Keeping up with retinal photoreceptors and horizontal cells

Labelling and mapping of cells in the normal and diseased embryonic chicken retina

MARIA BLIXT
The childhood eye cancer retinoblastoma originates from the retina and its development is initiated while the foetus is in the uterus. Retinoblastoma has a reported incidence of 1 in 15,000 to 18,000 live births, and approximately 90% of all patients are diagnosed before the age of 5. The occurrence of retinoblastoma is usually detected by the parents and the most frequent symptoms are leukocoria (white pupillary reflex), strabismus (squinting) or if the child complains of visual problems. Retinoblastoma is diagnosed by examination under anaesthesia and documentation by RetCam. It is treated with various cytostatic agents, or by laser. If the treatment is unsuccessful, or there is a risk that the tumour cells will spread and form metastases, the eye is removed.

Previous studies have indicated that the cell type from which the tumour arises, the cell-of-origin, may be the cone photoreceptors and/or their immediate interneuron, the horizontal cells. Determining the cell-of-origin for retinoblastoma is an important goal, however, understanding the molecular mechanisms that distinguish the photoreceptors and the horizontal cells from the other retinal cells may prove just as important for understanding this disease.

The aim of my project has been to develop, optimise and validate methods to label, map and target expression to photoreceptors and horizontal cells in the chicken embryonic retina. We have successfully established several methods that test the expression pattern of conserved, regulatory DNA sequences, and have performed short- and long-term expression of various genes that have been reported to be involved in cell cycle regulation and cell fate determination. One of my most important findings was that a region from the \textit{RXR} gene allowed us to specifically target the photoreceptors and horizontal cells. Our previous knowledge, together with the newly established tools, puts us an important step closer towards understanding the development and behaviour of the retinal photoreceptors and horizontal cells, however, further studies are of course needed.

\textbf{Keywords:} Chicken, electroporation, horizontal cells, photoreceptors, retina, piggyBac

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“Our real teacher has been and still is the embryo, who is, incidentally, the only teacher who is always right.”

Viktor Hamburger
List of Papers

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


III **Blixt, M.**, Konjusha, D., Ring, H., Hallböök, F. The zinc finger gene Nolz1 is controlled by retinoic acid and regulates the formation of chicken retinal progenitors and Lim3 expressing bipolar cells. Under review in *Developmental Dynamics*.


*These authors contributed equally to this work

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Preface

Proliferation, differentiation, migration, signalling, and apoptosis; the life of a cell is simple, yet simultaneously complicated. What mechanisms govern all these steps? What happens when these cellular steps deviate from the expected route? And how, if possible, does the cell correct these aberrations?

When a cell is unable to correct or compensate for errors that occur in the cell, diseases like cancer may arise. Retinoblastoma is a rare childhood cancer that originates from the retina. Much effort has been put into finding the type of retinal cell that gives rise to retinoblastoma. According to studies performed in mouse the horizontal cell has been proposed to be the cell-of-origin, whereas human studies indicate that it is the cone photoreceptor cell. We believe that it may be less important to find out what type of cell the tumour originates from, but rather to look at it from a mechanistic perspective. Why are these cells able to continue proliferating and eventually become neoplastic? Previous research performed in our group has added to our understanding of the horizontal cells, however, more studies are needed.

During my PhD I have focused my work on the types of retinal cells proposed to be involved in retinoblastoma formation. My aim has been to establish methods that allow us to gain a better understanding of the origin and developmental behaviour of photoreceptors and horizontal cells in the chicken embryonic retina.

When working with biological systems it is important not to underestimate the time, effort, patience, curiosity, ingenuity, troubleshooting and luck needed in order to accomplish your goals. I now have years of first-hand experience in this area and I believe that all my ups and downs have made me a better scientist.
Introduction

The vertebrate eye

The eye is an extension of the brain that detects light and converts it into electrical signals that are passed on to the visual cortex of the brain (Fig. 1). In the eye, the rays of light first encounter the cornea, a structure that both protects the eye and refracts the incoming light. A portion of the refracted light passes through the pupil, an opening that is surrounded by the coloured iris. The next refractive structure is the biconvex lens. The lens is attached to muscles allowing it to change its shape in order to produce a focused image at the back of the eye. The focused, incoming light passes through the vitreous body and falls on the retina, a thin sheet of neural cells that detect the light, converts it into neural signals that are transmitted to the brain via the optic nerve. At the centre of the human retina, on the temporal side of the optic nerve exit, is a highly pigmented yellow area called the macula. Located in the centre of the macula is a cone rich pit, the fovea, providing sharp central vision. The vitreous body is a clear central structure that supports the shape of the eye together with the distal sclera. Located between the retina and the sclera is the choroid, a vascular layer that together with the central retinal artery supplies the retina with oxygen and nourishment [1].

Figure 1. Schematic presentation of the human eye. Sagittal/horizontal section of the eye.
Development of the vertebrate eye

Embryonic development is initiated by the fusion of two haploid gametes (the sperm and the egg cell), forming a diploid zygote. From the zygote a bilaminar, and then a trilaminar, structure is formed that contains the three germ layers: endoderm, mesoderm and ectoderm. It is from the outer layer, the ectoderm, that the central nervous system eventually forms. Through a process referred to as neural induction, the ectoderm forms a thick structure called the neural plate. The cranial portion of the neural plate develops into the brain, whereas the caudal portion gives rise to the spinal cord. The primordial brain is divided into three brain vesicles that are converted into five secondary brain vesicles. The second of the five brain vesicles, the diencephalon, gives rise to the retina.

A bilateral evagination of the diencephalon leads to the formation of the optic pits. The optic pits enlarge giving rise to the optic vesicles (Fig. 2A). The vesicles are in contact with the surface primordial lens placode which leads to induction of the lens. The close interaction of the two structures results in an invagination of the optic vesicle, forming the bi-layered optic cup. The inner layer of the optic cup will give rise to the retina and the outer layer will give rise to the pigment epithelium (Fig. 2B and C). The developing eye is connected to the brain by the optic stalk which later on forms the optic nerve [2, 3]. Retinal differentiation begins around day 47 of gestation in humans, at embryonic day (E) 12 in mice and at E2 in chicken [3, 4].

The vertebrate eye is comprised of tissues with different embryonic origins. The retina, pigment epithelium, iris and ciliary body are derived from neural tissue, the lens is derived from the lens placode, the cornea is derived from the surface ectoderm and neural crest cells [2, 3], and the vitreous body originates from mesenchymal cells [5].

One group of genes important for the formation of the eye is the eye field transcription factors (EFTFs). The EFTFs are expressed in an anterior region of the neural plate. One of the EFTFs is the paired homeobox gene 6 (Pax6), which has been denoted the master regulator of eye formation. Misexpression of Pax6 induces formation of ectopic eyes [6], whereas Pax6 knock-out prevents the formation of eyes [7]. Conditional inactivation of Pax6 during early retinal development leads to an almost complete loss of the retina [8]. The orthodenticle homeobox 2 gene (Otx2) is another important transcription factor that regulates proper retinal development. Loss-of-function mutations lead to severe ocular malformations [9], whereas homozygous knock-out results in prenatal death of the embryo [10].
The retina

The retina, which is about 0.5 mm thick in humans, consists of both the neural retina and the retinal pigment epithelial (RPE) cells. The neural retina is comprised of five main neural cell types (Fig. 3); rod and cone photoreceptors, horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells, and the Müller glia cells [11-13].

The mature neural retina consists of three distinct histological cell layers: the outer nuclear layer (ONL) containing the cell bodies of the photoreceptors, the inner nuclear layer (INL) containing the cell bodies of the horizontal-, bipolar-, and amacrine cells and the ganglion cell layer (GCL) containing the cell bodies of the retinal ganglion cells. Each layer of cell bodies is separated by a layer of synapses, the outer plexiform layer (OPL) separates the ONL and the INL, and the inner plexiform layer (IPL) separates the INL from the GCL [11].

