ZT8) or 17:00 (ZT9), 20:00 (ZT12), 00:00 (ZT16), 04:00 (ZT20) and placed in RNA*later* until RNA extraction and precipitation for increased purity. Total RNA was extracted from eyes pooled per individual and precipitated over night using sodium acetate and ethanol. The extracted RNA was treated with DNase I and used for cDNA synthesis. cDNA was synthesised in both reverse transcriptase (RT) and non-RT reactions, and diluted to the equivalent of 10 ng/µl. In Paper III, cDNA from all time points was used except for 17:00 and in Paper IV, cDNA from all time points except 16:00 was used. 1 µl cDNA was used per reaction for qPCR experiments. In addition no template controls (NTCs) were performed for each primer pair and run as well as non-RT controls (NRTs) for each primer pair and each sample. qPCR primer pairs were designed using primer BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to span an exon-exon junction or to be located in different exons, to reduce possible amplification of residual genomic DNA.

The data obtained from qPCR experiments were exported and analysed using LinRegPCR 2014.6 [127] and StatPlus:mac v5 or GraphPad Prism version 5.00. Statistics were calculated using two-way ANOVA with Tukey HSD post hoc test for Paper III and one-way ANOVA with Tukey's post hoc test for Paper IV.

Results

Paper I

The five known visual opsin subtype genes [68,69,128] were identified in a variety of vertebrate genomes and phylogenetic analyses of the vertebrate visual *opsins* were performed. In our analyses, the previously cloned pouched lamprey (*Geotria australis*) visual *opsin* genes clustered within each vertebrate *opsin* subtype, LWS, SWS1, SWS2, RH1 and RH2 respectively, as observed by other investigators [128]. In the coelacanth (*Latimeria chalumnae*) genome we could identify three full-length and one fragmented visual *opsin* gene sequences. The full-length sequences cluster among the RH1, RH2 and SWS2 clades respectively. The RH1 and RH2 sequences have been identified previously [129]. The fragmented sequence appears to be a pseudo-gene sharing similarities to the SWS1 subtype and was previously reported as such based on two sequenced clones [129]. However, we identified that one of these clones included the coelacanth SWS2 gene sequence. In the spotted gar (*Lepisosteus oculatus*) genome assembly we identified seven visual *opsin* genes: one LWS, SWS2 and RH2 gene each and two SWS1 and RH1 genes. Of these only the intron-less RH1 gene has been identified previously in a gar species [97]. The two SWS1 genes in spotted gar appear to be the result of a local duplication. The two RH1 genes appear to be the *rhodopsin* and *exo-rhodopsin* genes that have previously been described in teleost fish [96] based on the lack of introns in the first and the presence of introns in the latter. This is the first time the full repertoire of visual *opsin* genes has been reported for these two species, which occupy crucial positions in the evolutionary tree of vertebrates.

We also expanded upon the initial synteny analyses by Nordström *et al.* (2004) and Larhammar *et al.* (2009) by analysing large chromosomal blocks in several vertebrate species containing the visual *opsin*-, *transducin* alpha subunit-, oxytocin/vasopressin receptor- and L-type voltage-gated Ca^{2+} channel genes. We analysed the phylogenies (both NJ and PhyML) of 34 neighbouring gene families out of which 23 support duplication in the same time-window as the 1R and 2R events. Some families also support duplication in 3R. Seven of the identified families did not have any annotated invertebrate orthologs, making relative dating of the duplications difficult. However, the vertebrate repertoires of these families are consistent with expansion in 1R and 2R. We also identified the corresponding linkage group re-

gions in the spotted gar genome. This analysis revealed high conservation of synteny between the spotted gar and the tetrapod genomes, but also major rearrangements in the teleost genomes after 3R, consistent with a global analysis of the spotted gar genome [25], as well as other studies analysing teleost genomes [28,41,45,55].

Paper II

In paper II the phylogenies and chromosomal locations of the vertebrate *transducin* subunit genes were analysed. We analysed the phylogeny of the GNAT gene family as well as the closely located (the same chromosomes) and closely related GNAI gene family. In no *GNAT3* gene in teleosts or amphibians were identified, but we did identify one *GNAT3* gene in the coelacanth genome. This would indicate that this gene emerged through gene duplication in the lobe-finned fish lineage. However, in studying the genomic locations and the phylogeny of the neighbouring GNAI gene family we concluded that all three family members of both the GNAT and the GNAI gene families have their origin in 1R and 2R, but that teleosts and amphibians have experienced independent secondary losses of the *GNAT3* gene. The most likely scenario for the evolution of the GNAT and GNAI genes involves an ancestral local duplication resulting in a GNAT-GNAI gene pair which was later duplicated in 1R and 2R, followed by the loss of one gene in each family. This is in line with previous data [71,72,76] and the results presented in Paper I. We did not identify any 3R duplicates of the GNAT family genes in the teleost genomes investigated.

