Loss of LMO4 in the retina leads to reduction of GABAergic amacrine cells and functional deficits.

Philippe Martin Duquette

This thesis is submitted as a partial fulfillment
of the M.Sc. program in Cellular Molecular Medicine
Faculty of Medicine

University of Ottawa
Ottawa, Ontario, Canada
June 4, 2011

ABSTRACT

LMO4 is a transcription cofactor expressed during retinal development and in amacrine neurons at birth. A previous study in zebrafish reported that morpholino RNA ablation of one of two related genes, *LMO4b*, increases the size of the eye in embryos. However, the significance of *LMO4* in mammalian eye development and function remained unknown since *LMO4* null mice die prior to birth. We observed the presence of a smaller eye and/or coloboma in ~40% of *LMO4* null mouse embryos. To investigate the postnatal role of *LMO4* in retinal development and function, *LMO4* was conditionally ablated in retinal progenitor cells using the *Pax6* alpha-enhancer Cre/*LMO4*flox mice. We found that these mice have fewer *Bhlhb5*-positive GABAergic amacrine and OFF-cone bipolar cells. The deficit appears to affect the postnatal wave of *Bhlhb5*+ neurons, suggesting a temporal requirement for *LMO4* in retinal neuron development. In contrast, cholinergic and dopaminergic amacrine, rod bipolar and photoreceptor cell numbers were not affected. The selective reduction in these interneurons was accompanied by a functional deficit revealed by electroretinography, with reduced amplitude of b-waves, indicating deficits in the inner nuclear layer of the retina. Thus, *LMO4* is necessary for normal GABAergic amacrine and OFF-cone bipolar cell development during retina development.

Table of Contents

List of Figures

List of abbreviations and gene names

ABC	AVIDIN-BIOTIN COMPLEX
Barhl2	BARH-LIKE 2
bHLH	BASIC HELIX-LOOP-HELIX
Bhlhb4	BASIC HELIX-LOOP-HELIX DOMAIN-CONTAINING PROTEIN, CLASS B, 4
Bhlhb5	BASIC HELIX-LOOP-HELIX DOMAIN-CONTAINING PROTEIN, CLASS B, 5
BMP	BONE MORPHOGENETIC PROTEIN
Brn3b	POU DOMAIN, CLASS 4, TRANSCRIPTION FACTOR 2; POU4F2
CB	CONE-BIPOLAR
cGMP	GUANOSINE MONOPHOSPHATE
ChAT	CHOLINE ACETYLTRANSFERASE
Chx10	CEH10 HOMEODOMAIN-CONTAINING HOMOLOG
CKO	CONDITIONAL KNOCK-OUT
CNS	CENTRAL NERVOUS SYSTEM
Cre	CRE RECOMBINASE
Crx	CONE-ROD HOMEOBOX-CONTAINING GENE
DNA	DEOXYRIBONUCLEIC ACID
E	EMBRYONIC DAY
ERG	ELECTRORETINOGRAM
Fgf	FIBROBLAST GROWTH FACTOR
Foxn4	FORKHEAD BOX N4
Flox	FLANKED BY LOXP

GABA	GAMMA-AMINOBUTYRIC ACID
GATA2	GATA-BINDING PROTEIN 2
GFP	GREEN FLUORESCENT PROTEIN
Hes	HAIRY/ENHANCER OF SPLIT
HPC-1	SYNTAXIN 1A
HEN1	NESCIENT HELIX LOOP HELIX 1; NHLH1
ICD	INTRACELLULAR DOMAIN
IHC	IMMUNOHISTOCHEMISTRY
INL	INNER NUCLEAR LAYER
IPL	INNER PLEXIFORM LAYER
Isl1	ISL LIM HOMEOBOX 1
KO	KNOCK-OUT
L cone	LONG WAVELENGTH CONE
Ldb1	LIM DOMAIN-BINDING 1
LIM	**L**IN11, **I**SL-1 & **M**EC-3
LIM-HD	LIM-HOMEODOMAIN
Lhx2	LIM HOMEOBOX GENE 2
Lhx3	LIM HOMEOBOX GENE 3
Lhx4	LIM HOMEOBOX GENE 4
Lmo4	LIM DOMAIN ONLY 4
Mash1	MAMMALIAN ACHATE-SCUTE HOMOLOG 1; ASL1

Math3	MAMMALIAN ATOH HOMOLOG 3; NEUROD4; ATH3
Math5	MAMMALIAN ATOH HOMOLOG 5; ATOH7
M cone	MEDIUM WAVELENGTH CONE
NeuroD	NEUROGENIC DIFFERENTIATION 1
Ngn2	NEUROGENIN 2
Nrl	NEURAL RETINA LEUCINE ZIPPER
Nre2e3	NUCLEAR RECEPTOR SUBFAMILY 2, GROUP E, MEMBER 3
OCT	OPTIMAL CUTTING TEMPERATURE
ONL	OUTER NUCLEAR LAYER
OPL	OUTER PLEXIFORM LAYER
Otx2	HOMOLOG OF ORTHODENTICLE 2
P	POSTNATAL DAY
Pax6	PAIRED BOX GENE 6
PBS	PHOSPHATE BUFFERED SALINE
PCR	POLYMERASE CHAIN REACTION
PFA	PARAFORMALDEHYDE
PKC	PROTEIN KINASE C
PH3	PHOSPHO-HISTONE 3
PKC	PROTEIN KINASE C
PNA	PEANUT-AGGLUTININ
POU	**P**IT1, **O**CT1, AND **U**NC-86

Prox1	PROSPERO-RELATED HOMEOBOX 1
Ptf1a	PANCREAS TRANSCRIPTION FACTOR 1, ALPHA SUBUNIT
Rax	RETINA AND ANTERIOR NEURAL FOLD HOMEOBOX GENE; RX
RPC	RETINA PROGENITOR CELL
S cone	SHORT WAVELENGTH CONE
SCL	STEM CELL LEUKEMIA HEMATOPOIETIC TRANSCRIPTION FACTOR; Tal1
shRNA	SHORT HAIRPIN RNA
Six3	HOMOLOG OF SINE OCULIS HOMEOBOX 3
Six6	HOMOLOG OF SINE OCULIS HOMEOBOX 6
TGFβ	TRANSFORMING GROWTH FACTOR, BETA
TH	TYROSINE HYDROXYLASE
Tlx	HOMOLOG OF TAILLESS; NR2E1
VEGF	VASCULAR ENDOTHELIAL GROWTH FACTOR
Vsx1	VISUAL SYSTEM HOMEOBOX GENE 1
Wnt	WINGLESS-TYPE MMTV INTEGRATION SITE FAMILY
WT	WILD-TYPE

Acknowledgments

First and foremost I would like to thank my parents. My mom for providing me with a stimulating environment as a child. Specifically, my interest in reading so many papers unrelated to my narrow line of work sometimes paid off. Useless knowledge can only be true for those who can predict the future. This, I attribute to my mother's interest in reading that has been passed on to me. The discipline and perseverance needed for science is something that I owe to my dad's everyday example. My school teachers and university professors, I thank you all. Specifically, Denis Rancourt who was my first year physics professor who taught me more about the real world than any curriculum could have done, besides transmitting his taste for physics. For this, I hope his position is reinstated at the University of Ottawa and the decision reconsidered for the sake of future students who will be deprived of a great professor. Another professor who helped me alot and became a friend is John Basso who taught me Genomics. Throughout my university years, I have not met a professor as altruistic as him. Finally, I would like to thank everybody who helped me with my lab work. Jin Xu, who initiated me to hands on science when I first join the Chen lab and helped a lot before he left and Farzaneh Safarpour who helped me in the beginning as well. The current lab members: Xun Zhou who helped me with a lot of experiments for this thesis, Mariana Gomez-Smith and Tina Qin who made sure I was taking care of my mice, and Erik Maclaren, a postdoc who is presently working with me on neurogenesis. Nida Lerma Yap helped me with constructs and Jesse Lu helped with the cell counts. I would also like to thank all my committee members, Marc Ekker, Valerie Wallace and Ruth Slack. A special thanks goes to Valerie Wallace's lab for reagents and technical help especially Chantal Medina and Ya Ping Wang. Also, Adam Baker from the Eye Institute helped me a lot technically for the electroretinography. Last but not least I would like to thank my supervisor Hsiao-Huei Chen who guided me through the years and stimulated my interest in science. I would really like to thank her for giving me the freedom to try new things. She provided me with a great learning environment with all the tools at my disposal to ensure my success, and for that I cannot thank her enough.

1. Introduction

1.1 The Retina

1.1.1 The Retina

The vertebrate retina is a light-sensitive tissue lining the inner surface of the eye. The optics of the eye create an image of the visual world on the retina[1, 2], which serves much the same function as the film in a camera. Light striking the retina initiates a cascade of chemical and electrical events that ultimately trigger nerve impulses. These are sent to various visual centers of the brain through the fibers of the optic nerve[1, 2].

1.1.2 Organization of the mouse retina

The retina consists of six classes of neurons and one type of glial cell interconnected into a highly organized laminar structure divided into three nuclear layers in which cell bodies reside and two plexiform layers made up of the different synaptic connections[1] (Figure 1a). The connections are also organized into different synaptic planes[1, 3-5] (Figure 1b). The light-sensitive photoreceptors are located in the outer nuclear layer (ONL) and transmit information to interneurons located within the inner nuclear layer (INL) via the outer plexiform layer (OPL)[4] (Figure 2). Within the inner nuclear layer, cell bodies of horizontal cells are located closest to the OPL, followed by the bipolar cells and amacrine cells that are closer to the inner plexiform layer (INL)[1, 5] (Figure 2). The inner plexiform layer is made up of synaptic connections between bipolar cells, amacrine cells and ganglion cells, the latter being located in the ganglion cell layer with displaced amacrine cells[4, 5] (Figure 2).

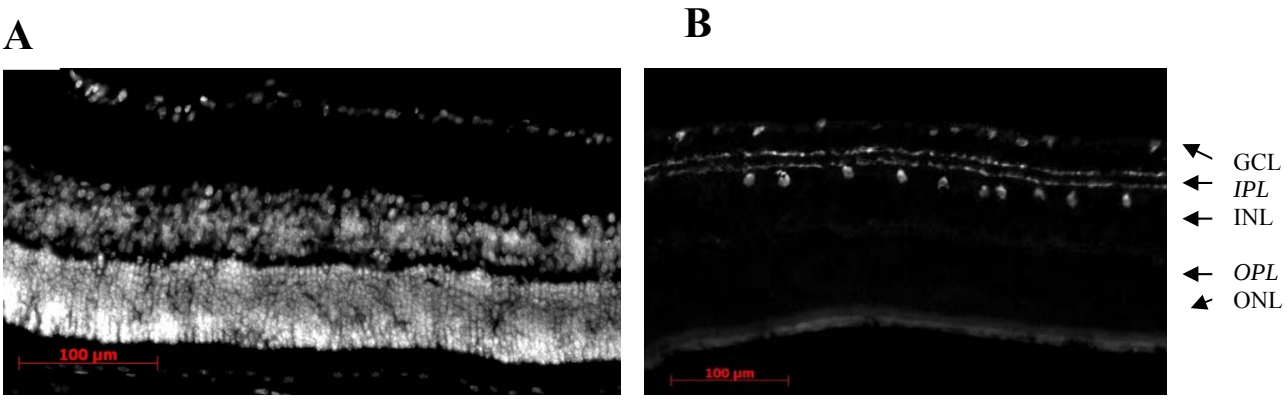


Figure 1: Organization of the retina. A) DAPI (4'-6-Diamidino-2-phenylindole) staining showing the different nuclear layers in a P15 (postnatal day 15) mouse retina. The space between the different layers are made up of synaptic connections. B) Example of the organization of the synaptic planes of cholinergic amacrine cells within the IPL. Staining was done with ChAT (Choline acetyltransferase) antibody.