The photoreceptors, located at the outer side of the retina, contain visual pigments that detect light of different wavelengths. Rods are used under dim light conditions and cones are used in bright daylight to mediate colour vision. There is only one type of rod cell, while the number of cone cell types varies depending on the species [14]. Cone cells are divided into subtypes based on the type of light-collecting opsin that they express [15]. Most mammals have two types of cones, green- and blue-sensitive, whereas primates have a third type, red-sensitive [14]. Chickens are tetrachromats and possess a fourth cone opsins, namely violet [16]. The chickens also possess coloured oil droplets in the cone cells, located between the inner and outer segments, which further increase the number of colours that can be discriminated [17-19]. In addition, the chickens have double cones that constitute about 40% of the total cone population [20, 21]. Only one of the cones in a double cone pair has an oil droplet [20, 21].
The signals generated by the photoreceptors are transferred via the bipolar cells, residing in the inner nuclear layer, to the ganglion cells. The bipolar cells have their apical dendrites in the OPL and their thinner axons in the IPL. Based on morphological characteristics, synaptic connections and receptor expression, up to 15 types of bipolar cells have been described [22-25]. Besides the bipolar cells, there are two additional types of cells present in the INL, the horizontal cells positioned in the outer part of the INL and the amacrine cells, positioned in the inner part of the INL.

The horizontal cells are a class of interneurons that modulate the signals between the photoreceptors and the bipolar cells [26]. They are subdivided into axon-bearing and axon-less subtypes. A comparative study on horizontal cell subtypes in a vast range of species revealed that the axon-bearing horizontal cell is universal, whereas the presence, and number, of axon-less subtypes may vary [27]. Most mammals have two distinct types of horizontal cells [28, 29], however, the rat and mouse have only one [30], and the chicken and humans have three [11, 31]. Studies performed in salamander revealed that the axon-bearing horizontal cells connect to rod and cone photoreceptors, whereas the axon-less horizontal cells connect primarily with cone photoreceptors [32].

The information carried from the bipolar cells to the retinal ganglion cells is modulated by the amacrine cells. The amacrine cells constitute a broad heterogeneous group consisting of over 20 different members [33].

The ganglion cells, the output neurons of the retina, reside on the inner side of the retina. They receive information from the bipolar- and amacrine cells and extend their axons through the optic nerve into the brain. There are at least 11 different subtypes of retinal ganglion cells [34]. Studies have revealed that subsets of ganglion cells are photosensitive. These intrinsically photosensitive retinal ganglion cells express melanopsin and they are important for circadian oscillation and pupil constriction [35-37]. It has also been suggested that melanopsin is expressed in subsets of bipolar and horizontal cells [38].

In the neural retina there is one major type of glia cell, the Müller glia, spanning across the entire retina [39]. There may also be astrocytes and microglia present [40]. The main function for the Müller cells is to maintain the homeostasis of the retina, recycle neurotransmitters and provide support to the neurons [41]. In addition, the Müller cells are able to take part in the visual process, by acting as optical fibres allowing light to passage from the retinal surface to the photoreceptor cells [42].

The retinal pigment epithelial (RPE) cells are located at the outer most part of the retina (Fig. 3). The RPEs phagocytose the outer segments of the photoreceptors and regenerate the visual pigment. In addition, they increase the optical quality by absorbing scattered light, they transport nutrients, ions and water, and secrete growth factors [43].
Proliferation and differentiation

Cell cycle regulation and apoptosis

Dividing cells progress through two main stages in the cell cycle, namely mitosis and interphase. Mitosis (M-phase) is the process of nuclear division. Interphase includes the DNA replication step (S-phase) and the two gap phases (G1 and G2) during which the cell prepares for replication (G1) or nuclear division (G2). During normal retinal development, the cell nuclei migrate along the apico-basal axis, a movement known as interkinetic nuclear migration (INM). During INM the cells undergo S-phase on the basal (inner) side of the retina and M-phase on the apical (outer) side [44]. The length of the cell cycle differs depending on the developmental stage and between species. At day one in embryonic development it takes five hours for the chicken retinal progenitor cells to complete a cycle, whereas at day six a cycle takes approximately ten hours [45].

Cell cycle progression is regulated by different checkpoints that ensure proper cell division. In response to DNA damage, the cell cycle is arrested to provide time for repair [46]. The DNA damage checkpoints are positioned at G1, before the cell enters the S-phase, in G2, after replication, and also during the S- and M-phases [47]. The DNA damage pathway includes activation of the transcription factor (a protein involved in the process of transcribing DNA into RNA) p53, which transcribes p21, leading to cell

Figure 3. Schematic presentation of the vertebrate retina. RPE: retinal pigment epithelium, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer, NFL: nerve fibre layer.
cycle arrest [48]. The activity of p53 is regulated by several different modulators, including the zinc finger containing transcription factor Zac1 (zinc finger protein that regulates apoptosis and cell cycle arrest). Zac1 is a tumour suppressor that interacts with and enhances the activity of p53 [49, 50]. The effect of Zac1 on the developing chicken retina was investigated in paper II.

The proto-oncogene cMyc encodes a transcription factor that plays a role in cell cycle progression. cMyc expression is virtually undetectable in quiescent (G0) cells, however, mitogenic stimulation leads to a G0 to G1 transition [51], and cMyc is thereafter expressed at equal levels, at all times, during the cell cycle [52]. Down-regulation of cMyc causes cells to accumulate in the G1 phase of the cell cycle and eventually die, whereas cMyc overexpressing cells initiate apoptosis without having arrested in the G1 phase [53]. Deregulated expression of cMyc has been detected in a wide range of human cancers [54]. Unlike cMyc, Nolz1 acts as a repressor of transcription and promotes cell cycle exit [55-57]. It has been reported to be expressed both in proliferating progenitor cells as well as in early differentiating post-mitotic neurons [56, 58, 59]. c-Myc and Nolz1 are investigated further in papers III and V.

Generation of new cells in the retina is tightly connected to apoptosis, which regulates the final number of cells. During chicken retinal development, two waves of apoptosis occur. The first wave peaks at embryonic days (E) 3.5-4 and is mostly localized around the optic nerve exit [60]. The second wave peaks at E10-14, and is localized to the inner nuclear layer and the ganglion cell layer [61, 62]. Depending on the species, it has been estimated that 40-90 % of all retinal ganglion cells that are born will die during development [63, 64]. Cell death of the ganglion cells may occur if their optic nerve fibers do not connect properly in the optic tectum [64]. In contrast to the retinal ganglion cells, there is evidence that the number of photoreceptors and horizontal cells is not regulated by apoptosis [61, 65].

Birth-dating of retinal cells

Despite the vast difference in the time required to form a functional retina in different species the cells are generated in a spatially and temporally conserved pattern. The birth of a cell refers to the time it withdraws from the cell cycle and initiates differentiation. For any given species the birth-order may vary slightly but there are some conserved aspects. The cells leave mitosis in close, overlapping succession (Fig.4). Ganglion cells are the first to withdraw from mitosis and bipolar cells are the last [66]. Cones, horizontal- and amacrine cells are born early whereas rods and Müller glia are born late [4, 67]. The first-born cells in the chicken retina leave the cell cycle on E2 and the last cells withdraw at E10-13 [4, 68]. In addition to the temporal distribution, the cells are generated in a central to peripheral wave-like
manner, originating from the optic nerve [4, 69]. Early-born cells in the peripheral part of the retina might therefore be born after late-born cells in the central part.

Figure 4. Birth-dating in the chicken retina. Schematic presentation of the generation of the retinal cells. E: embryonic day.

Cell-fate determination

Early progenitors divide symmetrically to generate new progenitor cells, whereas late progenitor cells divide symmetrically to generate post-mitotic cells. In addition, asymmetric divisions occur, generating one progenitor cell and one post-mitotic cell [70]. There is no universally accepted model to describe retinal cell-fate determination; however, several models have been put forward. One such model is the position-dependent model. According to this model, post-mitotic retinal cells migrate towards their final laminar position, and depending on their position they get exposed to position-dependent inductive signals that determine their differentiation [71]. The competence model, on the other hand, proposes that during retinal development, the progenitor cells pass through several competence stages where they have the ability to produce different cell types [72]. Both intrinsic and extrinsic cues are important for cell fate determination. Since gene expression varies amongst cells they will respond differently to extrinsic cues in the environment. However, a cell must be competent to respond to extrinsic signals. This view on retinogenesis has been challenged by the stochastic model [73, 74], suggesting that the retinal progenitor cells should be considered as equipotent and that cell fate determination has a partially stochastic component. The expression of key fate determinants can be viewed as probabilistic within the progenitor pool, generating a robust distribution of the mature cell types. Temporal competence generates windows that limit the cell types produced at each specific time point.
Methods to label and map neurons during development

Cell cycle markers

There are several different methods to investigate cell cycle progression, such as administration of DNA synthesis markers. The thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) gets incorporated into the DNA during S-phase and labelled cells are detected by a chemical reaction [75]. Another method to label dividing cells is by using an antibody against phosphorylated histone H3 (PH3). During mitosis the chromatin condenses and histone H3 is phosphorylated. The PH3 antibody therefore allows for specific detection of cells in late G2/M-phase [76]. Both methods are extensively used in our studies.