The human genome contains five GNB genes, *GNB1, GNB2, GNB3, GNB4* and *GNB5*. Our initial phylogenetic analyses indicated a pre-chordate origin for the ancestor of *GNB5*. Thus, we focused on the phylogeny of the vertebrate subfamily containing the *GNB1-4* genes. We found that they form four well-supported clusters with basally diverging tunicate (*Ciona savignyi*) and amphioxus (*Branchiostoma floridae*) orthologs in the PhyML analysis but that the tunicate ortholog clusters basal to the vertebrate *GNB3* sequences in the NJ analysis. To resolve this, we performed a detailed analysis of the genomic regions. We identified 11 neighbouring gene families (for details see Paper II) out of which eight were chosen for further phylogenetic analysis. These analyses show that the *GNB1-4* genes are located on four paralogous chromosomal regions that most likely originated in 1R and 2R. This suggests that the *GNB1-4* genes arose through these chromosome duplications. We identified 3R duplicates for *GNB1*, *GNB3* and *GNB4* in the investigated teleost genomes.

Finally the GNGT genes form a small vertebrate subfamily consisting of *GNGT1, GNGT2* and *GNG11*. *GNG11* is a mammal-specific local duplicate of *GNGT1*, which is not expressed in the retina [130]. These genes are locat-

ed in the well-studied *Hox* cluster paralogous chromosome regions, strongly indicating that the GNGT genes also originated through duplication in 1R and 2R. Zebrafish (*Danio rerio*) has two *GNGT2* genes, *gngt2a* and *gngt2b*, that probably are the result of duplication in 3R based on their locations in the *Hox* paralogon.

Paper III

In paper III we used RT-qPCR and ribo-probes for *in situ* hybridisation (ISH) experiments targeting the 3′UTR of the zebrafish (*Danio rerio*) *transducin* subunit genes. The probes were placed in 3'UTR in order to obtain specificity for each gene within the respective subfamilies. By performing ISH on adult zebrafish retinae we identified a conserved expression pattern between tetrapods and zebrafish for the different *transducin* orthologs. The *gnat1* and *gnat2* genes show clear expression in rods and cones, respectively as previously described by Brockerhoff *et al*. (2003) [131]. Both *gnat1* and *gnat2* could be observed in the adult pineal complex. During embryonic development we observed staining in the retina for both genes around 48 hours post fertilisation (hpf) and earlier expression, around 26 hpf, in the pineal complex.

The *gnb1a* and *gnb1b* genes showed similar expression to *gnat1* in the same rod cells throughout the adult retinae, as demonstrated by double ISH. Initial ISH experiments suggested lower expression of *gnb1b* than *gnb1a*. This was confirmed using RT-qPCR on several time points during the circadian cycle. Additionally, while *gnb1b* stayed at a constant level of expression during 24 hours, *gnb1a* varied and had peak expression during late afternoon and in during the night. In the adult pineal complex only expression of *gnb1a* was observed. In embryos the expression of *gnb1b* started around 48 hpf in the ventral retina and for *gnb1a* 52 hpf. Wider expression throughout the body was observed for both genes as previously been described by Xu *et al.* (2012) [132]. At around 26 hpf expression of the *gnb1a* gene, but not *gnb1b*, was observed in the pineal complex.

In contrast to *gnb1a* and *gnb1b*, the *gnb3a* and *gnb3b* genes show expression in different regions of the retina. Expression of *gnb3a* could not be detected in the adult retina using ISH. However, using RT-qPCR we detected low expression of *gnb3a* in adult eyes. The *gnb3b* gene was clearly detected by ISH in the cones of the medial and dorsal adult retina, but not in the cones of the ventral retina. In the adult pineal complex no expression of *gnb3a* or *gnb3b* was observed. When investigating the embryonic expression, both *gnb3a* and *gnb3b* started expression in the ventral retina around 48 hpf. The expression of *gnb3a* and *gnb3b* in the pineal complex starts around 26 hpf, and at 48 hpf no expression of *gnb3b* could be detected in this organ. As for *gnb1a* and *gnb1b*, wider expression throughout the body was detected.

The *gngt1* gene was detected in all rod cells of the adult retina and in the pineal complex, as described for *gnat1*. Expression started around 48 hpf in the ventral retina of embryos and around 26 hpf in the pineal complex.