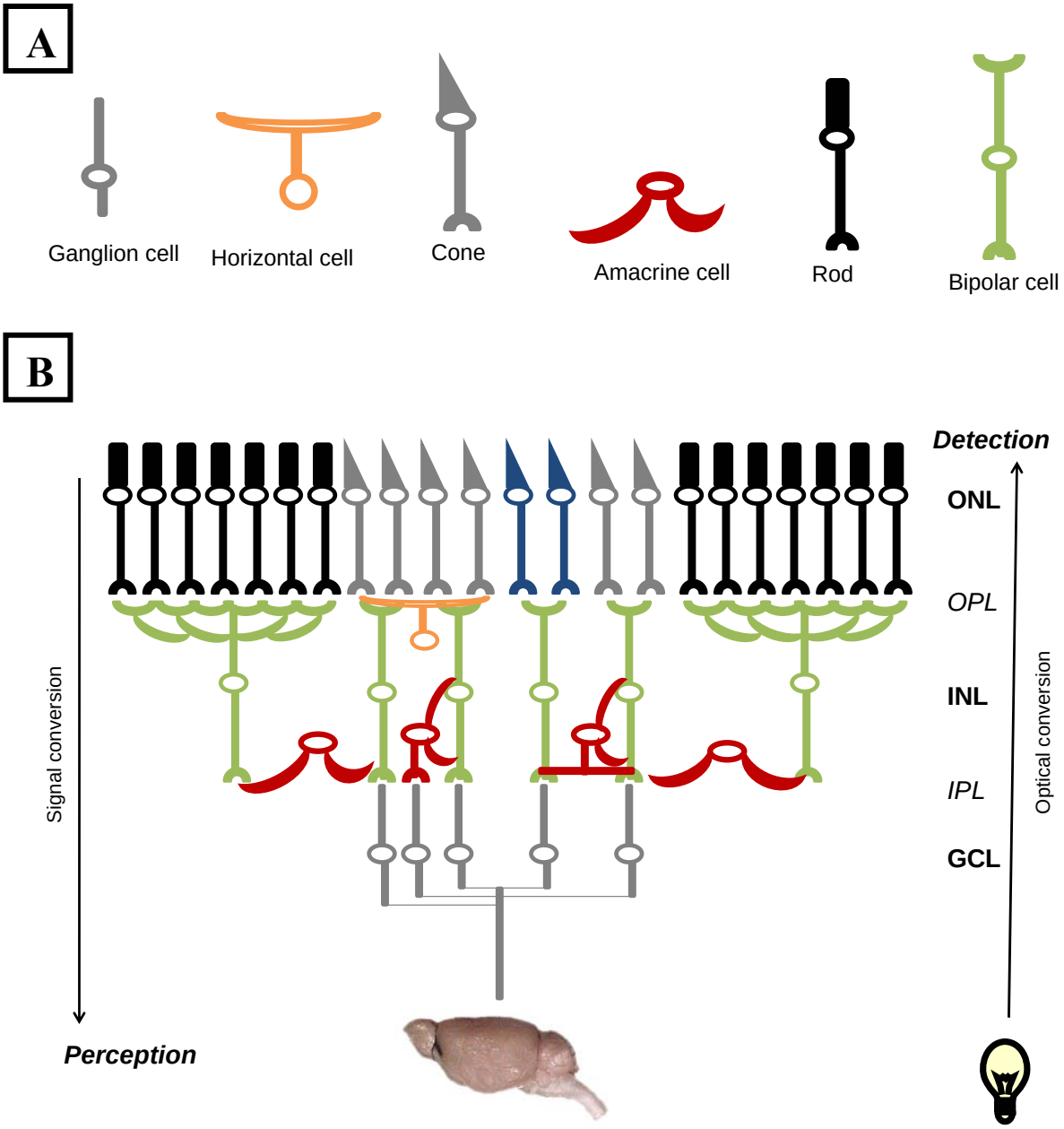


Figure 2: Cellular diversity in the retina. A) Six types of neurons exist in the retina: ganglion cells, horizontal cells, cone and rod photoreceptors, amacrine cells, and bipolar cells. See text for an explanation of their respective roles and developmental profile. **B)** Schematic view of the organization of the retina illustrating the connections between the different cells as well their relative positions. The optical conversion (refraction of light to be focused on the retina) of the light is made by the lens before hiting the back of the retina for detection. The signal is then converted into an electro-chemical signal by the photoreceptors and modified by the different cells within the retina before going to the brain. Notice the high convergence of rod photoreceptors onto one single bipolar cell, which transmits this amplified signal to the AII amacrine, which then relays that information to a cone-bipolar cell before being received by the ganglion cell which will send its axons to the brain for higher order perception. Müller glial cells are excluded from the diagram for simplicity.

1.1.3 Cellular diversity in the mouse retina

The identification and classification of neurons within the retina is almost complete[3]. This ambitious task initiated more than 100 years ago by Ramon y Cajal has given us the possibility of looking through a small window of the central nervous system (CNS), the retina. Two types of photoreceptors can be distinguished by the visual pigment in their outer segment that is excited by incoming photons as well as their cellular shape. Rods are the most abundant and contain more visual pigment relative to their evolutionary ancestor, the cone[1, 2, 5]. Mice are dichromatic but unlike most mammals, the mouse has only one type of cone co-expressing both the UV and M (medium wave-length) cone opsins[6]. Humans normally have three different cones: L cone (long wavelength), M cone (medium wave length), and S cone (short wave length).[2] All photoreceptors receive feedback from a third cell type, the horizontal cell[3] . Less than 1% of the total cell population of the retina consists of horizontal cells and only two subtypes have been identified[3, 7]. Once the information is encoded by the photoreceptors, it is then transmitted to an intermediate interneuron, the bipolar cell[1-3]. The diversity increases from 2 types of photoreceptors, 2 types of horizontal cells, to around 9-11 different types of bipolar cells[3]. Bipolar cells are in charge of the relay between the photoreceptors and the cell that forms the gateway to the brain, the ganglion cell[1, 2] (Figure 2). They can be distinguished by the neurotransmitter they synthesize, their synaptic partners and their morphology. Ganglion cells can be divided into 15-20 different subtypes[3, 7]. Their axons will form the optic nerve that will transmit the information to the brain for further processing and perceptual interpretation. By far the most diverse cell type in the retina are the amacrine cells with around 29 different

subtypes[3, 8]. They are also distinguished by their dendritic arborisation, the neurotransmitter they synthesize and their synaptic partners. These are the "master'' modulators of bipolar and ganglion cells. Just as horizontal cells feedback to photoreceptors, amacrine cells feedback to bipolar cells, but also transmit information to ganglion cells (Figure 2). Finally, the other cell type is the Müller glial cell. Being a glial cell, it does not have the same electro-chemical properties of neurons and is thus thought to serve as support for the neurons[2]. Müller cells are an anatomical link between the retinal neurons and the compartments with which these need to exchange molecules such as the retinal blood vessels.

1.1.4 Outer nuclear layer

1.1.4.1 Cone photoreceptors

As mentioned earlier, there are two different pigments located on a single cone in the mouse (dichromatic). The light-absorbing portion of the pigment is retinal. The absorbance spectrum of the chromophore depends on its interactions with the opsin protein to which it is bound; different opsins produce different absorbance spectra. There are three major steps in phototransduction[2]: 1) Activation of retinal initiates a chemical reaction that changes its conformation from 11-*cis* to all-*trans*, 2) all-*trans* activates cGMP phosphodiesterase to break down cGMP, and 3) the reduction in cGMP closes the cGMP-Gated channels. This reduces the current flowing inward and, consequently, hyperpolarizes cone photoreceptors. Thus, photoreceptors function in opposite direction to other neurons in that they are constantly being depolarized and when activated, they hyperpolarize (at rest, neurons are kept polarized and when stimulated, they depolarize).

This is due to their higher resting potential (-40mV for photoreceptors vs -70mV for other neurons). All photoreceptors release glutamate, and when they are stimulated by light, they release less glutamate.

1.1.4.2 Rod photoreceptors

It has been suggested that rods evolved from cones and function in a very similar way[3]. All rods contain the same photopigment rhodopsin and rods contain much more pigment than do cones. This renders them a lot more sensitive to dim light. Rods also amplify light signals more than cones. Rods outnumber cones 20 fold in the retina and many rods converge on a single bipolar cell[2, 3] (Figure 2). This and the fact that they have more pigment than cones enables them to detect a single photon of light. The way the rod system obtained its high sensitivity left it with low resolution of the visual world.

1.1.5 Inner nuclear layer

1.1.5.1 Horizontal Cells

All rods and cones receive feedback from horizontal cells. Horizontal cells are responsible for information flowing horizontally within the outer plexiform layer to allow photoreceptors to react to the activation state of other photoreceptors accordingly. They are said to enhance contrast between adjacent light and dark regions by feedback inhibition to neighbouring photoreceptors[1, 2].

1.1.5.2 Bipolar cells

Bipolar cells are responsible for transmitting the information from photoreceptors to ganglion cells and make up approximately 40% of the cellular population in the inner nuclear layer[3, 9, 10]. There is only one type of rod bipolar cell but at least 9 types of cone

bipolar cells[9, 11-13]. Since all photoreceptors respond by hyperpolarizing to light stimuli, it is the different postsynaptic receptors located on the dendrites of bipolar cells that are in charge of segregating the signal conversion into two different pathways: 1) the ON response and 2) the OFF response[9, 10, 13, 14]. The ON-bipolar cell mediates the ON response by depolarizing upon activation by a light-stimulated photoreceptor. It is the metabotropic receptor mGluR6 that is responsible for converting the light-invoked hyperpolarization of photoreceptors into a "traditional" depolarizing activation[9, 10, 12-14]. Since mGluR6 is negatively coupled to a cation channel by the second messenger cAMP, glutamate constantly released by photoreceptors in the dark keeps the cation channel closed and the ON-bipolar cell polarized[10]. Upon light stimulation, the reduction in glutamate released by photoreceptors causes less mGluR6 to be activated, thereby reducing the number of cation channels kept closed and allowing the entrance of cations into the cell and ultimately depolarizing it[10]. When photoreceptors are in the center of the receptive field, ON-center bipolar cells will respond by depolarization while OFF-center bipolar cells will hyperpolarize[1, 2, 13]. In contrast to the ON-bipolar cells, Off-bipolar cells respond in the opposite direction. The different response is mediated by the ionotropic receptors AMPA and kainate[9, 11, 12]. These receptors are cation channels and upon glutamate activation, a conformation change in the protein opens the channel and depolarizes the cell[2, 12]. Thus, when less glutamate is released after light stimulation, less channels are opened and they hyperpolarize[2, 12]. ON bipolar cells are also equipped with ionotropic receptors but it is the presence of mGluR6 that distinguishes ON bipolar from Off bipolar cells[9]. Photoreceptors located in the surrounding area of the ON-center will cause the opposite reaction in the bipolar cell through inhibition by horizontal cells[5, 9].

The axons of ON and OFF bipolar cells terminate within different layers of the inner plexiform layer depending on whether they make synaptic connections with ON-center ganglion cells or OFF-center ganglion cells[3, 4, 9, 13]. When bipolar cells are in the center of the receptive field, ON-center ganglion cells will respond by depolarization while OFF-center ganglion cells will hyperpolarize[1, 2]. The dendrites of OFF-center ganglion cells establish connections further from their somas than ON-center ganglion cells (Figure 3)[3, 4]. Thus, the synaptic planes closer to the inner nuclear layer (sublamina *a*) are comprised of Off-center ganglion cells and mediate the OFF responses while ON-center ganglion cells makes their connections proximal to their somas located in the ganglion cell layer (Figure 3).[3-5] This second synaptic plane (sublamina *b*) located further to the inner nuclear layer but closer to the ganglion cell layer mediates the ON response (Figure 3)[3-5]. Bipolar cells not only synapses with ganglion cells, but within the inner nuclear layer but also with different amacrine cells[3, 5, 11]. In particular, rod bipolar cells do not contact ganglion cells directly but use the cone bipolar cells to relay their information to ganglion cells via the AII glycinergic amacrines[3, 11, 13]. Bipolar cells are equipped with many different receptors to respond to the variety of neurotransmitters released by amacrine cells[10, 13]. The α1 glycine receptor unit has been localized to the postsynaptic membrane of the cone bipolar cell[15]. In addition, bipolar cells receive feedback from different amacrine cells. Bipolar cells are also equipped with D1 dopamine receptors that mediate their response to dopaminergic amacrine cells[3, 16]. Functional $GABA_A$ and $GABA_C$ receptors are present on the axon terminals of bipolar cells[17]. It is important to note that although GABA is an inhibitory transmitter (i.e. reduce the probability of an action potentials in its postsynaptic partner), the end result

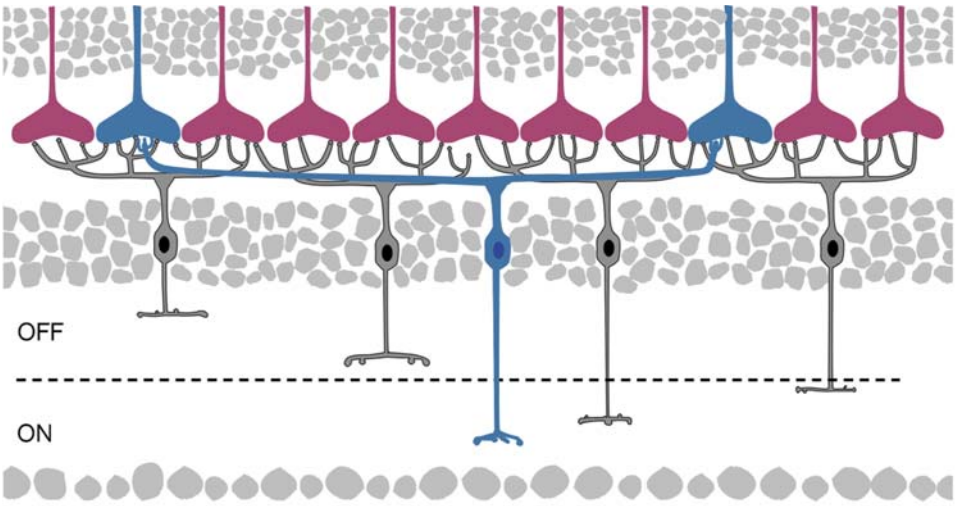


Figure 3: Connections between bipolar cells and ON/OFF-ganglion cells are made in different synaptic planes. Bipolar cells terminating in the "OFF" sublamina (sublamina a) will establish a connection with OFF-ganglion cells while those terminating in the ON sublamina (sublamina b) will synapse with ON-ganglion cells. (modified from ref 3)

of GABA release by amacrine cells might have an activation effect if this release targets a bipolar cell that activates an OFF-ganglion cell or another amacrine cell that inhibits a bipolar cell. This will be demonstrated later by a reduction in retinal activity correlated with a reduction in GABAergic amacrine cells in α-Cre/LMO4flox mice. It is worth mentioning that although the retina is a useful and relatively simple model of the central nervous system, its circuitry remains complex and not yet fully understood.