Cell-lineage tracing

To gain a better understanding of how different populations of cells are generated in the nervous system it is necessary to establish techniques that allow for labelling and mapping of the cells. Cell tracing can be performed by injection of detectable compounds that are transported with the cell or by the neurites, such as the horse radish peroxidase enzyme [69, 77], fluorescent-labelled dextran [78], and the lipid-soluble dye DiI [79]. In addition to these compounds, genetic markers such as GFP [80] and LacZ [81] can be transfected to the cells by electroporation of DNA constructs [82], or by viral vectors [83, 84]. In contrast to injected compounds, genetic markers have the advantage that they can be inherited by the progeny of the genetically altered cell, which allows for long-term studies of cell-lineages [85].

Cell-specific labelling by electroporation of gene-specific regulatory elements

One method for studying a specific gene or cell type is to introduce a reporter gene that will give expression exclusively in the cells that actively transcribe the gene of interest. To achieve gene-specific reporter gene expression, it is important to identify genomic elements that regulate the expression of the gene of interest. The ECR browser, a tool especially designed for comparison of multiple vertebrate genomes [86], identifies highly conserved genomic sequences in an animal’s genome. Since highly conserved regions most likely represent important regulatory elements, these sequences are potentially good candidates for driving reporter gene expression. In addition, transcriptional regulators have been shown to have conserved functions across vertebrate phyla [87]. The highly conserved sequences are analysed by insertion into an enhancer trap vector, such as Stagia3 [88, 89]. These techniques were used in paper IV.
Electroporation is a widely recognized method for transferring nucleic acids into cells, and it is possible to co-transfect multiple constructs simultaneously [82]. For studies of chicken embryogenesis, electroporation can be performed in ovo in the optic vesicle [90] or subretinally in the eye [91]. In addition, the eye can be enucleated and electroporated in a cuvette [92-94]. The method of electroporation has been used in papers I-V. By controlling the site of electroporation and selecting gene- or cell-specific conserved regions to drive reporter gene expression, specific cell types can be labelled and investigated [95]. In a recent study by Hadas et al (2014) they created a Chickbow, the equivalent to the mouse Brainbow [96], by electroporating plasmids with different Cre-reporter genes, driven by specific enhancer elements, into the chicken spinal cord [97]. This approach beautifully illustrates the advantage of combining gene- or cell-specific regulatory sequences with the electroporation technique.

Stable, long-term labelling with the Cre-LoxP piggyBac-technique

By using electroporation it is possible to transfecy cells at a specific time in development, and to target a restricted region, although it produces only a transient expression. However, in combination with a transposon system, which integrates the reporter gene into the host cell genome [98], robust long-term expression can be established, while still initiating expression at a specific time point. To achieve a cell- or gene-specific robust reporter gene expression three essential components are needed (Fig 5): 1) A trap vector that drives expression of Cre recombinase from a gene- or cell type-specific regulatory element [88]. 2) A “donor” reporter gene-construct with a transposon cassette that contains a strong ubiquitously active promoter, such as the cytomegalovirus early enhancer/chicken β actin (CAG) promoter [99], followed by a “floxed” STOP sequence [100]. 3) An episomal “helper” transposase vector that is ubiquitously expressed and catalyses the integration of the “donor” reporter construct into the genome of electroporated cells. Only cells that drive specific Cre expression will remove the STOP sequence from the integrated reporter, establishing a lineage with robust and stable reporter gene expression that is defined by the gene- or cell type-specificity.
The “helper” transposase works by cutting the genomic DNA, thereby generating a site into which the piggyBac “donor” reporter construct can be inserted. Studies performed both in the chicken DF1-cell line and in cattle revealed that the incorporation most often occurs in regions between exons and that the target sequences in the genome is TTAA [101, 102]. In transgenic chickens, the integration sites were found to be between genes or between exons [103]. It has also been shown that expression of the transposase alone does not induce recombination of endogenous transposon-like sequences [104], and it is therefore not expected to interfere with normal gene function. In addition, the transposase completely restores the integration site upon excision [105], thereby causing no permanent effect on the genome. The piggyBac system is capable of delivering inserts up to 100 kb [106], providing an advantage compared to the capacities of retroviral systems that are usually limited to 8 kb [107]. There are, however, drawbacks with the transposon technique, such as the lack of specificity in sequence integration and the need for assistance in order to achieve efficient cellular uptake [108].

The piggyBac system has been successfully used in a wide number of species such as mouse [109], rat [110], fruit fly [111], chicken [100, 112] and cow [102]. The system was used in papers IV and V.

Our current understanding of the photoreceptors and the horizontal cells

Cell-lineage studies indicate that all of the different cell types in the retina are derived from a common, multipotent retinal progenitor cell [113]. It has also been shown that the photoreceptors and the horizontal cells share an immediate progenitor [114]. A result further supported by our study where
we show that a 208bp regulatory sequence from the RXRγ gene directs expression to photoreceptors and horizontal cells [112].

The horizontal cells have been in focus for many years in our group and a number of important aspects regarding their development have been discovered. As previously mentioned, there are three types of horizontal cells in the retina, denoted H1, H2, and H3, distinguished by morphology and molecular markers [31, 115]. These are an axon-bearing subtype (H1) expressing Lim1, constituting 50% of the horizontal cells, and two axon-less subtypes (H2 and H3) expressing Isl1 and GABA or Isl1 and TrkA, respectively [115].

Several different genes, such as Ptf1a, FoxN4, Lim1, Prox1, Onecut 1 and 2 are pivotal for the generation of horizontal cells. Disruption of the FoxN4 gene abolishes the horizontal cells [116], whereas over-expression produces an increase [117]. The same pattern was seen for the Ptf1a and Prox1 genes [118-120]. Disruption of the Onecut 1 and 2 genes result in loss of horizontal cells [112, 122]. Lim1 has been reported to be important for cell migration as ablation of Lim1 produces ectopically positioned horizontal cells in the amacrine cell layer [123].

During normal retinogenesis the cell spans the entire apico-basal axis of the retina while the nuclei migrate, a behaviour referred to as interkinetic nuclear migration [44, 124]. The cells perform their M-phase on the apical/outer side where the future ONL will form. Studies on the axon-bearing Lim1 positive (+) horizontal cells revealed that they have a heterogenic final cell cycle that can be divided into three different behaviours [125]. Cells with behaviour “one” performed their final mitosis on the apical side. Cells with behaviour “two” performed a final mitosis on the apical side, followed by an additional S-phase while migrating to the basal side. These cells remained with a replicated genome, and were regarded as somatic heteroploids. DNA content variation is a condition also observed in neuronal tissues of other species such as mice and humans [126, 127]. Cells with behaviour “three” performed their final mitosis not on the apical side, but on the basal side [125], a feature also seen in the zebrafish retina [128].

Once the retinal progenitors have performed their last M-phase on the apical side of the neuroepithelium they withdraw from the cell cycle and migrate to their final layer [124, 129]. It has been observed that post-mitotic horizontal cells migrate to the basal/inner side where they align, before migrating back to their final position in the outer part of the INL [130], a path of migration that differs from the expected direct route. Both Lim1+ and Isl1+ horizontal cells display this migration, however, the Isl1+ cells perform their final M-phase and migration one day later. In addition, the horizontal cells possess the ability to migrate tangentially in the retina [131].

Following DNA damage, the cell cycle is arrested to provide time for repair [46]. Induced DNA damage in the horizontal cells suggested that they had a functional damage response pathway [62], but they were still able to
enter S-phase and complete mitosis [132]. Overexpression of the zinc finger transcription factors Zac1 and Nolz1 (papers II and III), both known to induce cell cycle arrest/exit [50, 57], did not have an effect on the number of horizontal cells generated [133]. These data suggest that the horizontal cells have the ability to progress through the cell cycle, even in the presence of factors/signals that should arrest them.