Finally, the *gngt2a* and *gngt2b* genes, like their tetrapod ortholog *GNGT2*, were expressed in cones in the adult retina. However, similar to *gnb3a* and *gnb3b* their expression was divided into different parts of the retina, i.e., spatial subfunctionalisation: the *gngt2a* gene was expressed in the cones of the ventral retina while *gngt2b* was expressed in the cones of the dorsal and medial retina. Expression of *gngt2a* but not *gngt2b* was detected in the adult pineal complex. During development, expression started around 48 hpf in the ventral retina for both *gngt2a* and *gngt2b.* In the pineal complex expression was found to start at 26 hpf for *gngt2a*. The expression of *gngt2b* is transient and was only present between 36 hpf and 3 dpf. Similar to the *gnb1a* and *gnb1b* genes, *gngt2a* showed wide expression throughout the body including the eye and pineal complex.

Paper IV

In Paper IV we used phylogenetic and comparative synteny analyses of the PDE6 catalytic and inhibitory subunit gene families in order to resolve their evolutionary history. Our phylogenetic analysis of the catalytic subunit genes is consistent with duplications in 1R and 2R followed by the loss of one gene, resulting in the three genes we see in extant gnathostomes. This scenario is consistent with previous analyses [71,81]. When analysing the chromosomal locations of the PDE6 genes we noted that they are located in the same paralogon as the NPY receptor genes [35] described previously [72]. To confirm this we identified 11 neighbouring gene families out of which eight were chosen for further analyses (three were excluded due to complex topologies or a multitude of members). Even though four of the identified families lack putative invertebrate orthologs, which prevents relative dating of the duplications, their chromosomal locations and species representation indicates an expansion in 1R and 2R. Three of the families had clear topologies in either NJ or PhyML or both, indicating an expansion in 1R and 2R. Taken together, these data and previous analyses suggest that the PDE6 catalytic subunit gene family expanded in 1R and 2R.

The PDE6 inhibitory subunit gene family has two members in amniotes, *PDE6G* and *PDE6H*, which are expressed in rods and cones respectively. Previous studies have not been able to identify any related genes in invertebrate genomes, but the sea lamprey (*Petromyzon marinus*) has two homologous genes [81]. We performed searches in a broad range of vertebrate and invertebrate genomes and confirmed the absence of PDE6γ in the available invertebrate genome sequences.

When searching the genome sequence of the spotted gar (*Lepisosteus oculatus*), coelacanth (*Latimeria chalumnae*) and Western clawed frog (*Xenopus (Silurana) tropicalis*), we identified additional duplicates relative to the two amniote genes. In teleost genomes even more genes were identified. The zebrafish for example, has five PDE6γ genes. Due to their short sequences and high sequence conservation, phylogenetic analyses of these genes are difficult. Therefore we analysed their genomic locations. Initial analyses revealed that they are located in the same chromosomal regions as the well-studied somatostatin 2-, 3- and 5 receptor genes (*SSTR2*, *SSTR3* and *SSTR5*). These genes and numerous neighbouring gene families have been shown to have expanded in 1R and 2R [41]. This indicates a similar evolutionary history for the PDE6γ genes. The spotted gar PDE6γ genes are located on linkage groups (LG) 10, 12 and 13, which carry *SSTR2*, *SSTR3* and *SSTR5* respectively. In chicken *PDE6G* is located close to *SSTR2* on chromosome 18 and *PDE6H* is located close to *SSTR3* on chromosome 1. The same is true for human *PDE6G*, but the *PDE6H* gene is translocated to a chromosome not carrying an SSTR gene, chromosome 12. This suggests that the additional PDE6γ gene located next to *SSTR5* on LG13 in the spotted gar genome, is a novel paralog of *PDE6G* and *PDE6H*. We suggest naming this novel gene *PDE6I*. When comparing the pattern of synteny between the spotted gar genes and those in coelacanth and Western clawed frog we were able to identify putative *PDE6G* and *PDE6I* genes in both genomes. In teleost genomes we identified one or more *PDE6G* and two or more *PDE6H* genes, depending on the species, and one *PDE6I* in the zebrafish (*Danio rerio* and Mexican cave tetra (*Astyanax mexicanus*). The teleost specific duplications are varied. The duplicate *PDE6G* genes are most likely the result of 3R and the same goes for some of the extra *PDE6H* genes, but this gene has also experienced additional local duplications in some teleost fish species. For the five identified zebrafish genes we propose the names *pde6ga*, *pde6gb*, *pde6ha*, *pde6hb* and *pde6i*.

When analysing the expression of these genes over the circadian cycle we saw a clear increase of *pde6ha* during the day, while *pde6ga* expression decreased. The *pde6hb* gene has a small peak of expression in the morning while *pde6gb* has minor peak of expression at night. Similarly to *pde6hb*, the cone specific catalytic subunit gene, *pde6c*, shows a small peak of expression in the morning. No major changes in expression were observed for the *pde6a* and *pde6b* genes.