1.1.5.3 Müller glial cells

In addition to providing support to retinal neurons[18], Müller glial cells maintain the correct electrochemical gradient of neurons by acting as a buffer of different ions. They also recycle the neurotransmitters secreted by neurons within the retina[1, 2, 7]. For this reason Müller cells are endowed with a wealth of different ion channels, ligand receptors, transmembraneous transporter molecules, and enzymes.

1.1.5.4 Amacrine cells

The majority of synaptic partners of ganglion cells are amacrine cells[3]. With over 29 types, they are responsible in carrying narrow tasks that finely control ganglion cells before information enters the brain[3, 8]. Amacrine cells are sometimes divided by the neurotransmitter they synthesize. Among these are glycinergic, GABAergic, cholinergic, and dopaminergic amacrines[8, 15, 17]. Most of the functions of the different amacrine cells have not yet been characterized. Among the known functions are the starburst cholinergic amacrines (they also release GABA) that promote responses to movement,

the dopaminergic amacrines that modulate the retina's responsiveness to light and the AII amacrines that relay the information coming from rod bipolar cells to cone bipolar cells[8]. These AII amacrine cells are in turn modulated by the dopaminergic amacrines and express the D1 dopamine receptor[3, 8]. All rods are connected to only one type of bipolar cells that does not contact directly to any ganglion cells[3, 11]. AII amacrine are responsible in relaying information from rod-bipolar cells to cone-bipolar cells[11]. There is however great diversity within amacrine cells synthesizing the same neurotransmitter. One study in particular classified 28 different amacrine cells in the rabbit retina and came up with four major classes[8]. The four major classes were divided by their dendritic field size (i.e. the area covered by their dendrites) and the pattern and depth of stratification within the inner plexiform layer. The four classes were 1) narrowly stratifying narrow-field cells, 2) broadly stratifying narrow-field cells, 3) medium-field cells, and 4) wide-field cells[8]. Dopaminergic and cholinergic amacrine cells fall within the fourth category of wide-field cells, supporting their global function that covers wide areas of the retina[8]. AII amacrine cells (glycinergic) fall in the "narrowly stratifying narrow-field cells" and "medium-field cells" suggesting that the relay of information from rod bipolar cells to cone bipolar cells can vary in distance[8]. Taken together, amacrine cells are by far the most diverse and least understood in terms of each subtype's function.

1.1.6 Ganglion cell layer

1.1.6.1 Ganglion cells

Ganglion cells are the output of the retina that convey information to the brain via trains of action potentials typical of neurons. Their myelinated axons form the optic

nerve that projects to the thalamus and the midbrain[2]. Just as bipolar cells respond differently to signals coming within their receptive field's center and its surrounding area, ON-center ganglion cells are excited when light is directed to the center of their receptive field and they are inhibited when it is directed to the surrounding area[2]. Off-center ganglion cells respond in the opposite direction. Around 15 different types of retinal ganglion cells have been identified based on electrophysiological recordings and morphological criterias[3]. All ganglion cells are located within the ganglion cell layer together with displaced amacrines.

1.2 Electroretinogram as a functional analysis of the retina

Neurons communicate via electro-chemical signals[2]. Chemical synapses are specialized regions where the axon terminal of a neuron releases a chemical, a neurotransmitter that will bind to specific receptors located on the dendrites of the receiving neuron[2]. From the dendrite, an electrical current is propagated to the soma where incoming signals are summed. If the neuron "decides" to respond with a signal of its own, an electrical current (action potential) will be sent along the axon and a signal will be transmited to another neuron[2]. Thus, given this fundamental property of the central nervous system, that is the change in electrical potential across the cellular membranes of active neurons, an electrical current can usually be measured in neighbouring tissues[19-21]. This is the case with the retina. When many cells are simultaneously active, an electric current can be measured on the cornea of the eye after light stimulation using the electroretinogram[19, 21]. A visual stimulus that consists of completely homogeneous luminance (ganzfield flash) is projected uniformly onto the

retina[19, 21]. The electrical current created by cells in the retina in response to the light stimuli is recorded by an electrode placed on the anasthesized cornea. The current recorded by the electrode is transmited to a computer software that sketches out the corresponding curve (the electroretinogram) onto a monitor[21]. When the retina is fully dark-adapted over-night, the initial response to the first weak stimulus is a negative potential thought to reflect the activity of amacrine and ganglion cells, resulting from the stimulation of rod photoreceptors[19-21]. This negative waveform is termed the "scotopic threshold response"[19]. For this study, the STR was not examined and only the two following waveforms were quantified. A total of 11 different flash intensities are projected onto the retina and the activity is recorded after each light flash with a growing intensity. After the STR, the next waveform to appear (when increasing the flash intensity) on the ERG is the b-wave. Once a flash with sufficient intensity is reached, the b-wave appears earlier on the ERG trace than the STR and has a positive waveform; it is a reflection of depolarizing bipolar cells, and to a lesser extent the other cells residing in the inner nuclear layer[12, 19, 20]. Thus, the b-wave is a good indicator of the integrity of the inner nuclear layer circuitry[19]. The dim flash is not sufficiently bright to record changes by the few hyperpolarizing rods stimulated (no a-wave) but allows the detection of the amplification occurring in the inner nuclear layer by the rod system. The use of higher intensity flashes allows the detection of the photoreceptor a-wave[19, 21]. The a-wave is an even earlier waveform than the b-wave with a negative form[12, 21]. When the flash reaches a high enough intensity, a sufficient number of photoreceptors are hyperpolarized to be recorded on the ERG. Therefore, the a-wave is a measure of the normal activity of the

photoreceptors while the b-wave indicates the activity of the inner nuclear layer (horizontal, bipolar & amacrine cells)[12, 19-21].

1.3 The retina as model to study the development of the central nervous system

One of the major challenge in neuroscience is determining how the overwhelming variety of cell types are produced in the central nervous system. With its six major neuronal cell types and one glial cell, the retina is an attractive and accessible model to study the genetics of CNS development. Furthermore, it is the only part of the CNS that can be visualized non-invasively. Since the retina is not essential to the viability of the animal, genetic manipulation can target the retina without affecting the viability of the animal. The possibility of culturing an intact (flat mount) or dissociated retina in a controlled environment makes it a powerful tool to study the molecular machineries necessary for CNS development since the lineage of the different cell types are known.

1.4 Molecular mechanisms controlling cell fate in the mouse retina

1.4.1 Retinal progenitors

Lineage studies have demonstrated that a single retinal progenitor cell can give rise to all cell types and is thus considered to be multipotent[22-24]. Differentiation of retinal cell types follows an overlapping sequence in that different cell types are produced

simultaneously but initiation of cell type production follows a conserved order. In other words, the first ganglion cell is born before the first amacrine cell but some amacrine cells are born before some ganglion cells, depending of the region of the retina. In addition, differentiation follows a central to peripheral gradient with the first ganglion cells being born in the central retina. Birth-dating experiments using a combination of BrdU, a nucleotide analog incorporated during DNA replication of proliferating cells, and different cell type markers showed that there is an overlap in birthdates between cell types[25, 26]. This is due to the central to peripheral gradient of differentiation such that some peripheral ganglion cells are born after central amacrine cells[27]. Thus, the sequence of initiation of cell types production starts with ganglion cells, then horizontal cells, amacrine cells, cone photoreceptors, rod photoreceptors, bipolar cells and finally Müller glial cells[25]. A combination of transcription factors that specify the eye field promotes proliferation of RPC (retinal progenitor cell) such as Rx1, Pax6, Six6, Six3, Tlx, and Lhx2[28]. Some genes have different and sometimes opposing roles depending on the development stage of the retina. Furthermore, retinal progenitor cells seem to restrict their competence as development proceeds to allow production of all cell types in the correct order[7, 27, 29, 30]. When culturing retinal progenitors *in vitro*, the lineage of each clone will follow the sequential order of cell production mentioned above[22, 23]. As retinal progenitor cells divide and start producing postmitotic cells, progenitors become responsive to different external cues to allow production of different cell types[24, 26, 27]. This is due to the activation of some genes that are responsible for acquiring a certain phenotype and the inhibition of others involved in acquiring a different one[22, 31].

1.4.2 Extrinsic mechanisms

A variety of signalling molecules have been shown to influence cell fate choice in the retina. The family of FGFs secreted from the optic stalk (which will become the optic nerve) have been shown to influence the expression of another key family of signalling molecules, the Hedgehogs[32]. One of its family members, sonic hedgehog is secreted by ganglion cells, the first postmitotic cells[33, 34]. This seems to promote proliferation of RPC and prevent differentiation into the same ganglion cell fate, although its role seems to diverge between species[33]. The Notch pathway is also important in maintaining a heterogeneous cell population during retina development[35]. Cells expressing the Notch receptor are activated by the ligand Delta and this culminates in the expression of the Hes family of bHLH transcription regulators that will repress the expression of proneural genes and that of Delta[36]. This prevents adjacent cells from differentiating at the same time because a cell that is differentiating will activate proneural genes such as Mash1 and Ngn2 together with Delta to activate Notch receptors in neighboring cells. This common developmental mechanism throughout the central nervous system is called lateral inhibition[36]. Many more signalling molecules are present and have been shown to impact proliferation and differentiation of RPCs such as Wnt[37],VEGF[38], TGFβ[39], and BMP[40].

1.4.3 Intrinsic mechanisms

Exposing early progenitors to a postnatal environment does not alter the proliferative capacity and the cell fate choice of early and late progenitors[41]. Furthermore, dissociated late retinal progenitors develop similarly in a serum-free (no

growth factors) and low density (virtually no cell-cell communication) environment than in a serum-containing retina explant[23]. The number of times RPCs divided, the cell type they generated, and the order in which they generated these cell types were very similar. This demonstrates that at least for late progenitors, an intrinsic mechanism predominates and the role of signalling molecules is minimal. It has been proposed that homeodomain factors regulate the layer specificity but not the neuronal fate while bHLH activators determine the neuronal fate within the homedomain factor-specified layers[42]. Thus, combinations of proper bHLH and homeodomain factors are required for neuronal subtype specification.

1.5 Genes that regulate cell fate during retina development

(in chronological order in terms of initiation of production - period of production in mouse in parentheses)

1.5.1 Ganglion cell development (*~ E11-P0*)

During retina development the basic helix-loop-helix transcription factor *Math5* is expressed in postmitotic cells and renders them competent to become retinal ganglion cells (RGC). Targeted disruption of the *Math5* gene leads to an 80% reduction of ganglion cells[43]. In the chick retina, misexpression of *Math5* failed to generate ganglion cells efficiently[44]. Further studies revealed that the POU domain transcription factor *Brn3b* and the LIM homeodomain transcription factor *Isl1* are expressed by newly born

ganglion cells and are essential for ganglion cell survival and maturation[45]. This role in terminal differentiation seems to be conserved in the spinal cord for *Isl1* and in the olfactory system in *Drosophila.* Specifically, in the spinal cord, *Isl1* forms a specific transcriptional complex with other LIM-HD transcription factors and other non-DNA binding LIM domain-containing cofactors such as *clim2* and the LIM-domain only (LMOs) family of cofactors such as *LMO4* to specify and segregate motoneurons from interneurons and subsequently promote proper axonal pathfinding. Similarly, in the *Drosophila* olfactory system, *Acj6* and *drifter*, two POU-HD transcription factors are required for proper dendritic targeting. It was also shown that these two classes of transcription factors can cooperate together in neuronal differentiation. In *C. Elegans,* the POU-HD transcription factor UNC-86 and the LIM-HD transcription factor MEC-3 dimerize on the *mec-3* promoter to maintain *mec*-3 expression which is required for touch receptor differentiation. It was shown that *Math5* regulates the expression of these two RGC-specific transcription factors necessary for RGC differentiation. Thus a combination of *Math5*, *Brn3b*, and *Isl1* is necessary for proper ganglion cell production. In addition, the Bar-class homeodomain transcription factor *Barhl2* has been show to act downstream of *Math5* and *Brn3b* to specify a subset of ganglion cells[46] (Figure 4).

1.5.2 Horizontal cell development (~ E12-E16)

Horizontal cell genesis is regulated by *Foxn4*[47], *Ptf1a*[48] and *Prox1*[49]. If any of these three genes are mutated, horizontal cell genesis is compromised. Forced expression of *Prox1* results in the generation of horizontal cells[49], indicating that *Prox1* is involved in horizontal cell production (Figure 4).