During retinogenesis, proliferation and apoptosis are tightly connected to regulate the final number of the different cell types. However, there is evidence that neither the final number of horizontal cells nor photoreceptors are regulated by apoptosis [61, 65], implying that they are able to resist the apoptotic machinery during embryogenesis. In the mature retina, photoreceptor cell death is a common feature in a number of eye diseases, such as retinitis pigmentosa, macular degeneration, and rod-cone dystrophy. However, a study performed on degenerating dog retinas revealed that terminally differentiated photoreceptors have the capacity to proliferate, despite carrying the degenerative mutation [134]. In addition, both the photoreceptors and the horizontal cells have been implicated to be involved in the formation of the retinal tumour retinoblastoma [135, 136]. Taken together, all these studies indicate that the photoreceptors and the horizontal cells possess behaviours that deviate from the normal cell cycle control, and further research should be aimed at elucidating the underlying cause and its effect on retinal development and disease initiation.

Retinoblastoma, a tumour originating from the retina

Retinoblastoma is a paediatric eye tumour that arises in the retina. It has a reported incidence of 1 in 15-18 000 live births. Tumour development is initiated in utero and approximately 90% of all patients are diagnosed before the age of 5 [137]. The most frequent symptoms of retinoblastoma are leukocoria (white pupillary reflex) and strabismus (squinting) [138]. Retinoblastoma is diagnosed by examination under anaesthesia and documentation by RetCam. It is treated with various cytostatic agents, or by laser. If the treatment is unsuccessful, or there is a risk that the tumour cells will spread though the optic nerve and form metastases, enucleation is performed (personal communication Dr. Charlotta All-Eriksson).

Retinoblastoma was first discovered to be initiated by a biallelic inactivation of the RB1 gene, the basis for Knudson’s “two-hit” hypothesis on tumour formation [139]. According to the hypothesis, retinoblastoma is a cancer caused by two mutational events, either the first mutation is inherited and the other occurs spontaneously in the somatic cells, or both mutations occur spontaneously in somatic cells. The RB1 gene is a tumour suppressor gene and its product is a chromatin-associated protein that regulates the transcription of cell cycle genes. The RB protein mainly acts by interaction with
the E2F transcription factor, thereby suppressing transcription of its targets, resulting in restricted expression of genes needed for cell proliferation [140].

In a recent study performed on 94 retinoblastoma samples it was shown that, although the loss of RB1 is common, a number of other gene losses and gains were detected, implying a more complex nature of the tumour than was first believed [141]. Furthermore, investigation of 1068 unilateral retinoblastoma tumours with intact RB1 genes (RB1+/+) revealed MYCN amplifications (MYCN^A) in approximately 1.4% of the cases [142], suggesting that a MYCN amplification alone is enough to cause retinoblastoma. MYCN^A tumours are aggressive and usually diagnosed earlier than the RB1^-/- tumours [142, 143]. The MYC family of proto-oncogenes consists of three MYC proteins, c-Myc, MYCN and MYCL, all encoded by separate genes. The family members are transcription factors that control the expression of certain cell cycle genes, thereby promoting proliferation [144].

In humans, RB1 mutations can be detected by sequencing of patient blood samples [145]. DNA sequencing can also be used to detect MYCN amplifications; however, as these amplifications are only reported in the retinoblastoma tumours, blood samples cannot be used for testing [145]. In order to genotype a tumour, a biopsy would have to be taken for analysis. As retinoblastoma is an aggressive tumour, taking a biopsy would potentially lead to leakage of tumour cells from the eye, with an increasing risk for metastases (personal communication Dr. Charlotta All-Eriksson). Retinoblastoma caused by a MYCN mutation is always unilateral and the eye is usually enucleated or treated with intraretinal chemotherapy, before the child is six months old.

Previous studies have indicated that the cell-of-origin for retinoblastoma may be the horizontal cell in a mouse model [135], and the cone photoreceptor cell in humans [136]. It has been shown in mouse that the cell-of-origin for retinoblastoma appears to be naturally death-resistant [146]; a behaviour that corresponds well with that of the photoreceptors and horizontal cells, whose final number in the retina is not regulated by apoptosis [61, 65]. Determining the cell-of-origin for retinoblastoma is an important goal, however, understanding the mechanisms that distinguish the photoreceptors and the horizontal cells from the other retinal cells may prove just as important.
The chicken embryo as a model organism to study neuronal development

A lot of our knowledge regarding human development and our biological processes comes from studies performed on model organisms. A model organism is an *in vivo* model that is used for research when human experimentation is unfeasible or unethical. Depending on which biological system needs to be studied certain model organisms prove more useful.

Research performed on chicken embryos has contributed to extending the knowledge in fields such as genetics, virology, cancer and immunology [95]. Already 2000 years ago the great philosopher and scientist Aristotle highlighted the chick as a great model for studying eye development. The chicken owes this status to a number of aspects such as the fact that *in ovo* studies are more easily accomplished than the corresponding *in vivo* studies of mammals. The eye is large in size and easily accessible for manipulations, such as *in ovo* electroporation [91], culture of retinal explants [92], whole mount *in situ* hybridisation [147] and retroviral infection [84]. In addition, the period over which the eye develops is relatively short and the different stages of chicken development, from the early cell divisions to hatching, have been extensively studied and described in detail [148, 149]. Based on distinct morphological criteria, Eyal-Giladi and Kochav (1976) defined 14 stages encompassing development taking place pre- and post egg-laying [148]. Hamburger and Hamilton (1951), on the other hand, described the development that ranged from the initiation of incubation until hatching. Embryos were classified into stages (st) or the corresponding embryonic days (E) [149].

The eyes of the chicken begin to develop early during embryogenesis and when the chickens hatch, all retinal cell types are present [4, 5]. Mice and rats, on the other hand, are blind at birth and a major part of their retinal development occurs postnatally [4, 150]. In the chicken embryo, immunological activity appears in the 2nd week of incubation [151], the chicken embryo can therefore be considered as immunodeficient during its early development. This enables injections and orthotopic transplantations without the risk of rejection. However, the lack of an active immune system may also make them more vulnerable to external agents, such as microbes, that could be introduced during experimental procedures.

Even though the chicken is not a mammal the initial embryological development in humans and chickens share similarities, and chickens are therefore good models for studies on retinal development. There are, however, differences between the human and chicken eye. Humans have a fovea (Fig. 1) with a high density of cones, whereas the rods display high density outside of the fovea [152]. The chicken retina appear to lack a fovea [153] and their cones are distributed across the entire retina [20]. In addition, humans have a vascularised retina, whereas the chicken retina is avascular
and instead relies on a structure termed pecten oculi, which plays an important role in the nourishment of the retina [154].

The sequence of the chicken genome is available. The first assembled draft of the chicken genome sequence was published in 2004 [155]. Having an available genome sequence allows for studies of gene families and homologues as well as gene regulation and evolution. The chicken was the first non-mammalian amniote to have its genome sequenced. The genome is about 40% the length of the human genome. Based on sequence comparison, about 60% of chicken protein-coding genes have a single human orthologue. Sequences expressed in the chicken brain proved to be more conserved than sequences expressed in any of the other tissues investigated [155].

As with all model animals there are drawbacks. One major drawback has been the inability to genetically modify the chicken. However, recent studies have reported the generation of transgenic animals [156] and the successful use of the genome editing tool CRISPR/Cas9 [157]. Although not the most commonly used animal model, the chicken has for a long time proved to be useful, and with the recent technical advances more people appear to have discovered the potential that lies within the chicken model system.
Aims of the thesis

The overall aim of my work was to broaden our knowledge regarding the behaviour of the horizontal cells, both in the normal and diseased retina. The aim was also to develop, optimise and validate methods for labelling and targeting of expression to photoreceptors and horizontal cells in the chicken embryonic retina.

Specific aims were:

**Paper I:** To develop a quick, reliable and reproducible method for investigating reporter gene expression of DNA plasmids.

**Paper II:** To investigate the effect of misexpression of the zinc finger protein Zac1 on the regulation of the cell cycle in photoreceptors and horizontal cells.

**Paper III:** To analyse the effects of the zinc finger protein Nolz1 on the generation of neuronal cells in the embryonic chicken retina.

**Paper IV:** To validate and optimize the piggyBac system, together with *in ovo* electroporations, as a method for labelling and mapping photoreceptors and horizontal cells.