Upon investigating the expression pattern of the zebrafish PDE6γ genes, we saw conserved expression of these genes in the same photoreceptor cells as their tetrapod orthologs. The *pde6ga* and *pde6gb* genes are both expressed in rod photoreceptor cells throughout the retina, while the *pde6ha* and *pde6hb* genes are expressed in cones. However, due to the low expression level of *pde6i* it could not be analysed further.

When investigating the expression of these genes in embryos we found that all but *pde6ha* start expression in the ventral retina around 48-72 hpf and around 24 hpf in the pineal complex. The expression of *pde6ha* started in both the retina and pineal complex around 48 hpf.

Discussion

Evolution of the vertebrate phototransduction cascade components (Papers I, II and IV)

The analyses in Papers I, II and IV build upon observations made previously in our research group [71,72] and expand these with detailed analyses of a wide selection of vertebrate species and a large number of neighbouring gene families. Here we present extensive evidence that the visual *opsin* genes, *transducin* subunit genes and the PDE6 subunit genes expanded concomitantly in the early vertebrate whole genome duplications 1R and 2R, and some also in the teleost-specific whole genome duplication 3R.

Previous phylogenetic analyses of the visual *opsins* displayed a stepwise duplication pattern [68,128] rather than the double bifurcating topology that one would expect from a gene family that was duplicated in two whole genome duplications (Figure 3). However, analyses of conserved synteny in the human genome suggested that the visual *opsins* are located in four related chromosomal regions [71,72] often referred to as a paralogon [133]. To resolve this issue we analysed the genomes of different vertebrates and compared the paralogous regions containing the visual *opsins*. Our analyses reported in Paper I support an expansion as proposed by Larhammar *et al.,* (2009) with two ancestral *opsin* genes located on the same chromosome, one short-wavelength receptor gene (proto-SWS) and one long-wavelength receptor gene (proto-LWS). This ancestral pair was duplicated in 1R and 2R to form two subfamilies with four members each. The SWS family consisted of the ancestors of SWS1, SWS2, RH1 and RH2. The LWS ancestor lost all duplicates except one. This scenario is supported by the presence of four paralogous regions in both lobe-finned fish (human and chicken) and a ray-finned fish (spotted gar). Synteny between the spotted gar (*Lepisosteus oculatus*), human and chicken (*Gallus gallus*) genomic regions housing the visual *opsin* genes is well conserved. This is consistent with more global analyses of the spotted gar genome showing high conservation of synteny with tetrapods [25]. We observed major rearrangements in the zebrafish (*Danio rerio*) genomic regions housing the visual *opsin* genes, which is in line with previous studies of chromosomal regions in the teleost lineage after 3R [28,41,55]. These rearrangements have made previous analyses of conserved synteny between lobe-finned fish and teleost fish difficult. The conservation of synteny between human, chicken and spotted gar, representing a more

basal lineage of ray-finned fish that has not undergone 3R, provides compelling evidence for the expansion of the visual *opsins* and these genomic regions in 1R and 2R.

The data presented for the *transducin* alpha subunit gene family in Paper II were performed before Paper I and received additional support by the more extensive analysis of their chromosomal regions in Paper I. This scenario, with an ancestral pair of proto-GNAT and proto-GNAI located on the same chromosome that later expanded in 1R and 2R is also consistent with previous analyses [71,76]. Paper II also describes analyses of the beta subunit genes *GNB1-4* genes as well as the gamma subunit genes *GNGT1* and *GNGT2* reports that they too were duplicated 1R and 2R as part of two separate paralogons. In addition the *transducin* beta and gamma subunit genes were further duplicated in 3R. The two PDE6 subunit gene families were both duplicated in 1R and 2R and the PDE6γ gene family was duplicated further in 3R (Paper IV). When analysing the PDE6γ genes of non-amniote vertebrates we identified a novel PDE6γ gene, which after paralogon analyses appears to be a 2R paralog of the rod-specific *PDE6G* and cone-specific *PDE6H* genes. Data from both lobe-finned and ray-finned fish supports this conclusion. We suggest naming this gene *PDE6I* to be consistent with the current naming of the inhibitory subunit genes. It is possible that this gene is present in more teleost species since we identified additional PDE6γ genes, albeit their true orthology identity could not definitely be assigned. The function of the newly discovered *PDE6I* gamma subunit gene is still unknown, and further investigations would be interesting to pursue.