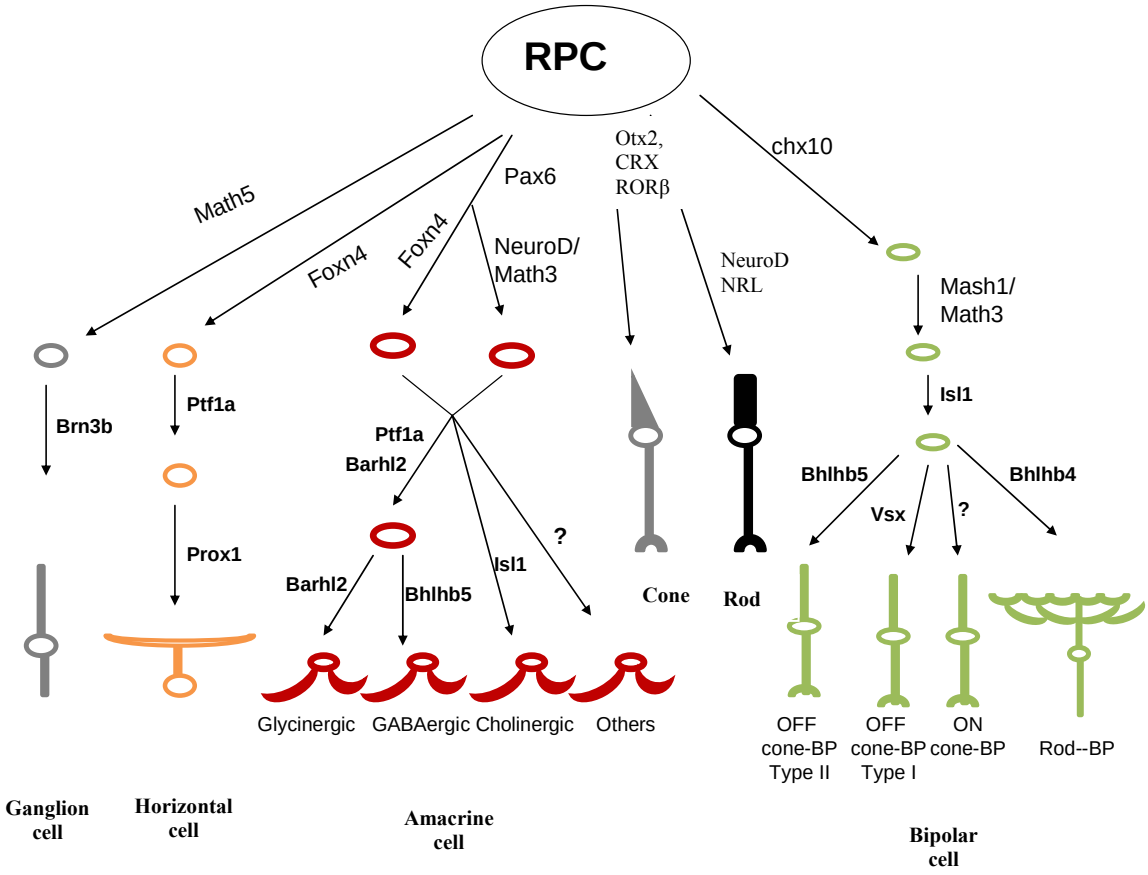


Figure 4: Neurogenesis in the retina. Retinal progenitor cells are multipotent and give rise to all cell types. A combination of homeodomain and bHLH transcription factors are necessary for fate specification. Studies are beginning to identify the factors necessary for subtype specification within a major neuronal type. For example, the specification of glycinergic, GABAergic and cholinergic amacrines are regulated by different factors downstream of *Pax6*, *NeuroD*/*Math3*, and *Foxn4*.

1.5.3 Amacrine cell development (*~ E12-P2*)

The neural bHLH genes *Math3* and *NeuroD* are expressed in retinal progenitors at embryonic stages when amacrine cells are generated. Even though *Math3/NeuroD* double mutant embryos exhibit selective loss of amacrine cells, misexpression of either *Math3* or *NeuroD* only promotes the generation of rod photoreceptor cells[42, 50]. Although misexpression of *Math3* or *NeuroD* alone fails to generate amacrine cells, misexpression of Pax6 together with *Math3* or *NeuroD* promotes amacrine cell genesis[50], indicating that the combination of the bHLH and homeobox genes is critical for amacrine cell fate determination. Thus, these bHLH proneural genes expressed throughout the developing CNS are not just involved in the binary choice "glial versus neuron" but is necessary to further segregate neuronal cell types within the neuronal lineage. The forkhead/winged helix transcription factors constitute a large family of important proteins characterized by a 110 amino acid DNA binding domain that folds into a variant of the helix-turn-helix motif that consists of three α helices flanked by two large loops (wings)[47]. *Foxn4* is a recently identified member of this gene family that is highly expressed during retina development[47, 51]. *Foxn4* is expressed by a subset of lineage-biased retinal progenitors and it controls the genesis of amacrine and horizontal cells by regulating the expression of downstream factors such as *NeuroD*/*Math3* and *Ptf1a*[48, 51]. Targeted disruption of the winged helix/forkhead transcription factor *Foxn4* largely eliminated amacrine cells while overexpression using a retroviral vector strongly promoted the amacrine fate[47]. It is still unclear whether *Foxn4* functions upstream of *NeuroD*/*Math3* or in parallel but it has been suggested that the expression of *Foxn4* in progenitors renders them competent to become amacrine cells. Downstream of *Foxn4*, the bHLH transcription factor *Ptf1a* also plays a

crucial role in amacrine cell development[48]. *Ptf1a* plays a crucial role in pancreatic development in mice[52]. *Ptf1a* has also been shown to be necessary in GABAergic neuronal specification in the cerebellum[53]. In *Ptf1a* null retina, amacrine cells are greatly reduced[48]. In a separate study, it was found that inhibitory glycinergic and GABAergic amacrines were completely lost in *Ptf1a* knock-out retinal explants[54]. It was shown that *Mash1* controls the expression of *Ptf1a* and that *Mash1* is expressed in sensory progenitors and controls the balance between excitatory and inhibitory cell fates in the developing mouse spinal cord[55]. Further downstream, the same LIM-HD transcription factor necessary for RGC development, *Isl1*, is necessary for amacrine cell production since loss of *Isl1* results in almost a complete loss of cholinergic amacrine cells[56]. In a parallel differentiation pathway, *Bhlhb5* seems to control GABAergic amacrine production since *Bhlhb5* targeted deletion leads to their selective loss[57]. *Bhlhb5* is a member of the Olig subfamily of bHLH transcription factors[57]. It has previously been shown to be expressed in CNS development[58], sensory organs[59], kidney and hair follicles[60]. Due to its inability to bind DNA alone or in combination with other bHLH proteins, *Bhlhb5* is thought to function as a negative regulator of other bHLH proteins[61]. *In vitro* evidence has shown that *Bhlhb5* represses *Pax6* promoter activity through a non-DNA-binding mechanism[62]. *Bhlhb5* is predominantly expressed in post-mitotic cells in the developing mouse retina[57]. Co-localization of *Bhlhb5* and retinal cell type-specific markers revealed that Bhlhb5

expression is restricted to GABAergic amacrine and Type 2 OFF-CB cells[57]. Targeted deletion of *Bhlhb5* leads to a loss of Type 2 OFF-CB and GABAergic amacrine cells[57]. Expression studies of early embryonic retinas have demonstrated that *Bhlhb5* is mostly

expressed in cells of the *NeuroD*+ and *Math5*+ lineage[57]. These studies strongly argue for the crucial role of *Bhlhb5* as a factor downstream of the bHLH-class of retinogenic factors in the specification of amacrine and bipolar subtypes.

On the other hand, forced expression of the Bar-class homeodomain transcription factor *Barhl2* in retinal progenitors promotes the differentiation of glycinergic amacrine cells, whereas a dominant-negative form of Barhl2 has the opposite effect (inhibited differentiation)[63]. In a "partially" contradicting recent study, it was found that both glycinergic **and** GABAergic amacrine cells were reduced together with ganglion cells in Barhl2 null retina[46]. Thus, the mechanisms regulating amacrine cell genesis are starting to be clarified (Figure 4). With over 29 subtypes, much work remains to be done to clarify the precise transcriptional mechanisms regulating subtype specification.

1.5.4 Photoreceptor cell development (*cones ~ E12-E17, rods ~ E14-P10*)

The homeobox gene *Crx* and *Otx2* are key molecules regulating photoreceptor cell development[64, 65]. *NeuroD* is a candidate bHLH gene with the ability to actively drive retinal progenitors toward a photoreceptor cell fate since *NeuroD* misexpression results in enhanced rod cell genesis[50]. *Nrl*, a basic leucine zipper transcription factor regulates fate decisions between rod and cone through activation of the orphan nuclear receptor *Nr2e3* which activates rod-specific genes[66] (Figure 4).

1.5.5 Bipolar cell development (~ *E15-P10*)

Mutation in the homeobox gene *Chx10* (also known as *Vsx2*) results in a complete loss of bipolar cells[67]. *Chx10* is a homeobox gene containing a CVC domain. Another

CVC-containing gene expressed in bipolar cells is *Vsx1*[68]. These two CVC-containing genes have been shown to be expressed by two distinct bipolar subtypes[69, 70]. Rod-bipolar cells express *Chx10*[71] while cone bipolar cells express *Vsx1*[68, 70]. In addition, in *Mash1-Math3* double knock-out mice, *Chx10* expression did not disappear despite a complete loss of bipolar cells[72]. Misexpression of *Chx10* was not sufficient to generate mature bipolar cells[42]. Similarly, misexpression of *Mash1* or *Math3* alone did not induce bipolar cells but inhibited Müller cell production[72]. Interestingly, when *Chx10* is misexpressed together with *Mash1* or *Math3*, there is induction of bipolar cell development[42]. Although initial bipolar cell specification is largely unaffected in *Isl1*-null retina, bipolar cells are greatly reduced by the time the retina reaches maturity indicating a role in subtype specification and/or maturation[56]. Of the known factors regulating subtype specification, *Bhlhb5*[57] and *Vsx1*[68] are necessary for OFF-cone bipolar cell genesis and maturation, respectively, while *Bhlhb4*[73] is necessary for rod bipolar cell genesis. Interestingly, *Vsx1* mutant mice have defects in their cone visual pathway while their rod visual pathway remains unaffected[68]. Thus, *Vsx1* seem to be necessary to specify cone bipolar cells and *Bhlhb5* acts downstream to specify OFF-CB while *Bhlhb4* functions downstream of *Chx10* to specify rod-bipolar cells. Thus, initial bipolar cell genesis is regulated by two different classes of transcription factors, the bHLH genes *Mash1* and *Math3*, and the homeobox gene Chx10 (Figure 4) while subtype specification is further regulated by different combinations of bHLH and HD transcription factors.

1.6 Gliogenesis (*Müller glial cell development ~ E17-P11)*

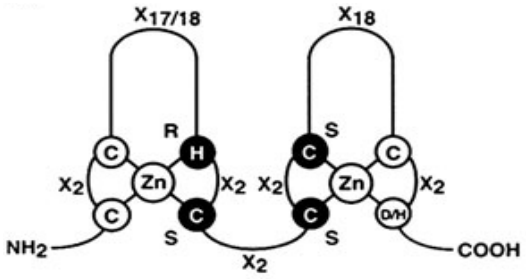
Initially, immature Müller glial cells are important for the proper histological organization and wiring of neuronal circuits[18]. They also serve as a migration substrate for young neurons[74]. It has been shown that under certain conditions young Müller cells can re-enter the cell cycle to produce new neurons and/or Müller cells[75]. Notch signaling is known to maintain cells in an undifferentiated state[76]. Rax is a homeobox gene implicated in Müller glial cell genesis[76]. Misexpression of *Rax*, *Hes1*, or the *Notch1* ICD (intracellular domain) efficiently promotes the generation of Müller glial cells[76]. The maturation of Müller glial cells works in concert with the maturation of retinal neurons. After neurons establish their synapses, Müller cells send side branches towards newly formed synapses and express glutamate uptake transporters in their membrane to serve their neurotransmitter recycling role. In addition, Müller glial cells control the vascularization of the retina[77] and form the blood-retina barrier[78].