**Paper V:** To investigate the short- and long-term effect of overexpression of cMyc and MYCN during retinogenesis.
Results and discussion

Paper I

We aimed to establish a protocol that allowed us to perform, quick, easy and reproducible analysis of DNA plasmids and chemical reagents in chicken embryonic retinas. Based on previous experience, the invasive nature of subretinal injections and in ovo electroporations sometimes generated a high frequency of malformations, and may even have affected the survival of the embryo. To avoid these potential complications we developed an ex ovo culturing protocol. Chicken embryonic eyes were enucleated, the sclera and pigment epithelium removed, the explants were then electroporated ex ovo in a plasmid solution (Fig. 6A), and cultured in an incubator. After 24 hours of culturing whole retinal explants that had been electroporated with a GFP-expressing vector, GFP positive cells were visible in a large part of the retina (Fig. 6B). Sectioning of the retina showed GFP positive cells distributed across the apico-basal axis of the retina (Fig. 6C), displaying the efficiency of the protocol.

Figure 6. Electroporation of whole retinal explants. (A) Schematic of electroporation of whole retinal explant using two electrodes. When applying a current the electrodes will transfer the DNA plasmids into the retinal cells (B) Whole retinal explant after 24 hours of culturing. (C) Fluorescence micrograph of sectioned retina 24 hours after electroporation. ON: optic nerve.

The protocol could also be used to treat retinal explants with chemical reagents, such as those affecting the cell cycle. The whole retinal explants were then stripped of their sclera and pigment epithelium and directly placed in retina culture medium in the incubator, followed by addition of chemicals.

Advantages with our established ex ovo protocol is that it allows for more accurate control of the concentration of DNA plasmids and chemicals, and that retinal explants from a wide range of developmental stages can be used. In addition, it is possible to treat one eye and use the other as a control, without the risk of systemic effects via the blood stream.
One disadvantage with our protocol is that the retinal explants can only be cultured for a limited time. Already after 24 hours of culturing we detected apoptotic cells in the retinas. It is also unclear how development of the retinal cells is influenced by enucleation, and our retinal explants were therefore cultured for a maximum of 24 hours. This time-restriction still allowed enough time for the DNA plasmids to produce detectable reporter gene expression. In contrast to our study, others have cultured retinal explants for longer time-periods [158, 159]. However, it should be noted that the retinal explants resemble an injured retina where the retinal ganglion cells have been axotomized, leading to ganglion cell degeneration [159, 160], which undoubtedly has an effect on the retina.

The *ex ovo* electroporation protocol presented in paper I is a good complement to experiments performed *in ovo*, it allows for quick, and reliable investigation of DNA plasmids, and serves as an important tool in our research. A similar protocol was developed where retinal explants were electroporated, dissociated and used for primary cultures [93], showing the versatility of *ex ovo* electroporations.

**Paper II**

To ensure proper cell division the cell cycle is regulated by a number of different checkpoints, positioned throughout the various phases of the cell cycle [47]. In response to e.g. DNA damage the DNA damage response pathway arrests the cell cycle and allows time for repair [46]. It has been shown that a subtype of horizontal cells, the Lim1 expressing, is able to enter mitosis even in the presence of DNA damage [132], despite having a functional DNA damage response pathway [62]. One of the proteins involved in the damage response pathway is the tumour suppressor and transcription factor Zac1. Our results showed that overexpression of Zac1 in the chicken retina induced p53-dependent expression of p21. A previous study also showed that Zac1 enhances the activity of p53 [49]. Induced p53-activity increase transcription of p21, and p21 can in turn block the G1/S- and G2/M-transitions [161]. We investigated whether Zac1 could affect the retinal progenitor cells and found that overexpression of Zac1 reduced the number of cells that entered S- or G2/M-phase (Fig. 7A-C), thereby indicating that Zac1 promotes cell cycle arrest or exit. However, the Lim1+ horizontal cells were able to enter both S- and G2/M-phase while overexpressing Zac1 (Fig. 7D and E). This supports our hypothesis that the Lim1 horizontal cells are less sensitive to events that trigger the p53 system.
Figure 7. The effect of Zac1 overexpression on cell cycle progression. (A) Relative density of EdU+ cells in electroporated st22 (E3.5) retinas after 24 hours. (B) Relative density of PH3+ cells in electroporated st22 (E3.5) retinas after 24 hours. (C) Fluorescence micrographs showing PH3+ cells in retinas electroporated at st22 (E3.5) with the Zac1 overexpressing construct. (D) Fluorescence micrographs showing Myc-tag (mZac1), Lim1, EdU in retinas electroporated at st25 (E4.5). (E) Fluorescence micrographs showing Myc-tag (mZac1), Lim1, PH3 in retinas electroporated at st27 (E5). Arrow heads denote double- or triple positive cells. Student’s t-test, * p <0.05, **p <0.01, n ≥4, mean ± SD. Scale bar: 10µm in all figures. Control: GFP vector, gcl: ganglion cell layer, mZac1: mouse Zac1.

As the horizontal cells appeared to be less sensitive to Zac1 overexpression we also investigated if Zac1 could affect their number in the retina. Our results showed that the relative density of Lim1+ horizontal cells did not change after Zac1 overexpression (Fig. 8A). The horizontal cells are generated at approximately the same time during retinogenesis as the photoreceptors [68], and they are derived from a common retinal progenitor cell [114]. We therefore also investigated if the number of photoreceptors changed after Zac1 overexpression. Our results showed that the relative density of visinin+ photoreceptors decreased (Fig. 8B), indicating that they are affected by events triggered by Zac1. It has also been reported that Zac1 induce apoptosis in cell lines [50], an effect that we also noted in the embryonic chicken retina (Fig. 8C). The results presented in paper II shows that the HCs have a capacity to withstand the effect caused by Zac1, thereby adding further knowledge regarding their cell cycle regulation.
Paper III

In paper III we investigated the effect of the zinc finger gene Nolz1 on the development of the chicken retinal progenitors. First we looked into the endogenous expression of Nolz1 during retinogenesis by performing qRT-PCR, *in situ* hybridisation, and western blot analysis. The results showed that Nolz1 was expressed in two waves, the first in retinal progenitor cells and the second in early differentiated neurons. There was a sustained expression of Nolz1 in the inner part of the inner nuclear layer, indicative of expression in amacrine cells.

Previous studies have shown that Nolz1 promotes cell cycle exit [56, 57]. We therefore overexpressed Nolz1 in st22 (E3.5) retinal progenitors and analysed the fraction of cells that entered S-phase by 5-ethynyl-2-deoxyuridine (EdU) incorporation [75, 162], as well as the fraction of cells that entered G2/M-phase by staining for phospho-histone H3 (PH3) [76]. The results showed a decrease in the EdU+ or PH3+ cells (Fig. 9A and B), indicating that Nolz1 causes progenitors to extend or withdraw from the cell cycle. This was further confirmed by the increased fraction of p27+ cells (Fig. 9C). The cyclin-dependent kinase inhibitor 1B (p27) causes cell cycle withdrawal, and its expression precedes cell cycle exit [163, 164].
Figure 9. Nolz1 decreased the number of cycling retinal progenitor cells. (A) Bar graph showing fraction of EdU+ cells following overexpression of Nolz1 for 24 hours. (B) Bar graph showing fraction of PH3+ cells following overexpression of Nolz1 for 24 and 72 hours. (C) Bar graph showing the fraction of p27+ cells following overexpression of Nolz1 for 72 hours. Student’s t-test, * p< 0.05, ** p< 0.01, n = 4, mean ± S.D.

We performed both short- and long-term overexpression of Nolz1 and analysed its effects on the formation of the different retinal cell types. Overexpression for 72 hours decreased the formation of visinin+ photoreceptors, Brn3a+ retinal ganglion cells, and Lim3+ photoreceptors (Fig. 10A). This is consistent with the proposed role of Nolz1 as a repressor of transcription [165, 166]. Long-term overexpression lead to a decrease in the number of Lim3+ cells (Fig. 10B). In the chicken retina, Lim3 is expressed in both photoreceptors and bipolar cells [67]. Since there was no effect on the Lim3+ photoreceptors we concluded that the decrease was confined to the bipolar cells.

Based on the overexpression we can conclude that Nolz1 has an effect on the Lim3+ cells, which is in line with previous results obtained from chicken spinal cord studies [166]. The discrepancy between the short- and long-term studies may be due to the time point at which the different cell types are generated. Photoreceptors are generated early, whereas bipolar cells are generated later during retinal development (Fig. 4) [4]. The decrease in Lim3+ photoreceptors may be compensated for by retinal progenitor cells. However, as the bipolar cells are among the last cell types to be generated there are no progenitor cells left in the retina that can compensate for the loss. The discrepancy may also be due to other factors that affect the expression and effect of Nolz1 in the retina, such as other transcription factors or necessary co-factors.