Failure to identify a homolog to the PDE6γ subunit genes in invertebrate genomes is consistent with previous studies [81], even though more genome assemblies of invertebrate species are available now than previously. Both Hidden Markov Model-based searches (HMMER) and BLAST searches were performed tested without success. Thus, it seems as if the ancestral PDE6γ subunit gene arose in the common ancestor of vertebrates and was later duplicated in 2R resulting in the *PDE6G, PDE6H* and *PDE6I* genes. The sudden emergence of an inhibitory subunit for PDE6 in vertebrates has been suggested to have facilitated a higher catalytic activity needed for rapid visual responses by being able to rapidly inhibit activity in the absence of light [79].

No clear 3R duplicates for the visual *opsins*, *transducin* alpha or the PDE6 catalytic subunit genes could be identified. Nevertheless, the visual *opsin* genes are known for their many local duplications in different vertebrate lineages. There are local duplicates for most of the visual opsin genes in teleosts [95], and in humans the medium wavelength sensitive green *opsin* gene emerged as a local duplicate of the red *opsin* gene [134]. In some species of teleosts we were able to identify local duplicates of the *PDE6H* genes, although not in zebrafish. We could also identify 3R duplicates for the PDE6 inhibitory subunit *PDE6G* and *PDE6H* genes. For the *transducin*

beta subunit genes we identified 3R duplicates of the rod specific *GNB1* gene and the cone specific *GNB3* gene. For the gamma subunits of *transducin* we identified 3R duplicates of the cone specific *GNGT2* gene. This is noteworthy since the 3R event has been proposed to have driven the early expansion and diversification of teleost fishes [54,135]. Our results indicate that this important event in the evolution of teleosts also contributed to the diversification of phototransduction systems and visual specialisations in this lineage.

Subfunctionalisation of 3R duplicates (Papers III and IV)

Expression in the adult retina

In zebrafish (*Danio rerio*) we were able to see different types of specialisation for the different gene duplicates generated in 3R. We demonstrate that the two rod-specific *transducin* beta subunit genes, *gnb1a* and *gnb1b*, co-localise in rods. The cone specific *gnb3a* and *gnb3b* genes show signs of spatial subfunctionalisation by expression of *gnb3b* in the dorso-medial retina but not in the ventral retina. The expression of *gnb3a* was too low to be detected by ISH. We propose that this lower *gnb3a* expression may have a protective function in the ventral retina by lowering light sensitivity. Differences between the dorso-medial and ventral retina have been observed in photoreceptor number, outer segment (OS) length and pigment epithelium thickness [136]. The ventral retina has also been found to be more resistant to light induced damage than the dorsal and medial retina [137].

For *GNGT2*, the differential expression patterns between paralogs in the zebrafish retina also indicate spatial subfunctionalisation where one paralog, *gngt2a* is expressed in the ventral retina and the other *gngt2b* is expressed in the dorso-medial retina. This is probably due to subfunctionalisation through differential loss of regulatory elements between the duplicates [59]. However, to fully understand the mechanism and what regulatory elements this concerns, more extensive analyses are needed. Similarly to the *gngt2a* gene expression in the ventral retina other research groups have shown expression of some visual *opsin* genes in zebrafish, including the LWS opsin *lws-1* and the RH2 opsin *rh2-4* [62].

Expression analyses of four of the zebrafish PDE6γ subunit genes using ISH in adult retina showed that the *pde6ha* and *pde6hb* genes are expressed in cones and the *pde6ga* and *pde6gb* genes are expressed in rods. The *pde6i* gene could not be amplified properly from eye cDNA, which indicates low expression or expression elsewhere. The *pde6ga* and *pde6gb* genes seem to be co-expressed in the same rods, and the *pde6ha* and *pde6hb* genes in the same cones throughout the retina.

After analysing the expression of the PDE6γ and *transducin* subunit genes in the adult pineal complex and during development, we observed expression of all except *gnb1b* at some time point, indicating further subfunctionalisations when it comes to expression patterns in the eye and pineal between different 3R duplicates. Of the investigated genes, *gnat1*, *gnat2* *gnb3b*, *gngt1*, *gngt2a*, *pde6a*, *pde6c* and *pde6gb* have previously been shown to be expressed in the pineal complex of zebrafish [138,139]. This, in combination with the data presented here for the *gnb1a*, *gnb3b*, *gngt2b*, *pde6ha*, *pde6hb* and *pde6ga* genes suggests that many of the components of the visual phototransduction cascade are also used for photoreception in the zebrafish pineal complex at some point during development or in the adult. When performing WISH analyses in embryos we observe an earlier expression of these genes in the pineal complex than in the eyes. This agrees with studies of *opsin* expression in the sea lamprey (*Petromyzon marinus*) [140], green and UV *opsin* in the Atlantic halibut (*Hippoglossus hippoglossus*) [141,142] and red *opsin* in zebrafish [143]. In Atlantic halibut this earlier expression has been proposed to be involved in hatching [141].