1.7 LMO4

LMO4 consists of two LIM domains or four tandem zinc fingers (Figure 5). The LIM domain, an evolutionary conserved motif, is made of two cysteine and histidine-rich zinc fingers[79-81]. The LIM domain is characterized by a double zinc finger structure and is found in proteins that play different critical functions in cell fate determination,

LIM only domain 4 (LMO4)

LIM domain



LMO

Figure 5: Protein structure of LMO4. The LIM domain consists of two cysteine and histidine-rich zinc fingers. LMOs consists of two tandem LIM domains, or four tandem zinc fingers.

differentiation, and cytoskeleton organization[79]. It is found in a variety of nuclear and cytoplasmic proteins, including LIM-only (LMO), LIM kinase, and focal adhesion proteins[79]. LIM domains may occur by themselves such as LMOs or in association with functionally divergent domains such as in LIM-homeodomain (LIM-HD) transcription factors consisting of a LIM domain and a DNA binding homeodomain[79]. One of the central functions of the LIM domain is to mediate protein-protein interactions, which may have either positive or negative effects on gene transcription[79, 80]. The LMO subclass of LIM proteins comprises four members (LMO1 to LMO4), each of which is defined by two LIM domains[82]. The *LMO1* and *LMO2* genes were originally identified by their translocation in acute T-cell leukemia, and their overexpression in transgenic mice leads to T-cell tumors[82]. *LMO2* has been established to have a critical function in early hematopoiesis[83] and angiogenesis[84]. *LMO3* is the least understood member of the LMO family. It was first cloned on the basis of its sequence homology and was found to be highly expressed in the brain and some vestibular ganglion cells . Then the expression of *LMO3* was found to be upregulated in astrocytes treated with dopamine[85]. Another study has shown that LMO3 is expressed at high levels in subsets of neuroblastoma and is a neuronal-specific oncogene in neuroblastoma cells[86]. *LMO4* was identified by virtue of its interaction with the ubiquitous cofactor protein Ldb1/NLI/CLIM2 and in an expression screen using autologous serum from a breast cancer patient[87, 88]. It is the most divergent member of the family and is widely expressed in both embryonic and adult tissues, including thymus, skin, and distinct regions within the brain[87-89]. The *LMO4* gene is also highly expressed in the proliferating mammary gland and is overexpressed in more than 50% of primary breast cancers, underscoring its

importance in the regulation of cell growth[90]. *LMO4* is widely expressed during central nervous system development[88, 89, 91-96] but remains poorly explored even though its importance is evident from the severe exencephaly phenotype[97-99], abnormal motor development in the spinal cord[93, 95, 100] and abnormal cortical development[92, 94, 99], resulting from the loss of the two alleles (*LMO4* -/-). In the spinal cord *LMO4* is differentially expressed in adductor and sartorius sensory neurons and it was suggested that it regulates the activity of LIM-HD transcription factors to regulate cell fate choice and proper axonal pathfinding to the different muscles[89]. It was also shown to be crucial in segregating motoneurons from V2 interneurons in the spinal cord[95]. Being expressed in motoneuron progenitors, LMO4 prevents the transcriptional complex (the V2-tetramer) necessary for V2 interneuron differentiation from forming by sequestering LIM-HD[95]. Without LMO4, the V2 tetramer is more favourable due to its lower energy in comparison to the Motoneuron-hexamer which needs another transcription factor. Because of LMO4, the less favourable transcriptional complex can thus form and activate motoneuron-specific genes. This is an example of a negative regulation. Within the V2 interneurons, LMO4 controls the balance between excitatory and inhibitory interneurons by forming a one-to-one complex with SCL, Gata2, and Clim2 to promote V2b inhibitory interneuron formation[93]. Thus, LMO4 promotes motoneuron differentiation by inhibiting a competing V2 interneuron transcription complex and later, within the V2 interneuron precursors, it forms a transcriptional complex to activate V2b inhibitory interneuron differentiation. In addition, LMO4 augments the transcriptional inhibitory activity of the bHLH factor HEN1 in cultured hippocampal neurons and controls neurite outgrowth[101]. Mice deficient in LMO4 die prior to birth and 50% of embryos display exencephaly[97-99].

Ablation of *LMO4* in cortical neurons impairs thalamocortical projections to layer 5[94]. Ablation of one of the two paralogs of *LMO4* in zebrafish increases the size of the eye[102]. In the chick, LMO4 augments Wnt signaling in the progenitors of the ciliary marginal zone of the retina[103].

LMO4 is expressed in the amacrine cell layer of the postnatal day 0 mouse retina[96], but the functional importance of *LMO4* in the development of the mammalian retina has not been studied.

In characterizing the phenotype of LMO4 deficient mice we noticed a structural defect in the eye. This defect, coloboma, results from incomplete closure of the optic fissure during development. To understand the functional significance of *LMO4* in mouse retinal development, we conditionally ablated a floxed *LMO4* allele in the retina using the *Pax6* alpha enhancer driven *Cre* recombinase. We chose the alpha enhancer because it is expressed early during retina development in retinal progenitor cells in the ventral region of the retina but almost nowhere else. This allowed for the normal development of the embryos except for the targeted region, the retina. Whereas these mice do not develop coloboma and have normal-sized eyes, using neuron subtype-specific markers, we observed a reduction in GABAergic amacrine and OFF-cone bipolar cells. Electroretinograms revealed a functional deficit in these mice.

1.8 Hypotheses

LMO4 is necessary for cell fate choice during retina development

1.9 Objective

To characterize the role of LMO4 in cell fate specification in the mouse retina.

1.10 Summary of findings

We observed the presence of a smaller eye and/or coloboma in ~40% of *LMO4* null mouse embryos. To investigate the postnatal role of LMO4 in retinal development and function, LMO4 was conditionally ablated in retinal progenitor cells using the Pax6 alpha-enhancer Cre/LMO4flox mice. We found that these mice have fewer Bhlhb5-positive GABAergic amacrine and OFF-cone bipolar cells. The deficit appears to affect the postnatal wave of Bhlhb5+ neurons, suggesting a temporal requirement for LMO4 in retinal neuron development. In contrast, cholinergic and dopaminergic amacrine, rod bipolar and photoreceptor cell numbers were not affected. The selective reduction in these interneurons was accompanied by a functional deficit revealed by electroretinography, with reduced amplitude of b-waves, indicating deficits in the inner nuclear layer of the retina.

2. Materials and Methods

2.1 Animals

All experiments in animals were approved by the University of Ottawa animal care ethics committee, adhering to the guidelines of the Canadian Council on Animal Care. The *Pax6* α-Cre transgenic mice with a downstream GFP reporter tag were obtained from P. Gruss [104] and were maintained on a C57BL/6 background. α-*Cre*/wt mice were crossed to homozygote *LMO4*flox/flox mice [99] to generate F1 heterozygotes that were backcrossed to produce α-*Cre*/LMO4flox homozygote mutant or littermate control (*LMO4*flox/flox) mice. *LMO4* null mice were described previously[97]. Genotyping for the α-Cre transgene, *LMO4*flox or *LMO4* null alleles was performed by PCR using forward primer CCG CAG AAC CTG AAG ATG TTC and reverse TCA TCA GCT ACA CCA GAG ACG .

2.2 Immunohistochemistry

Embryos and dissected eyes plus optic nerves from animals younger than P7 were fixed in 4% paraformaldehyde (PFA) overnight in 0.1 M phosphate buffered saline (PBS) pH 7.4. Older animals were transcardially perfused with 4% PFA prior to dissection of the eyes and to overnight incubation at 4°C. Following PBS washes, samples were cryoprotected overnight in a 30% sucrose/PBS solution. Tissues were embedded in a 1:1 mix of 30% sucrose and OCT (Tissue-Tek, Japan) and frozen by immersion in -30°C 2-methyl butane. Embedded tissue was sectioned on a cryostat at 14 μm. Sections were

transferred onto Superfrost Plus coated slides (Fisher Scientific, USA) and stored at -80°C. IHC was performed according to the protocol for fluorescence detection described previously[105-107]. The following primary antibodies were used: goat polyclonal anti-Bhlhb5 (1/100), anti-LMO4 (c15) (1/50), anti-Brn3b (c13) (1/100, Santa Cruz Biotechnology), anti-choline acetyltransferase (ChAT) (1/2000, Millipore); sheep polyclonal anti-Chx10 (1/2000, Exalpha Biologicals, Inc); rabbit polyclonal anti-calretinin (1/2000, Swant), anti-tyrosine-hydroxylase (TH) (1/2000, ImmunoStar), anti-Prox1 (1/3000, Chemicon International), anti-phosphorylated histone 3 (Ser-10) (1/400, Upstate) and anti-activated caspase 3 (Asp175) (1/100, Cell Signaling; kind gift from David Park); mouse monoclonal anti-Pax6 (1/50; Developmental Studies Hybridoma Bank), anti-PKC (1/100, BD Pharmingen), anti-calbindin (1/500, Sigma), anti-rhodopsin (1/3; Developmental Studies Hybridoma Bank), anti-peanut agglutinin (PNA) (1/500, Santa Cruz Biotechnology), anti-HPC (syntaxin)(1/2000, Sigma). Antibodies were visualized with appropriate secondary antibodies conjugated with Cy3 and Cy2 and visualized on a Zeiss Z1 fluorescence microscope. For LMO4, Pax6, Brn3b and PKC immunostaining, antigen was unmasked with microwave treatment in 10 mM citrate buffer (in 1L water add 42g citric acid, 21 g Sodium hydroxide, pH=6) using a 1000 watt microwave set at power 6 for 10 min and sections were cooled on ice for 10 min before processing for immunostaining.

To compare gene expression and differentiation, immunostained positive cells in the same region of the peripheral retina at the optic nerve plane were counted with Zeiss AxioVision automatic software. For PKC+ rod bipolar and Bhlhb5+ amacrine and cone bipolar cells, cells were counted manually. At least 3 comparable retinal sections of each

mouse and 3 mice of age-matched genotype were used for statistical analysis by Student t test and $p<0.05$ were considered to be significant.

2.3 *In situ* hybridization

Tissues were processed for in situ hybridization with digoxigenin-labeled antisense or sense riboprobes, as previously described[108]. For dual in situ hybridization and immunohistochemistry, biotin-labeled LMO4 antisense riboprobes were used, followed by biotin/avidin ABC kit (Vector laboratory) and cy2- conjugated streptavidin (Jackson laboratory) together with anti-Bhlhb5 goat antibody and cy3-conjugated anti-goat secondary antibody (Jackson laboratory). For more reliable comparisons of gene expression patterns, wild-type and mutant tissues were processed on the same slides.

2.4 Electroretinography (ERG)

The electroretinogram is a non-invasive technique and the mice usually wake up a couple of hours after the experiment (some mortality occurs probably due an overdose of the anaesthetic but the majority survives). Since the mice cannot be returned to the animal facility after being taken out for this experiment, the mice were sacrificed in order to extract the retina for histological experiments. First, the mice are taken out of the animal facility at around 3:00pm and dark-adapted overnight. The following morning, usually from 9:00am to 1:00pm, the electroretinograms of 12 mice are recorded and in the afternoon, the retinas are extracted. ERG was performed using the ESPION system (Diagnosys LLC, Littleton, MA) as previously described[109, 110]. Briefly, mice that had been dark-adapted overnight were anaesthetized under safe light conditions using an

intraperitoneal injection of avertin at 250 mg/kg. Eyes were dilated using both 1% tropicamide and 2.5% phenylephrine hydrochloride (Alcon Canada). To ensure constant body temperature during testing, mice were put on a warming source. Silver wire loop electrodes were placed on both corneas with a drop of 0.3% hypromellose (Novartis) to maintain corneal hydration. A gold minidisc reference electrode was placed on the tongue and a ground needle electrode was placed subcutaneously in the tail. The animal's head was positioned under the center of the Ganzfeld dome. Single flash stimuli (4 ms duration) were presented at 11 increasing intensities ranging from 0.001 to 25 cd s/m2. Five ERG traces were obtained and averaged for each luminance step. The minimum negativity occurring between 10 and 40 ms post-stimulus was defined as the a-wave. The maximum positivity occurring between 40 and 80 ms poststimulus was defined as the b-wave[110]. Differences during ERG analyses were determined using ANOVA statistical analysis.

2.5 Bhlhb5 promoter activity assay

Using a mouse targeting vector Bhlhb5-lacZ[57] (gift of Dr. Lin Gan, University of Rochester, NY, USA), a 2,110 bp fragment containing sequence from –1,898 to +212 relative to the start site of transcription was amplified by PCR using sequence-specific oligonucleotides Bhlhb5-fwd 5'-TTCTCGAGGGAGCCTCTCATTAGC-3' and Bhlhb5-rev 5'-ATAAGCTTCCAGCCGACGGTGCT-3'. The PCR product was cloned between the XhoI and HindIII sites of the pGL3-basic vector and transfected into F11 neuronal cells using Lipofectamine 2000 as described previously[107] with control shRNA or shRNA specific to LMO4. LMO4shRNA (clone ID TRCN0000084375) and control scrambled non-silencing shRNA were purchased from OpenBiosystems. Transfection efficiency

was normalized using a CMV enhancer-driven beta-galactosidase reporter plasmid as described[111].

3. Results

3.1 LMO4 null mice have developmental eye defects.

In phenotyping *LMO4* null mice, we identified several features of altered eye development including smaller eyes and the presence of a coloboma phenotype in ~40% of embryos (Figure 6). Although exencephaly was often associated with the eye phenotype, defective eye development was noted in some *LMO4* null mice without exencephaly (Figure 6B, rightmost panel), indicating that these phenotypes are independent or only partially penetrant.