In summary, overexpression of Nolz1 in the chicken embryonic retina decreased the number of cycling retinal progenitor cells, possibly by extending or arresting the cell cycle, as there was no indication of apoptotic activity. Short-term overexpression decreased the photoreceptors and the retinal ganglion cells, whereas long-term overexpression decreased the number of bipolar cells. The differences between the short- and long-term experiments highlights the importance of performing studies with varying time points in order to better comprehend the complete story.
**Figure 10.** The short- and long-term effect of Nolz1 overexpression on cell type specificity in the chicken embryonic retina. (A) Bar graph showing the fraction of Lim1 (horizontal cells), visinin (photoreceptors), Ap2α (amacrine cells), Brn3α (retinal ganglion cells), Lim3 (photoreceptors and bipolar cells), and Lim3 (PR) (apical Lim3+ cells, photoreceptors) expressing cells in retinas electroporated with the Nolz1 overexpression vector at st22 (E3.5), and analysed after 72 hours. (B) Bar graph showing the fraction of cells following overexpression of Nolz1 from st22 (E3.5) to st40 (E14). Student’s t-test, * p< 0.05, ** p< 0.01, *** p< 0.001, n = 4, mean ± S.D.

**Paper IV**

*In silico* analysis and comparison of genomic sequences is frequently used to find highly conserved cis-regulatory elements in the genome of different species. Computer models may be helpful in predicting the effect of a conserved genomic sequence during embryonic development, however, to fully understand its role it is important to analyse the sequence *in vivo*. We, and others [167, 168], have shown the high potential of the chicken embryo when it comes to the analysis of promoters and enhancers. In paper IV we combine *ex ovo* electroporation of retinal explants with *in ovo* electroporation and long-term studies as tools to evaluate the possible activation of, and specificity of, genomic enhancer/promoter sequences.
In paper IV we focused on sequences/genes known to be important for photoreceptors and/or horizontal cells. Analysis of genomic sequences from the *Pax6*, *Otx2*, and *RXRγ* genes was presented. For stable, long-term reporter gene expression we took advantage of the piggyBac transposon system, which allows for integration of the reporter gene into the chicken genome [100]. The plasmids (Fig. 11) containing the *RXRγ* sequence and the GFP reporter gene were introduced, by subretinal injection and *in ovo* electroporation, into retinal progenitor cells at st22/25/28 (E3.5/4.5/5.5). Analysis was performed at stage 40 (E14), when all the different cell types have been generated [68], or at stage 44 (E18), when the opsins are expressed [169]. Our results showed that it was only the *RXRγ* sequence that produced an expression profile that was restricted to the photoreceptors, horizontal cells and their common progenitor (Fig. 12A).

**Figure 11. Diagram of plasmids used for piggyBac incorporation and *RXRγ*-specific gene expression.** GFP: green fluorescent protein, CAG: cytomegalovirus early enhancer, chicken β-actin promoter, RXRγ: retinoid X receptor γ, RAGE: LoxP-STOP-LoxP.

**Figure 12. Activity of the *RXRγ* sequence in chicken retina.** *(A)* Fluorescence micrograph of a st40 (E14) retina. *(B)* Bar graph showing the distribution of GFP positive cells in sectioned st40 (E14) retinas. Mean ± SD, n=4 per stage. *(C)* Fluorescence micrograph of a st40 (E14) retina showing pairs of photoreceptors (arrow heads) and a pair with a photoreceptor and a horizontal cell (arrows). St: stage, E: embryonic day, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer.
Our results showed that the regulatory sequence from the \textit{RXR}γ gene directed reporter gene expression (GFP) to a progenitor cell that differentiated into cones, rods, and all three subtypes of horizontal cells. Approximately 80% of the GFP positive cells were located in the outer nuclear layer where the photoreceptors reside, and 20% were located in the outer part of the inner nuclear layer where the horizontal cells reside (Fig. 12B). Cross sections of the retinas revealed that the GFP positive cells were sometimes generated in pairs with two photoreceptor cells or a photoreceptor and a horizontal cell (Fig. 12C), confirming the previous notion that these two cell types originate from a common progenitor [114]. We also noted that a fraction (about 1%) of the GFP positive cells were located in the ganglion cell layer. The occurrence of displaced photoreceptors and horizontal cells has been previously described [170, 171].

To perform morphological analysis of the GFP+ cells we flat-mounted st44 (E18) retinas. The results showed the presence of all three horizontal cell subtypes (Fig. 13A), as well as photoreceptor cells (Fig. 13B). The photoreceptor cells were not further subdivided in the flat-mounted retinas due to the high difficulty in subdividing them based strictly on morphology. Based on work done by us, and others, we proposed a model for the potential roles of \textit{RXR}γ and \textit{Lim1} in photoreceptor and horizontal cell genesis (Fig. 13C).

\textbf{Figure 13. The \textit{RXR}γ sequence drove expression in photoreceptors and all three types of horizontal cells.} Z-stacked confocal fluorescence micrographs of flat mounted st44 (E18) retinas showing the presence of all three types of horizontal cells (A) and photoreceptors (B). (C) Schematic model of the role of \textit{RXR}γ and \textit{Lim1} in photoreceptor and horizontal cell genesis. Asterisk (*) denotes axon terminal.

A great advantage with the combination of the piggyBac integration system and the \textit{in ovo} electroporation is that we can direct stable expression of reporter genes to a specific developmental stage and a specific region. Our results showed that when we introduced the \textit{RXR}γ sequence into to optic vesicle of st12 (E2) embryos GFP+ cells were found in all retinal layers.
across the apico basal axis of the retina. By performing the in ovo electroporation at later time points this obstacle was circumvented. A non-specific expression pattern would also have been expected if transgenic animals were used, further highlighting the advantage of the piggyBac technique.

The RXRγ sequence used in our study may not produce an expression pattern that is identical to that of the endogenous RXRγ gene. As the sequence has been inserted into a DNA plasmid it is no longer surrounded by the same DNA sequence as in the chicken genome. This may affect the expression profile and needs to be considered following analysis of regulatory sequences in general. However, in this specific case, our aim was to identify a sequence that would drive expression specifically in the photoreceptors and horizontal cells, thereby allowing us to perform directed expression of other genes, such as modulators of the cell cycle, specifically to these cell types. By establishing the restricted expression pattern for the RXRγ regulatory sequence we are able to perform studies directed specifically at these cells, without affecting the otherwise normal environment of the retina.

Paper V

In paper V we investigated the effect of overexpression of the mutationally stable cMyc (cMyc-T58A) and MYCN (MYCN-T58A) on chicken retinal progenitor cells. Short-term overexpression of cMyc-T58A and MYCN-T58A was performed on st22, 25 and 28 (E3.5, 4.5, 5.5) embryos. Retinas were analysed 48 hours after electroporation. We looked at cell cycle progression, apoptosis and generation of Lim1+ horizontal cells and visinin+ photoreceptor cells. To investigate a potential change in cell cycle progression we analysed the fraction of cells in S-phase EdU incorporation [75, 162], and cells in late G2/M-phase, by staining for PH3 [76]. The results showed no change in the number of cells that entered S-phase or G2/M-phase. Apoptosis was investigated by staining for cleaved caspase 3 (CC3) [172, 173]. Increased apoptosis was observed in a few of the samples. In animals electroporated at st22 (E3.5) there was an increase in the number of Lim1+ horizontal cells. In animals electroporated at st25 (E4.5) or 28 (E5.5) there was no change in the number of Lim1+ cells. We did, however, see an effect on the distribution of the Lim1+ cells. At st30 (E6.5) the majority of the Lim1+ cells are located at the apical side of the retina in untreated animals. Following overexpression the Lim1+ cells were localised across the apico-basal axis of the retina, indicating an effect on their migration. For visinin, there was no change detected at either developmental stage.

We also performed stable, long-term overexpression by taking advantage of the piggyBac integration system, previously used in chicken [100, 112].
Embryos were electroporated at st22 (E3.5) and analysed at st40 (E14), a stage when all the different cell types are present [68]. Overexpression of cMyc-T58A and MYCN-T58A lead to an increase in the number of visinin+ photoreceptor cells. The visinin+ cells were not localized to the outer nuclear layer but rather appeared in clusters. The results showed a general increase in cell proliferation in the retina as evident by the increase in PH3+ cells. We also confirmed the presence of PH3+ photoreceptor cells. In addition, we investigated if visinin+ photoreceptors in st44 (E18) retinas showed expression of rhodopsin, red/green opsin, or blue opsin, markers of more mature photoreceptors that are first detectable between st40-42 (E14-E16) [169]. There were very few visinin+ cells that also stained positive for any of the opsins.