Rhythmic changes of expression levels

Like previous studies have reported [107,110,111] we were able to observe retinomotor movements in the adult zebrafish (*Danio rerio*) retina by comparing different time points during the 24 hour period, as well as changes of expression levels for some of the zebrafish *transducin* and PDE6 subunit genes. The rod-specific *gnb1a* and *gnb1b* genes show different expression during the circadian cycle: *gnb1a* fluctuates quantitatively in a rhythmical fashion, with peaks of expression in the afternoon and during the night, whereas *gnb1b* remains constant. The functional consequence could be a dosage effect, as the two proteins have high amino acid sequence identity (they differ by only 3 amino acid residues), indicating that their interactions with other proteins are likely to be identical. This observation provides evidence that the 3R duplicates of *gnb1* have undergone temporal subfunctionalisation. Similarly, for the PDE6γ genes, some of the 3R duplicates vary significantly in expression between day and night, *pde6ha* but not *pde6hb* and *pde6ga* but not *pde6gb*. The *pde6ha* gene shows high daytime expression while *pde6ga* shows low daytime expression. We propose that the increased levels of *pde6ha* during the day helps prevent cones from bleaching by reducing their light sensitivity. This would be accomplished by the gamma subunits' inhibition of the PDE6 catalytic subunits. Rods on the other hand are bleached during the day and a reduced expression might save metabolic costs. The observed changes in expression of the PDE6 subunit genes could also be similar to the changes in expression observed for visual *opsins* that has been attributed to replenishment of proteins to prepare for cone OS shedding during the night in chicken (*Gallus gallus*) [106] or peaks in visual

sensitivity in zebrafish [107]. However, this does not explain why some of the investigated genes do not show any clear changes in expression.

Comparisons with the spotted gar

The differential expression patterns and/or expression levels observed for the 3R duplicates of the *transducin* and PDE6 subunit genes are most likely due to changes in regulatory elements [59]. The divergence of expression between gene duplicates has been previously described for many genes in zebrafish (*Danio rerio*), for example the arrestin genes *arr3a* and *arr3b* [60]. The optimal way to study what happens with gene duplicates after 3R is to study how the non-duplicated genes are expressed in for example the spotted gar (*Lepisosteus oculatus*), a holostean ray-finned fish, compared to the teleost paralogs. There are not yet many studies that have carried out such comparisons, but an investigation of the Paired-related homeobox (*Prrx*) genes of the spotted gar has been reported [144]. No clear subfunctionalisation could be seen for the teleost genes with regard to expression patterns and the authors concluded that the data showed neither sub- or neofunctionalisation [144]. However, they did observe differential expression levels with the *prrx1a* gene, always being expressed at a higher level than the *prrx1b* gene in both eel and zebrafish [144]. This is similar to our observations for the duplicated *GNB1*, *PDE6G* and *PDE6H* genes in zebrafish. The most likely cause of expression level differences are changes in the regulation of gene expression, which could be a sign of dosage-related subfuctionalisation. Another possibility would be increased mRNA degradation.

The cyclostome divergence (Paper I)

There is now considerable convincing evidence that the common ancestors of all jawed vertebrates experienced two rounds of whole genome duplication [44,45,56] and that a third round of duplication occurred in a common ancestor of teleost fish [25,28,29,47,48]. However, it is still debated whether cyclostomes diverged before or after the 1R and 2R events. Common among studies of whole genome sequences of lampreys is that the data supports that the cyclostome lineage has at least gone through two rounds of duplication, presumably the same ones as the gnathostomes [9], and possibly a third genome duplication [10]. However, a recent study suggested that also the two first whole genome duplications in the Arctic lamprey (*Lethenteron camtschaticum*) might have occurred independently of those in gnathostomes [145]. However, this study by Nah *et al.* (2014) did not address the difficulty of assigning orthologs using phylogenetic analyses between cyclostomes and gnathostomes [36,57,146,147]. Paper I presents data from the chromosomal regions housing the visual *opsin* genes in selected gnatho-

stome genomes. We concluded that there were two ancestral vertebrate visual *opsin* genes in the same chromosomal region, and that this genomic region was duplicated in 1R and 2R, giving rise to the current gnathostome repertoire of five visual *opsin* subtypes. These same subtypes have been identified in the pouched lamprey (*Geotria australis*), a cyclostome. Thus, the most parsimonious explanation for the origin of the lamprey opsins is through the same sequence of duplication events, i.e., 1R and 2R. Our conclusion is in line with the conclusions made previously by Kuraku *et al.* (2009) whose dataset of several gene families including the visual opsins [57]. The visual *opsins* probably illustrate this shared evolutionary history particularly well because they rapidly found new functions during this time period.