3.2 LMO4 is expressed in the retina during development.

Given its well documented role in CNS development and the observed eye phenotype, we tested the possibility of *LMO4* being expressed in the retina during development**.** Unfortunately, our *LMO4* antibody did not detect any *LMO4* protein at embryonic day 14.5 in the retina; this is likely due to the low expression levels of LMO4 protein that is below detectable levels. The *LMO4* mRNA transcript was detected starting at E14.5 by *in situ* hybridization (Figure 7A). From E14.5 to P0, *LMO4* seems to be restricted to the neuroblast layer where progenitors and nascent horizontal and amacrine cells are located and excluded from ganglion cells within the ganglion cell layer (Figure 7A). Only at P0 was *LMO4* detected in the ganglion cell layer, suggesting that *LMO4's* expression in the

ganglion cell layer might only be in displaced amacrine cells (Figure 7A). A sense control (with the same purine/pyrimidine composition and the same sequence as the *LMO4* mRNA transcript) was used to confirm the specificity of the probe (Figure 7B) Given our past experiences with this particular antibody being unable to detect lower levels of expression in the embryonic brain, we tried detecting the mRNA transcript of the *LMO4* gene by *in situ* hybridization. At postnatal day 0 (P0), *LMO4* was found to be highly expressed in amacrine cells in the inner nuclear layer, in either (or both) displaced amacrine cells and ganglion cells in the retinal ganglion cell layer and lower levels were detected in retinal progenitor cells (Figure 7C). In contrast to embryonic stages, at postnatal day 0 (P0), *LMO4* protein was detectable using our antibody. Immunostaining at P0 showed that *LMO4* is co-expressed with the amacrine cell marker Pax6 in the inner nuclear layer (arrowheads, Figure 8) and in displaced amacrine cells (and/or ganglion cells) in the retinal ganglion layer (arrows, Figure 8), confirming our initial findings by *in situ* hybridization . LMO4 protein expression was again below detectable levels at P15 when the retinal layers and neuronal subtypes are fully developed (data not shown).

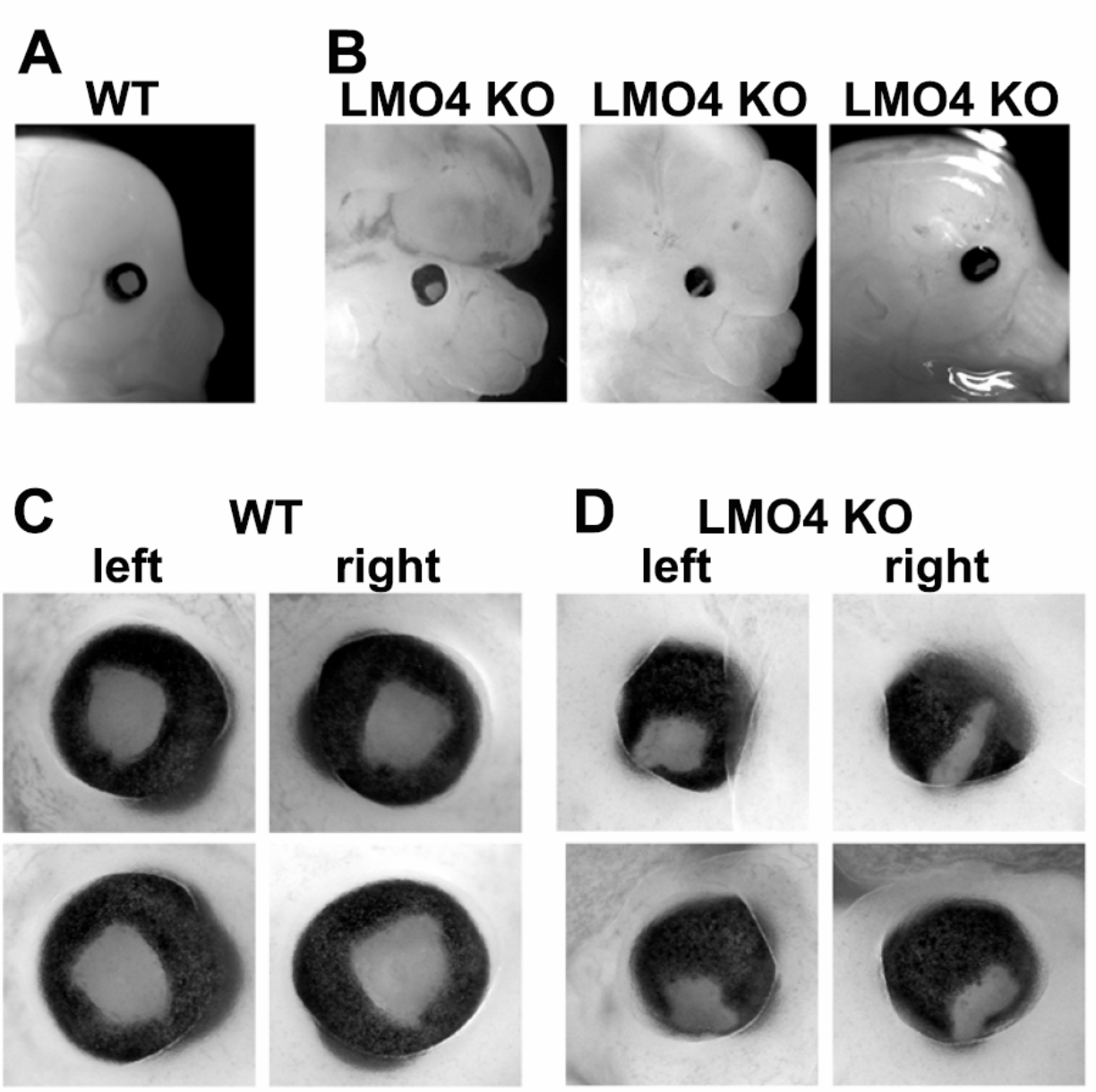


Figure 6: LMO4 null mice have a partially penetrant neural tube closure defect and coloboma.
(A) Wild type mice at embryonic day 14.5 (E14.5) show normal forebrain and eye development. (B) Three phenotypically divergent LMO4 null (LMO4 KO) mouse embryos reveal exencephaly with relatively normal eye (left panel), exencephaly with a small eye and coloboma (middle panel), and non-exencephalic phenotype but with coloboma. (C & D) Close up views of the left and right eyes of wild type and LMO4 KO mice, respectively.

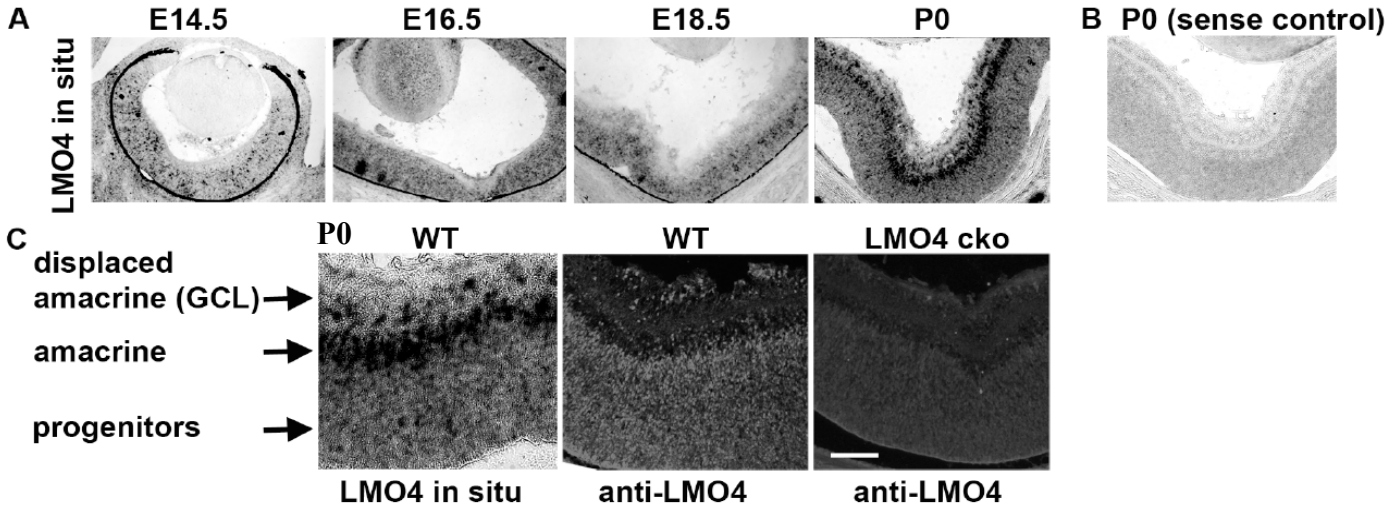


Figure 7: . Pattern of LMO4 expression during retinal development.
(A) In situ hybridization reveals gradually elevated expression of LMO4 mRNA from E14.5 to postnatal day 0 (P0). (B) Sense control probe shows background staining to be compared to the antisense probe used in (A). (C) Close-up view of P0 wild type (WT) retina shows high LMO4 mRNA levels in the displaced amacrine and amacrine layers and lower diffuse expression in the retinal progenitors. Immunofluorescence with anti-LMO4 antibody shows associated pattern of LMO4 expression in a P0 wt retina section and background staining in the LMO4 α-Cre conditionally ablated retina (LMO4 cko). Scale bar, 100 μm.

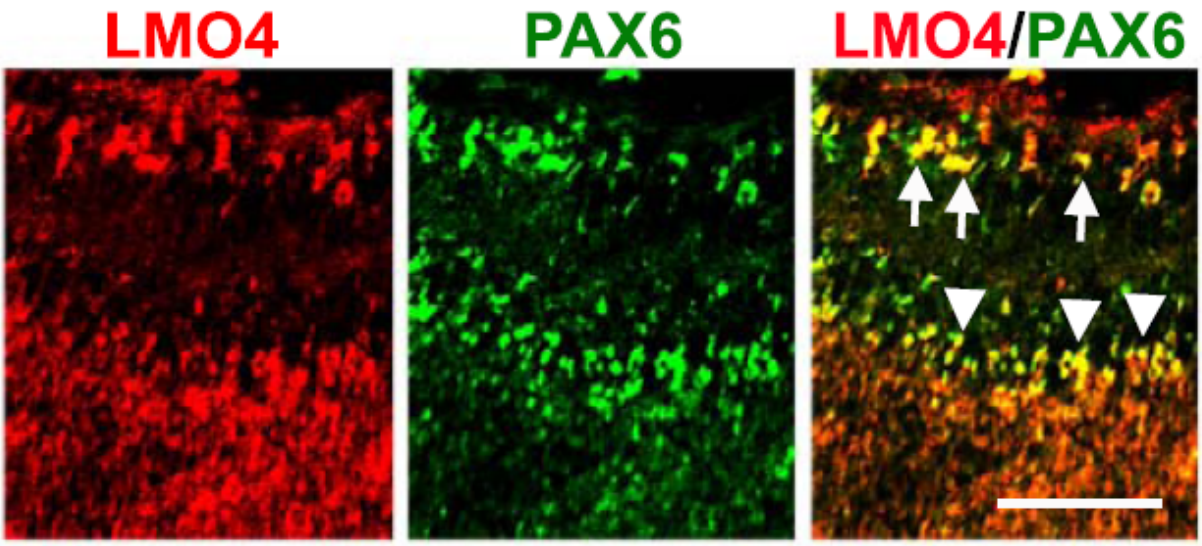


Figure 8: LMO4 is colocalized with Pax6 in amacrine and displaced amacrine cells. Dual immunofluorescence staining reveals LMO4 cells in red, Pax6 cells in green and dual labeled cells appear yellow. Amacrine cells are indicated by arrowheads and displaced amacrine cells in the retinal ganglion cell layer by arrows. Scale bar, 50 μm.

3.3 Conditional ablation of LMO4 in the retina

Retinal cell differentiation begins at around E12 and continues into the first postnatal week[7]. Since *LMO4* expression increases as the retina develops and seems to be maximal at P0, we wanted to study further LMO4's role in retina development and hypothesised a potential role in cell fate choice of RPC. Given that *LMO4* null mouse embryos die before birth, it was impossible to determine how LMO4 is required for postnatal retinal development and function. Thus, we conditionally ablated *LMO4* in the developing retina by crossing LMO4flox mice with α-Cre-IRES-GFP transgenic mice where the Cre recombinase and a GFP reporter are driven by the retinal neural progenitor-specific *Pax6* enhancer (*Pax6* alpha enhancer)[112]. α-*Cre* transgene is active in the peripheral retinal progenitors of the embryo and stably expressed in mature amacrine cells[104]. We confirmed expression of the GFP reporter predominantly in the ventral peripheral retinal progenitor cells at E14.5. By P0, all amacrine cells expressed the GFP reporter (and by inference the Cre-recombinase) (Figure 9). All GFP+ cells were Pax6+, indicating their amacrine identity (data not shown). Immunostaining showed that LMO4 is ablated in the peripheral retina of the α-Cre/LMO4flox mice at P0; to simplify, we abbreviate these as LMO4 conditional knockout (LMO4 cko) mice (Figure 7C).

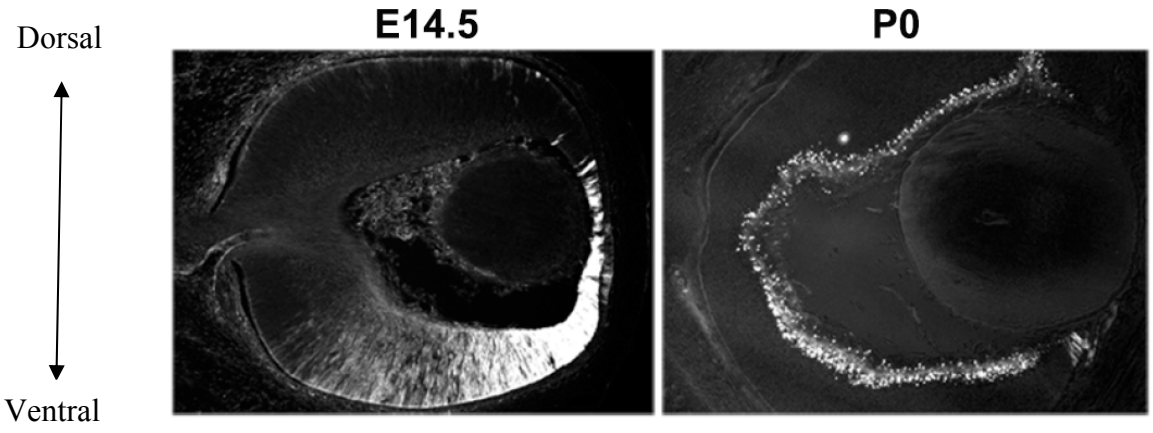


Figure 9: Expression of the green fluorescent protein (GFP) reporter from the Pax6 α-enhancer in αCre-IRES-EGFP mice.
At embryonic day 14.5 (E14.5), GFP is highly expressed in the peripheral retina, predominantly in the ventral compartment. By postnatal day 0 (P0), GFP expression is widespread in the amacrine cell layer. GFP expression is an indicator of where Cre-recombinase is expressed.