Our results indicate that long-term overexpression of the mutationally stable cMyc and MYCN had a clear effect on proliferation and distribution of the visinin+ photoreceptors, however, the cells did not appear to have reached a more mature stage of development.
Conclusion and future directions

The overall aim of my work has been to broaden our knowledge of the development and behaviour of the photoreceptors and horizontal cells in the chicken embryonic retina, as previous studies have revealed that these two cell types display properties that distinguish them from the other retinal cell types. A major focus has been on the development and refinement of methods that allow us to label and map these specific cell types, for both a short- and long-term perspective.

One of my most important achievements was the establishment of a protocol that allow us to perform stable, long-term labelling of the photoreceptors and horizontal cells by combining the RXRγ sequence with the piggyBac integration system and in ovo electroporation (Paper IV). Now that we possess these highly valuable tools we can direct expression of other genes, such as cell cycle regulators, to these cells, without affecting the surrounding retina. As it has proved challenging to establish transgenic chicken lines, in ovo electroporation and genomic integration using the piggyBac transposon technique offers an easy and highly reproducible alternative.

Besides developing methods to study these cell types I have also evaluated the effect that the two zinc finger transcription factors Zac1 and Nolz1 have on the developing retinal cells. In paper II we showed that overexpression of Zac1 promotes cell cycle arrest and/or exit and that it can trigger apoptosis, via activation of the p53-p21 pathway. However, the horizontal cells appeared to withstand the effect of Zac1 overexpression demonstrated by their ability to progress through the cell cycle (Fig. 14). Overexpression of Nolz1 (paper III) also had an effect on the retinal cells; however, there was no change in the number of Lim1+ horizontal cells.
Figure 14. Schematic of the effect of Zac1 on retinal cells. Overexpression of Zac1 in the chicken embryonic retina induces cell cycle arrest and/or apoptosis by upregulation of p53 and p21. The horizontal cells do not respond to these signals and progress through the cell cycle. RPCs: retinal progenitor cells, HPCs: horizontal progenitor cells, DDR: DNA damage response pathway. Apical (outer) and basal (inner) side of the retinal neuroepithelium. Adapted from Blixt et al [174].

As we have now established protocols for stable, long-term labelling of photoreceptors and horizontal cells we can perform more detailed studies on these cell types specifically while located in the normal, unaffected retina. It would be very interesting to continue the investigation of the effect of factors involved in cell cycle regulation, differentiation, and apoptosis in these two cell types. Hopefully, our continued studies can expand our current understanding of the development and behaviour of the photoreceptors and the horizontal cells.
Materials and methods

Table 1. Methods used in the papers

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Animals
Fertilized White Leghorn chicken (*Gallus gallus*) eggs were obtained from Ova Production. Eggs were incubated at 37°C in a humidified incubator (Grumbach). Embryos were classified into stages (st) or the corresponding embryonic age in days (E) according to Hamburger and Hamilton [149]. Animal experiments were performed according to the guidelines given by the Association for Research in Vision and Ophthalmology and were scrutinized and approved by the local animal ethics committee in Uppsala.

DNA constructs
Plasmids used in the papers presented here are listed in Table 2. Primers used to amplify sequences for cloning are listed in Table 4.
Table 2. DNA plasmids used

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\(^1\)RAGE: LoxP-STOP-LoxP

Electroporations

Retinal explants

Eyes were enucleated and the sclera and pigment epithelium were removed. Eyes were put in a cuvette containing the DNA solution (0.1 μg/μl) in 1x Dulbecco’s Phosphate Buffered Saline (DPBS) +Mg\(_2\)Cl + CaCl\(_2\). Five 50 ms 15 V pulses were applied using an electro square porator ECM 830 (BTX, Harvard Apparatus). After electroporation, retinal explants were cultured in medium containing 1:1 DMEM:F12 Nutrient mix, 10% foetal bovine serum, 10 U/mL penicillin-streptomycin, 5 μg/mL insulin and 2 mM L-glutamine for 24 hours at 37°C, 5% CO\(_2\) on a rotator shaker with a constant speed of 50 rpm.

In ovo

The DNA plasmids (5 μg/μl) were mixed at the ratio of 1:1 with 1xPBS and 0.1M MgCl\(_2\). A dye was added to the mix to help visualize the injection site. Approximately 0.2 μl solution was injected into the subretinal space (≥st22) of the eye or into the optic vesicle (st12), and five 50 ms 12 V (st12) or 15 V
(≥st22) pulses were applied using an electro square porator ECM 830 (BTX, Harvard Apparatus). After electroporation, eggs were sealed with tape and but back into the incubator to allow for further development.

Cell cultures
Chicken fibroblast DF1- or COS-cells were electroporated using the Gene Pulser II (BioRad), set to 250 V and 250 μF. For DF1-cells, 10 μg of each construct at a 1:1:1:1 ratio of helper construct:donor construct:Cre recombinase construct:RFP control construct was used. The electroporated cells were cultured in DMEM containing 12% foetal bovine serum, 2% L-glutamine and 100 U/mL penicillin-streptomycin at 37 °C and 5% CO₂.

COS-cells, grown to confluence in 10 cm petri dishes, were electroporated in a 4 mm cuvette using the Gene Pulser II (BioRad), set to 380 V and 250 μF. 10 μg DNA in 600 μl 1xPBS was used to test the antibody. 2.5 μg DNA and 400 μg morpholino in 600 μl 1xPBS was used to assess the ability of the morpholino to down-regulate the expression of the Nolz1 protein. 24 hours post electroporation the cells were harvested and used for western blot.

Western blot
Retinas were placed in Radio Immuno Precipitation Assay (RIPA) buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH = 8.0) containing 1 x Halt Protease and Phosphatase inhibitor cocktail (78440, Thermo Fischer Scientific), vortexed for 45 sec and rotated for 30 minutes at 4°C and 10 rpm before being centrifuged at 13000 rpm for 15 minutes. Protein concentration was measured with the DC Protein Assay (5000114, Bio-Rad). For protein separation, 35 μg of protein, for each sample, was mixed with an equal part Laemmli lysis buffer (38733, Sigma-Aldrich) and separated on a 10% Mini-Protean TGX Stain-Free Precast gel (4568033, Bio-Rad) for 50 min at 200 V. Proteins were transferred to Immun-Blot PVDF membranes (1620174, Bio-Rad) for 1 hour at 100 V and blocked for 1 hour at room temperature in 5% BSA in TBS/T buffer (20 mM Tris-Base, 136 mM NaCl, 0.1% Tween-20). Primary antibodies were diluted in 1% BSA in TBS/T and incubated overnight at 4°C. After three washes of 10 minutes each with TBS/T, membranes were incubated with secondary antibodies for 1 hour at room temperature. Chemiluminescence was detected with the ChemiDoc MP Imaging System (Bio-Rad) and quantified with the Image Lab software (Bio-Rad). For densitometry each sample was normalized to its respective Actin level. Primary antibodies used were; Nolz1 (1:2000, rabbit, kind gift from the CHDI Foundation [56]) and actin (1:1000, rabbit, A2066, Sigma-Aldrich). Secondary antibody used was; rabbit IgG-HRP conjugated
(1:10000, donkey, ab97064, Abcam). The ladder was visualized using precision protein streptactin-HRP conjugate (1:10000, 1610380, Bio-Rad).

Tissue collection and Immunohistochemistry
Retinal explants and enucleated eyes were fixed in 4% paraformaldehyde (PFA) in 1x PBS at 4°C for 15 minutes, and cryoprotected in 30% sucrose in 1xPBS. The tissue was embedded in OCT (NEG50, Richard-Allan Scientific) and 10 μm sections were collected on Superfrost Plus slides (J1800AMNZ, Menzel-Gläser). DF1-cells were fixed in 4% PFA.

For immunohistochemistry, retinal sections were rehydrated in 1xPBS for 15 min and incubated in blocking solution (1% foetal calf serum, 0.02% Thimerosal and 0.2% Triton X-100 in 1xPBS) for 30 min. Primary and secondary antibodies were diluted in blocking solution and incubated on slides in a humidified chamber overnight at 4°C and for 2 hours at room temperature, respectively.