Conclusions

The data presented in this thesis support the previous hypothesis about the origin of rods and cones and their respective phototransduction cascades before the emergence of gnathostomes in the early vertebrate tetraploidisations [2,71,72]. This means that the vertebrate ancestors that lived more than 500 million years ago during the Cambrian period most likely had a single vertebrate phototransduction cascade that drove the photoreception in a single cone-like type of photoreceptor cell, indicated by the existence of two *opsin* subtypes before 1R and before the appearance of *rhodopsin*, and the origin of *rhodopsin* in 2R from a green *opsin* ancestor [68]. The two tetraploidisation events quadroupled also other genes encoding this ancestral signalling cascade. This allowed for different mutations in both coding and non-coding parts of genes, which lead to diverging expression patterns, both temporally and anatomically, as well as diverging protein functions. This resulted in the formation of two different types of photoreceptor cells, proto-cones and proto-rods. A lifestyle in clear surface waters favoured colour vision and the selection of four distinct cone *opsins* with specific spectral sensitivities that are present in all current vertebrates, with a few exceptions due to secondary losses [2,84]. In addition the third tetraploidisation event in the teleost ancestor resulted in an increased gene repertoire in the *transducin* beta and gamma subunit families as well as in the PDE6γ gene family. Our analyses of expression of these extra genes resulting from 3R in zebrafish (*Danio rerio*) discovered strong evidence for further specialisations with different spatial expression or expression levels of the gene duplicates. Furthermore, the observed expression of most visual genes also in the pineal complex suggests that this light-sensitive organ in zebrafish utilises most of the phototransduction cascade components used in rods and cones. Strongly suggesting a shared origin for the eye and the pineal.

Future perspectives

The analyses of expression performed in this thesis studying the variation of gene expression of the *transducin* and PDE6 genes in a normal light-dark cycle indicate that it would be of interest to examine if these changes are of a circadian nature. To do this, gene expression analyses using zebrafish (*Danio rerio*) kept in constant light or constant darkness would have to be performed. If expression level changes persists under these conditions, this may indicate that they are under circadian control.

Work is currently in progress to investigate the evolution of the cyclic nucleotide gated channels (CNGs), which are expressed in rods and cones. These channels close when levels of cGMP decrease in the cell upon PDE6 activation. Thus they are the final step in the activation part of the phototransduction cascade. CNGs have CNGA1 and CNGB1 subunits in rods and CNGA3 and CNGB3 subunits in cones. The CNGA genes form a vertebrate subfamily, which in preliminary analyses appear to have expanded in 1R and 2R. The CNGB family is still inconclusive with regard to the vertebrate whole genome duplications. The phylogeny appears to support this type of duplication, but the genes are not located in a clear paralogon. The zebrafish has putative 3R duplicates of all genes in both of these gene families and we are currently investigating the expression of these genes in the eye and pineal complex.

In addition to the above-mentioned analyses, it would be of interest to investigate the phototransduction cascade gene repertoire of the pouched lamprey (*Geotria australis*), since it lives in brightly lit shallow waters and has retained all five vertebrate visual *opsin* subtypes. It is possible that the pouched lamprey has retained more members of the *transducin* and PDE6 subunit gene families than the more deep-water living sea lamprey (*Petromyzon marinus*) [81,148]. The sea lamprey and the Arctic lamprey (*Lethenteron camtschaticum*), the only lamprey species with sequenced genomes, have been shown to only have two functional visual *opsin* genes, RH1 and LWS [149], and thus also most likely has suffered secondary losses in many of the genes encoding the other proteins of the cascade. By characterising the pouched lamprey genes we might be able to better determine if the cyclostomes diverged before, in-between or after the early vertebrate whole genome duplications. The possible lamprey specific 3R might have, as seen in the teleost fish, contributed to further specialisations in the lamprey retina if they too have extra duplicates of any of the genes.

Svensk sammanfattning (Swedish summary)

Tidigt i ryggradsdjurens evolution, för ca. 500 miljoner år sedan, fördubblades arvsmassan (genomet) två gånger, i händelser som kallas 1R och 2R. Därvid fick många gener extra kopior som bildade familjer av gener. Efter fördubblingarna kunde många genkopior genomgå evolutionär selektion och få nya funktioner, exempelvis genom att uttryckas i nya celltyper eller vid olika tidpunkter. Analyser av flera genfamiljer som används i nervsystemet har visat att dessa fått fler medlemmar i 1R och 2R. Senare i utvecklingslinjen som ledde till de äkta benfiskarna (teleoster), som är strålfeniga fiskar, skedde ytterligare en genomfördubbling kallad 3R, vilket har lett till att många genfamiljer i dessa fiskar har fler medlemmar än i övriga ryggradsdjur. I denna avhandling har jag undersökt hur synsystemet har uppkommit under ryggradsdjurens evolution genom att studera genfamiljer som kodar för proteiner i ögats signalkaskad. Kunskap om detta bidrar till ökad förståelse av hur ögat fungerar.