3.4 Reduced Bhlhb5+ amacrine and bipolar cells in alpha-Cre/LMO4flox mice

By immunostaining with cell-type specific markers at P15, we further determined how conditional ablation of *LMO4* in retinal progenitors affects development of the peripheral retina at the optic nerve plane (Figure 10). Nuclei stained with DAPI showed no obvious anatomical difference in the retina of the *LMO4* cko mice compared to littermate controls. The amacrine and ganglion cell marker Pax6 showed a slight (15%) reduction of amacrine cells in *LMO4* cko mice. Using amacrine subtype specific markers, we found a 30% reduction in the number of GABAergic (Bhlhb5^{+}) amacrine cells (Figures 10 & 11A), but no difference in the number of cholinergic (ChAT^{+}) or dopaminergic (TH^{+}) amacrine cells (Figures 11A & 12). In addition to amacrine cells localized at the inner region of inner nuclear layer, Bhlhb5 also labels subsets of cone bipolar cells localized at the outer region of the inner nuclear layer [57]. There was also a 30% reduction in the number of Bhlhb5+ cone bipolar cells in LMO4 cko mice compared to littermate controls (Figure 11A). In contrast, there was no difference in the number of rod bipolar cells that are immuno-positive for protein kinase C (PKC) (Figures 11B & 12). Since we observed a 30% reduction in the number of cells labelled with a pan-bipolar cell marker Chx10 (Figures 10 & 11B), this result suggests that in addition to Bhlhb5+ OFF-cone bipolar cells, LMO4 is also required for differentiation of other Bhlhb5-negative cone bipolar cells.

No difference in the number of retinal ganglion cells (Brn3b+), horizontal (calbindin+), rod (rhodopsin+) or cone (peanut agglutinin+, PA+) cells was observed (Figure 12). In addition, the number of photoreceptor nuclei in the outer nuclear layer

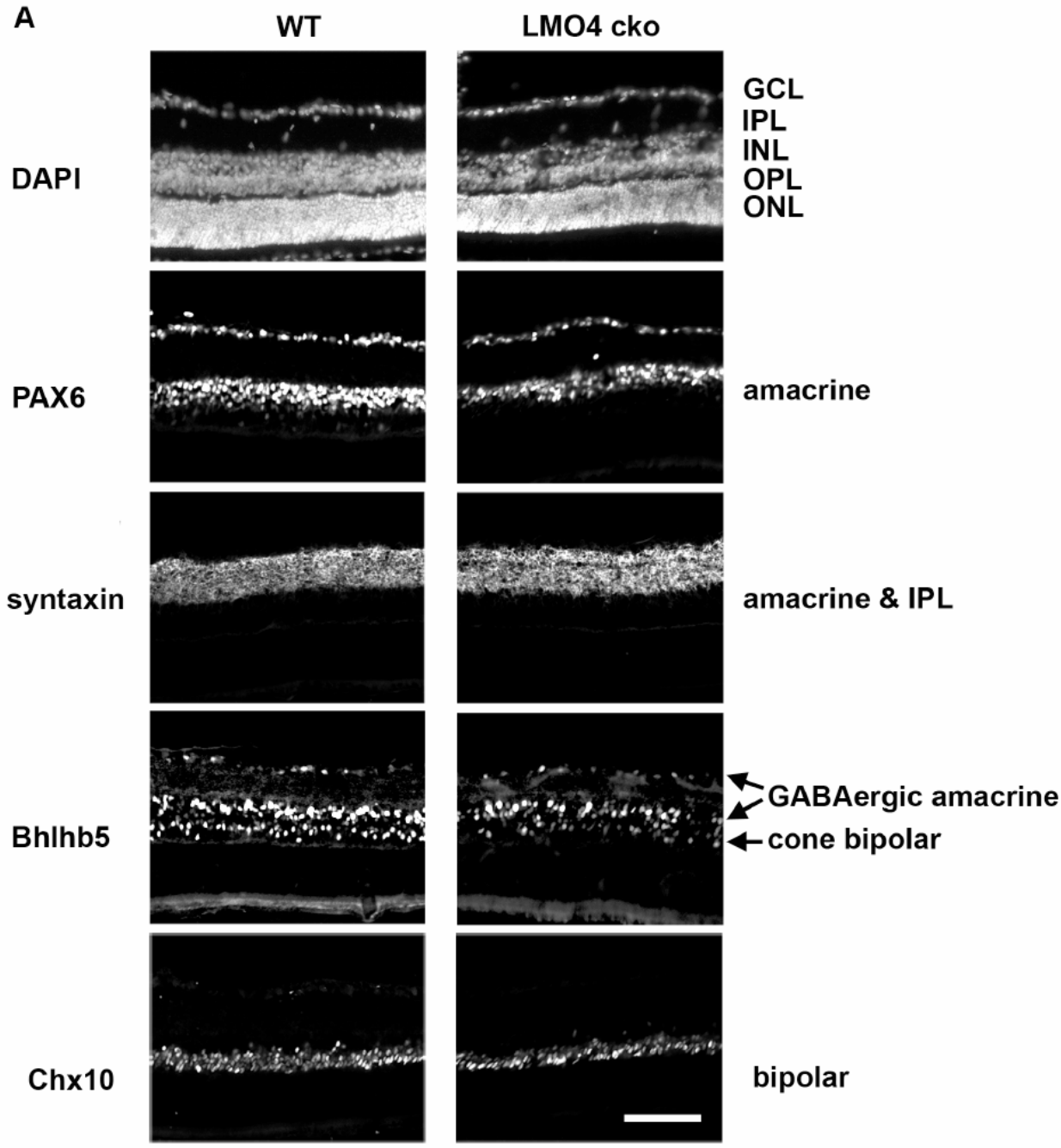


Figure 10 Selective loss of Bhlhb5 amacrine cells and cone bipolar cells in LMO4 cko mice. Neuron-specific markers were used to phenotype in P15 retinas the functional consequences of LMO4 ablation in retinal progenitors. DAPI stained all retinal nuclei of the ganglionic cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL); Pax6 labeled all amacrine cells; Syntaxin labels amacrine cell bodies and the IPL; Bhlhb5 labels GABAergic amacrine cell and OFF-cone bipolar cell nuclei; Chx10 labels bipolar cell nuclei. A significant reduction in Pax6+, Bhlhb5+, Chx10+ cells was observed in LMO4 cko mice. Scale bars, 100 μm.

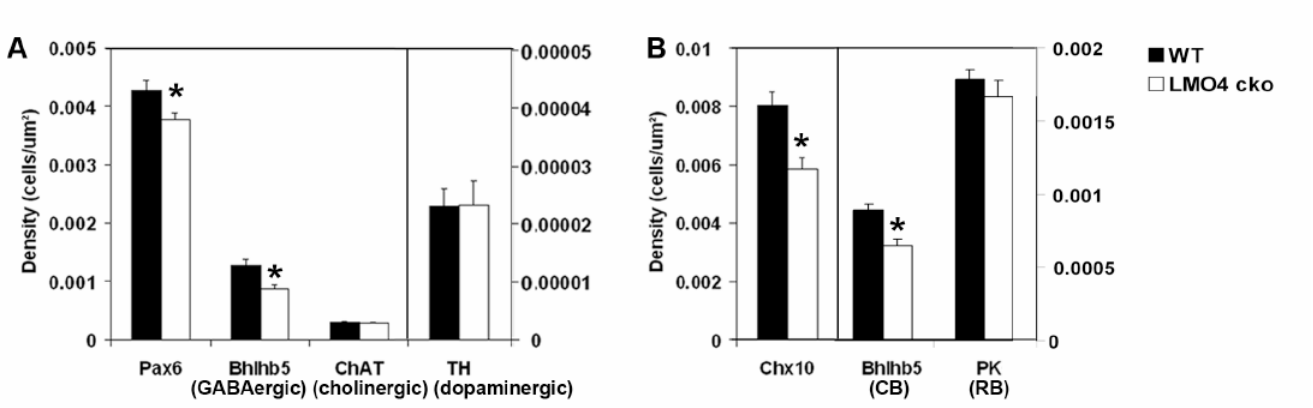


Figure 11: Selective loss of Bhlhb5 amacrine cells and cone bipolar cells in LMO4 cko mice is statisticaslly significant.

(**A**) A reduction in the density of amacrine cells labeled with the pan-amacrine Pax6 antibody and Bhlhb5 antibody that labels GABAergic amacrine cells was measured in LMO4 cko mice compared to littermate controls. No difference in cholinergic (ChAT+) or dopaminergic (TH+) amacrine cell density was observed. (**B**) A reduction in the density of bipolar cells labelled with the pan-bipolar Chx10 antibody and Bhlhb5+ cone bipolar (CB) cells was observed in LMO4 cko mice. However, PKC+ rod bipolar (RB) cell density was not different. Filled bars are for littermate controls and open bars are for LMO4 cko mice. The bars indicate s.e.m. and the asterisks indicate $p<0.05$.

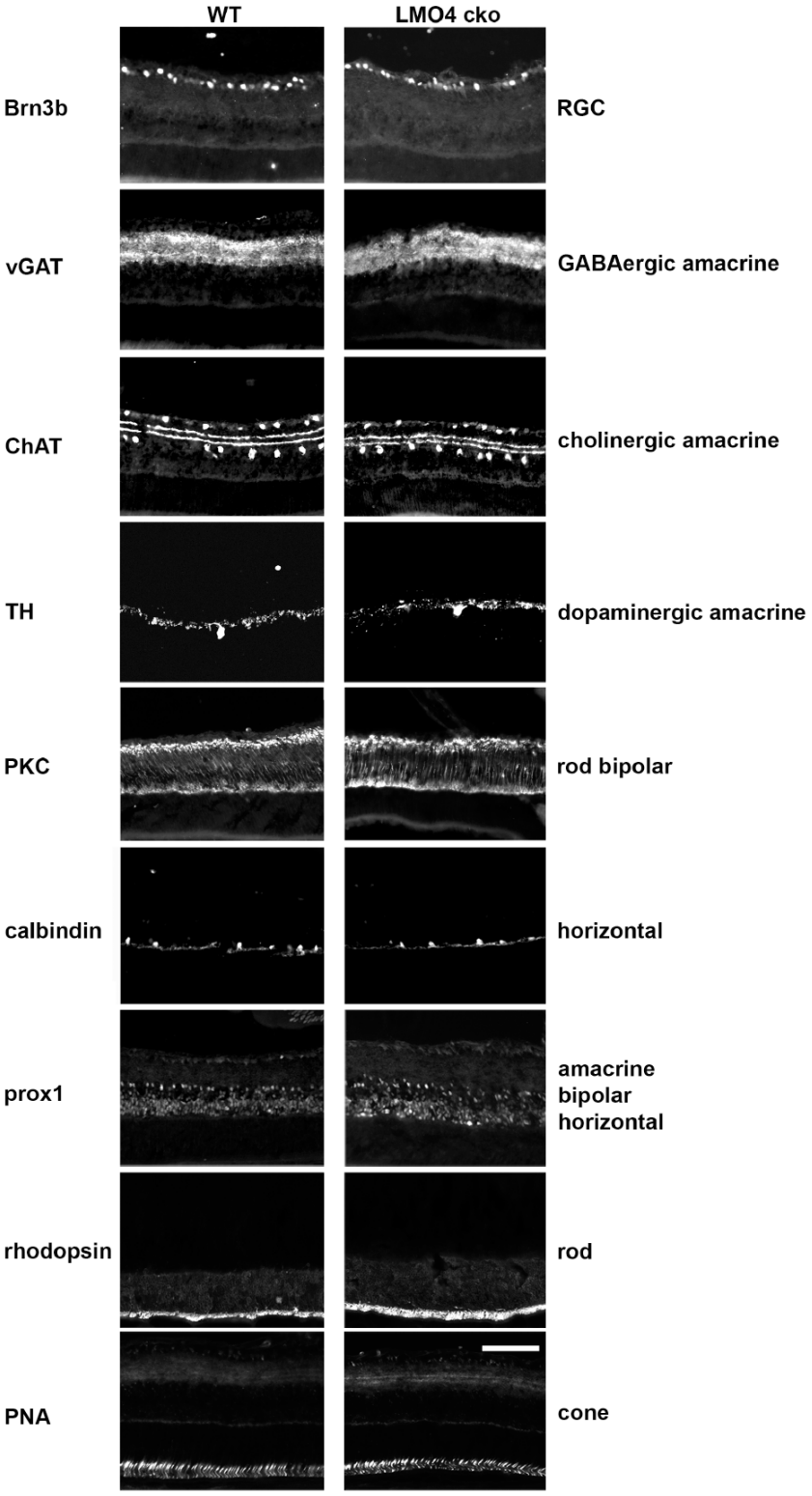


Figure 12: No significant difference was seen with other immunostaining markers Brn3b, retinal ganglion cells (RGC); vesicular GABA transporter (vGAT), GABAergic neurons; choline acetyltransferase (ChAT), cholinergic amacrine cells; tyrosine hydroxylase (TH), dopaminergic amacrine cells; PKC, rod bipolar cells, calbindin, horizontal cells; prox1, amacrine, bipolar and horizontal cells; rhodopsin, rod photoreceptors; peanut agglutinin (PNA), cone photoreceptors. Scale bars, 100 μm.

was not different (data not shown). Taken together, these results indicate a role for LMO4 in the development of Bhlhb5+ (inhibitory GABAergic) amacrine and cone bipolar cells.