Primary antibodies are listed in Table 3. Secondary antibodies were obtained from Invitrogen. ProLong Gold (P36935, Life Technologies) with DAPI was used to visualize the nuclei. Cells stained with EdU (Click iT EdU imaging kit C10337, Life Technologies) were visualized according to the manufacturer’s protocol.

Table 3. Primary antibodies used for immunochemistry. PRs: photoreceptors, HCs: horizontal cells, BPs: bipolar cells, ACs: amacrine cells, RGCs: retinal ganglion cells, MC: Müller Glia.

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<th>Company</th>
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<td>Santa Cruz</td>
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\(^1\) Developmental Studies Hybridoma Bank, \(^2\) J.D. Ochriotor, T.P. Moroz, P.J. Linser, The 2M6 antigen is a Muller cell-specific intracellular membrane-associated protein of the sarcolummal-membrane-associated protein family and is also TopA, Molecular vision, 16 (2010) 961-969.

**Intraocular injections**

To specifically inhibit p53 [175] electroporated eyes were injected with 175 ng cyclic Pifithrin-α (3843, Tocris) 6 hours prior to analysis. Cisplatin (2251, Tocris) induces formation of DNA adducts and activates the DNA damage response pathway [176]. In paper II, stage 29 eyes were injected with 1.5 µg Cisplatin 2, 4, or 6 hours prior to analysis.

**EdU injections**

The thymidine analogue 5-ethynyl-2-deoxyuridine (EdU; Click iT EdU imaging kit C10337, Thermo Fischer Scientific) was used to visualize cells in S-phase [75, 162]. Yolk sac injections were performed with 50 µg EdU.

**Treatment with retinoic acid**

Eyes from st25 (E4.5) or 35 (E8) chicken embryos were enucleated, the sclera and pigment epithelium removed with fine forceps, and placed in 1xPBS on ice. Whole retinal explants were used as this ensures a uniform exposure, thereby decreasing the variability between animals that may occur following intraocular injections [92]. For acclimatization, explants were incubated in a 1 ml 1:1 mixture of DMEM/F12 (31331028, Invitrogen) and F12 nutrient mix (31765027, Invitrogen), supplemented with 10% foetal bovine serum, Penicillin/Streptomycin (10 U/ml; P7539, Sigma-Aldrich) and L-Glutamine (2 mM; 25030024, Invitrogen) in a 24-well plate on a rotator shaker with a constant speed of 50 rpm in an incubator with 37°C and 5%
CO₂. Following acclimatization for 1 hour, retinoic acid (1 μM; R2625, Sigma-Aldrich) or vehicle (DMSO) was added and the explants were incubated for an additional 2 hours or 24 hours. Following treatment, explants were transferred to ice cold 1xPBS and retinas were removed using fine forceps. Dissected retinas were dissociated in Trizol for qRT-PCR analysis.

**Morpholino**
For knockdown experiments in paper III, morpholinos were electroporated into the eye of the chicken or in the COS-cells, as explained in the electroporation section. 300 nmol morpholino oligo (Gene Tools) was made into a 1 mM solution by dissolving the powder in distilled water. The Nolz1 oligo sequence was a follows: tgacccaaacacctgaataaat (XM_001233456.2). As control, the following oligo was used: tcaaaagtcctcacaacgcag. The morpholino solution, mixed with Fast Green to help visualize the injection, was introduced into the retinal cells at st22 (E3.5). Embryos were sacrificed 72 hours post electroporation, thus allowing the knockdown to proceed during several cell cycles.

**TUNEL**
TUNEL (G3250, Promega) was used to visualize DNA fragmentation, which indicates apoptotic cell death, according to the manufacturer’s protocol.

**In situ hybridisation**
Dissected eyes from st26, 29, 35, 38, 40 and 43 (E5-17) embryos were fixed in 4% paraformaldehyde (PFA) in 1x PBS for 1 hour at 4°C, and cryo-protected in 30% sucrose in 1xPBS. Fixed eyes were embedded in OCT (NEG50, Richard-Allan Scientific) and 10 μm sections were collected on Superfrost Plus slides (J1800AMNZ, Menzel-Gläser). *In situ* hybridization analysis was performed as previously described [177]. In short, a complementary RNA probe was made using the DIG RNA labelling kit (11277073910, Roche Diagnostic GmbH). The *in-situ* probe, detecting the 5’UTR and part of the coding sequence, has been previously used [147]. The probe was hybridized to untreated sections over night at 68°C under conditions containing 50% formamide and 5X SSC in a humidified chamber. The DIG labelled probe was detected by using an alkaline-phosphatase conjugated anti-DIG antibody (11093274910, Roche Diagnostic GmbH) followed by incubation with BCIP/NBT developing solution (11383221001, 11383213001, Roche Diagnostic GmbH) for 3 hours at 37°C.
Quantitative reverse transcription PCR
Retinas were stripped of pigmented epithelium and collected for the quantitative reverse transcription PCR (qRT-PCR). For ≥st30, the central part of the retina was collected to avoid bias imposed by the centro-peripheral aspects of retinal development. For all treatments/stages, a minimum of four animals were analysed. The mRNA was extracted with Trizol reagent (15596018, Life Technologies). The mRNA batches were treated with DNase (M6101, Promega) for 30 minutes at 37°C before 1 µg of mRNA from each batch was used to prepare cDNA with the high capacity RNA-to-cDNA kit (4387406, Life Technologies). The qRT-PCR was run using the IQ SyBr Green Supermix (1708882, Bio-Rad). The initial mRNA levels were normalized to β-actin and the TATA box binding protein (TBP). Control reactions containing primers but no cDNA were analysed in parallel. The primers were designed with either Primer Express v2.0 (Applied biosystem) or Primer3 Input version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). Primers used are listed in Table 4.

PCR
To amplify genomic DNA or DNA from vectors, polymerase chain reaction (PCR) was used. The PCR was performed using hot start at 95 °C for 1 minute, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at Tm-5 °C for 30 seconds and elongation at 72 °C for 1 minute/1000 bp, followed by a final elongation at 72 °C for 10 minutes. Primers used are listed in Table 4. All PCR products were verified with sequencing (Eurofins Genomics).

Table 4. List of primers used for PCR or qRT-PCR

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<th>Gene</th>
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</table>
Cell culture of MYCN T58A cells

E14 embryos electroporated with the piggyBac-MYCN T58A constructs at st22 were sacrificed and the part of the retina that contained GFP+ cells was dissected and used for cell culture. The MYCN T58A cells were manually dissociated by repeated pipetting and seeded onto a 35 mm dish containing RPMI1640 supplemented with 10% FBS and 1% PenStrep. After 7 days in vitro (DIV), non-adherent cells were transferred to a 100 mm dish. The adherent cells were kept in the original 35 mm dish. Medium was changed twice a week in both culture conditions. To allow for immunocytochemistry of adherent cells, cover slips were placed in the dish before seeding (for fixation and staining see immunocytochemistry). Non-adherent cells were transferred to a microscope slide, stained with DAPI and visualised by microscopy.

Orthotopic explants

MYCN T58A cells grown in cell culture were resuspended in 1xPBS, mixed with fast Green (F7252, Sigma-Aldrich) to help with visualization, and injected intraocularly into the right eye. For st25 embryos, approximately 20 000 cells were injected into the vitreous body or subretinally.

Quantification of cells

At least four sections per eye from four different embryos per treatment and antibody combination were used for cell counting. Cells were quantified as cells/mm² (relative density) or cell fraction of double positive cells. The mean number (+/- SD) for each combination of labelling and stage was calculated and the data analysed in GraphPad Prism (v3.02, GraphPad
software Inc.). Analysis of variance was done one-way ANOVA followed by Tukey’s multiple comparison post-hoc test or Student’s $t$ test and statistical significance was set to $P < 0.05$.

**Image analysis**

Images were captured using a Leica M165FC stereo microscope (Leica Microsystems) equipped with a Leica DFC495 camera, a Zeiss Axioplan 2 microscope (Carl Zeiss Vision GmbH) equipped with an AxioCam C camera or a Zeiss LSM 510 Meta confocal microscope. Confocal images were acquired with laser line 633 nm using BP filter 505-530 and a Plan-Apochromat 20x/0.8 or 63x/1.4 oil DIC objective lens. Figures were assembled in Adobe Photoshop CS4 (Adobe Systems Incorporated).
Acknowledgements

After a lot of hard work I have finally put together my thesis. Although I could not have done it on my own and I would therefore like to acknowledge and thank the following people:

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