I ryggradsdjurens näthinna sköter tappar seende i starkt ljus, som dagsljus eller kraftigt månljus, och stavar seende i mycket svagt ljus, som stjärnljus. Dessa två celltyper har en liknande reaktion på ljus, kallad signalkaskad, som börjar vid ljuskänsliga proteiner på cellernas yta som kallas opsiner: *rhodopsin* i stavar och olika färgopsiner i tappar. I opsinet sitter en molekyl A-vitamin som ändrar form när den träffas av en ljuspartikel (foton). Detta gör att opsinet aktiveras. Det aktiverade opsinet aktiverar sedan i sin tur proteinet *transducin* som består av tre subenheter: alfa, beta och gamma. När *transducin* aktiveras separerar alfa-subenheten från beta-gamma. Alfa-subenheten aktiverar därefter enzymet fosfodiesteras, PDE6, genom att ta bort dess två hämmande subenheter från de aktiva subenheterna. PDE6 sänker nivåerna av molekylen cGMP, en så kallad sekundär signalmolekyl i cellen. Detta leder till att kanaler för laddade partiklar (joner) på cellens yta stängs, cellens elektriska egenskaper förändras och den slutar att signalera. I slutändan leder det till signaler till hjärnan som gör att vi kan se.

Proteinerna i denna signalkaskad skiljer mellan tappar och stavar, men de är besläktade med varandra: tappar och stavar har varsin uppsättning. Tidigare analyser i vår forskargrupp har visat att de gener som används i tappar respektive stavar har gemensamma förfäder som troligen fördubblades i 1R och 2R. Denna avhandling beskriver detaljerat evolutionen för genfamiljerna som innefattar opsinerna, *transducin*-subenheterna och PDE6-subenheterna samt uttrycket av generna i näthinnan och tallkottskörteln hos modellorgan-

ismen zebrafisk. I egentliga benfiskar och många andra djur är tallkottskörteln också ett ljuskänsligt organ, nästan som ett ”tredje öga”.

Evolutionen har undersökts med bioinformatiska metoder för att ta reda på släktskap mellan gener från ett representativt urval av ryggradsdjur. Uttrycket av generna har undersökts med laborativa metoder såsom *in situ* hybridisering och kvantitativ Reverse Transkriptas-PCR.

Våra analyser visar att genfamiljerna för opsiner, *transducin* och PDE6 fick nya medlemmar i dessa tidiga genomfördubblingar före ryggradsdjurens uppkomst. Några genfamiljer expanderade ytterligare i 3R hos de äkta benfiskarna. För att ta reda på hur 3R har påverkat ögats evolution i de äkta benfiskarna undersökte vi uttrycket av de duplicerade generna i zebrafiskens näthinna och tallkottskörtel, men även under utvecklingen. Vi fann att vissa av de zebrafiskspecifika generna uppvisar ett annorlunda uttrycksmönster än sina människosläktingar. Hos beta-subenheterna hos *transducin* så såg vi att de duplikat som är uttryckta i stavar finns i samma celler i näthinnan men att de skiljer sig åt i uttrycksnivåer under dygnsrytmen på så sätt att den ena kopian varierar under dygnet medan det andra håller en nästan konstant nivå. För de beta-subenheter som är uttrycka i tappar så fann vi att den ena kopian hade mycket lågt uttryck och att den andra enbart var uttryckt i ryggsidan och ut mot sidan av näthinnan. Liknande observerades för gamma-subenheterna hos *transducin* som är uttryckta i tappar, där de är uttryckta i olika delar av näthinnan. När vi undersökte uttrycket för gamma-subenheterna hos PDE6 så fann vi att de tappspecifika generna båda är uttryckta i tappar i hela näthinnan men att en av dem varierar kraftigt i uttryck, höga nivåer på dagen och låga på natten. De stavspecifika generna är båda uttryckta i stavar i hela näthinnan men likt de tappspecifika generna så varierar en i uttryck mellan dag och natt, med låga nivåer på dagen. Vi fann att alla gener som undersöktes utom en också är uttryckta i tallkottskörteln.

Sammantaget tyder våra data på att de tidiga genomfördubblingarna spelat en avgörande roll i utvecklingen för uppkomsten och evolutionen av tappar och stavar i ryggradsdjur och att 3R bidrog till ytterligare specialiseringar i näthinnan hos äkta benfiskar. Våra analyser tyder också på att tallkottskörtelns ljusreceptor celler till stor del troligen använder samma komponenter i deras signalkaskader i zebrafisk.

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