Since the LMO4 antibody that worked well in our hands is raised in goat, as is the Bhlhb5 antibody, to compare their expression we performed dual in situ and immunostaining, using biotinylated LMO4 anti-sense mRNA and Bhlhb5 antibody. We found that Bhlhb5 protein was co-expressed with LMO4 mRNA in peripheral developing retinal cells at P0 (Figure 13A). These "peripherally localized" Bhlhb5 amacrine cells were significantly reduced in LMO4 cko mice at P0 (Figure 13B). However, no reduction in Bhlhb5-immunopositive retinal cells was observed at E14.5 in LMO4 cko mice (Figure 14), indicating that loss of LMO4 impairs Bhlhb5^{+} amacrine cell differentiation between E14.5 and P0. To test the possibility that GABAergic amacrine and OFF-cone bipolar subtypes were initially generated but later died of apoptosis in *LMO4* cko retinas, retinal sections were immunostained with anti-activated-caspase-3. No increase in apoptotic cells in *LMO4* cko retinas was detected at P0 (Figure 15). Taken together, targeted deletion of LMO4 specifically diminished the generation of selective Bhlhb5+ OFF-cone bipolar cells and GABAergic amacrine cells.

3.5 Bhlhb5 promoter activity assay

To address whether reduced expression of Bhlhb5 was related to loss of LMO4, we cloned a fragment of the mouse *Bhlhb5* promoter and tested the effect of LMO4 knock-down on a luciferase reporter by transient transfection of cultured neuronal cells (Figure 16). *Bhlhb5* promoter activity was specifically reduced by 50%

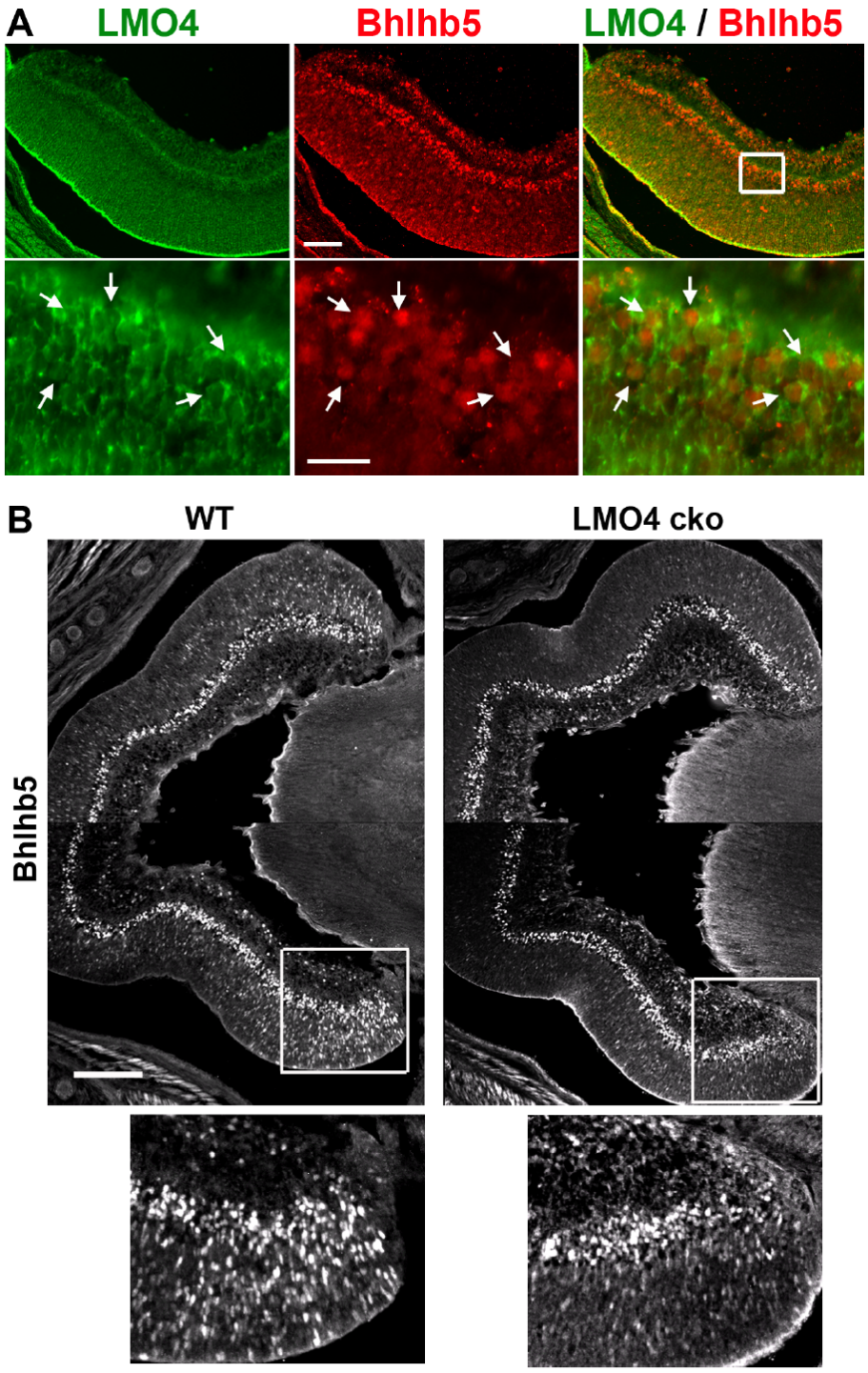


Figure 13: Loss of Bhlhb5+ cells in P0 LMO4 cko retinas.
(A) LMO4 is colocalized with Bhlhb5 in amacrine cells (arrows). LMO4 mRNA in the cytoplasm was revealed by in situ hybridization with an antisense probe labeled with biotin (green). Bhlhb5 in the nuclei was revealed by an antibody to Bhlhb5 (red). Inset is enlarged in the lower panels. Scale bars=100 and 25 μm, top and bottom panels, respectively. (B) Bhlhb5 immunostained cells were significantly fewer in the peripheral retina of LMO4 cko mice compared to littermate controls. Inset is enlarged in the lower panels. Scale bar=200 μm.

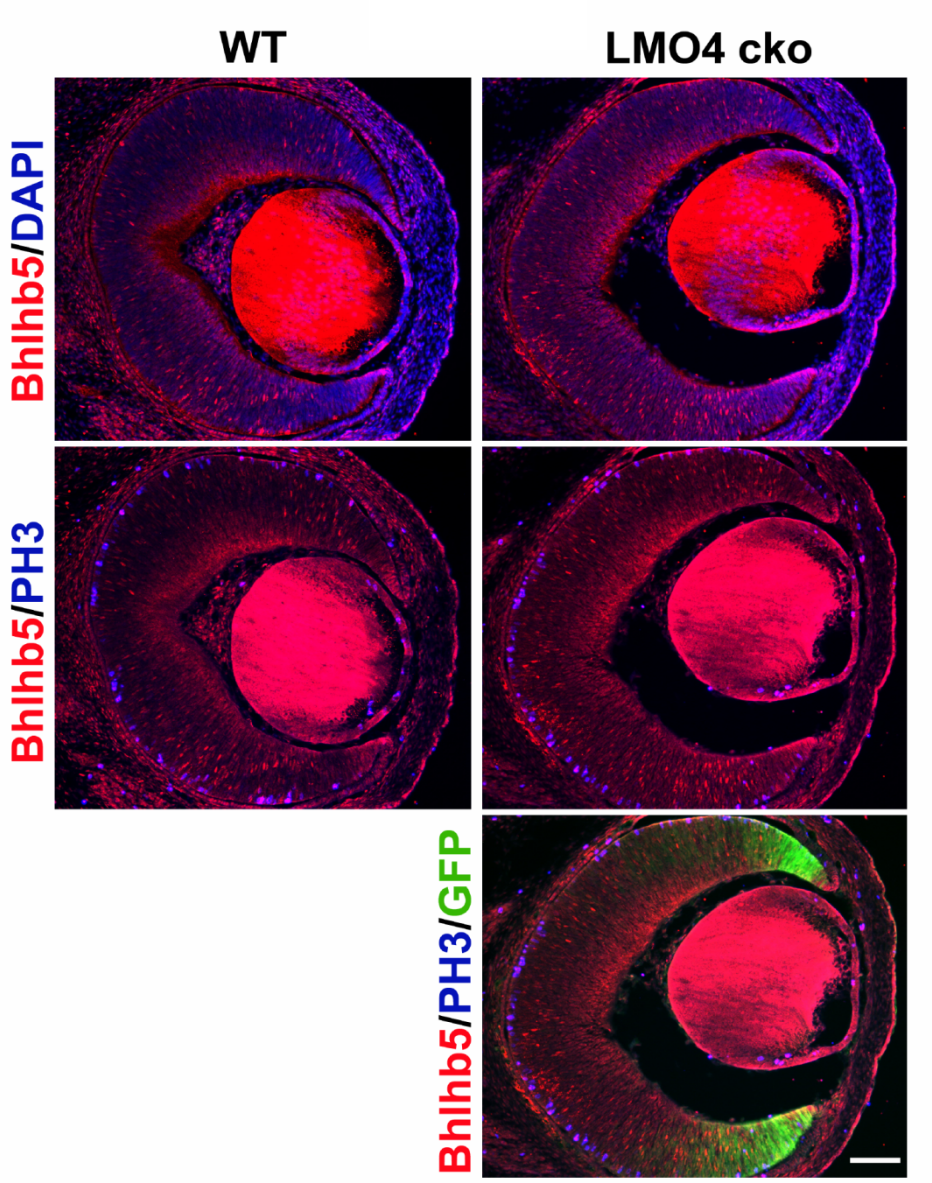


Figure 14: Bhlhb5 expression at E14.5 is not different in LMO4 cko mice. Immunofluorescent labeling of Bhlhb5 retinal neurons is compared from wild type (WT) and LMO4 cko retinas at E14.5. Bhlhb5 in red; DAPI, and mitotic marker phosphorylated histone 3 (PH3), in blue as indicated; GFP reporter expression driven by the Pax6 α-enhancer, in green. Most of the Bhlhb5 expression was detected in non-mitotic cells. No significant difference was observed in Bhlhb5+ differentiated cells or PH3+ mitotic cells in LMO4 cko retinas. Scale bar, 100 μm.

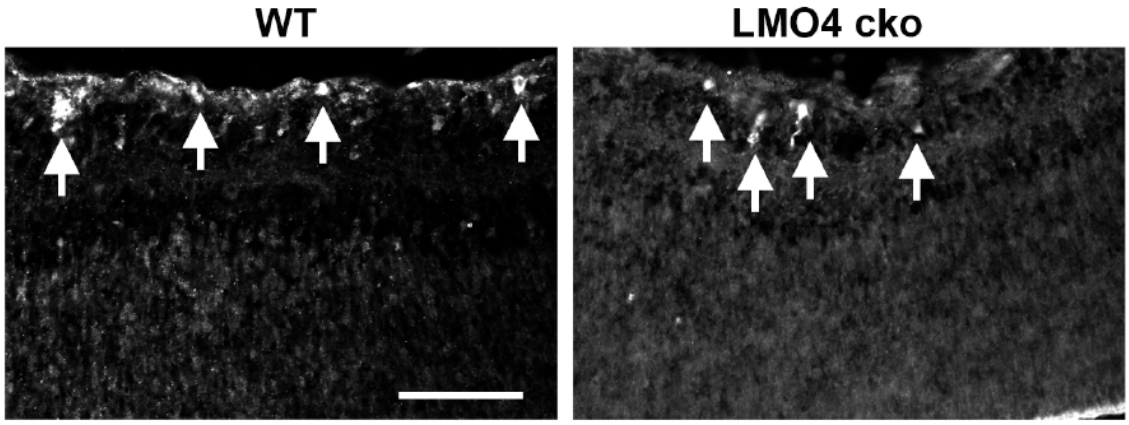


Figure 15: No evidence for increased cell death at P0 in LMO4 cko retinas. Antibody to activated caspase 3 revealed a similar number of apoptotic neurons in the retinas of littermate control (WT) and LMO4 cko mice (Arrows). Scale bar, 100 μm.

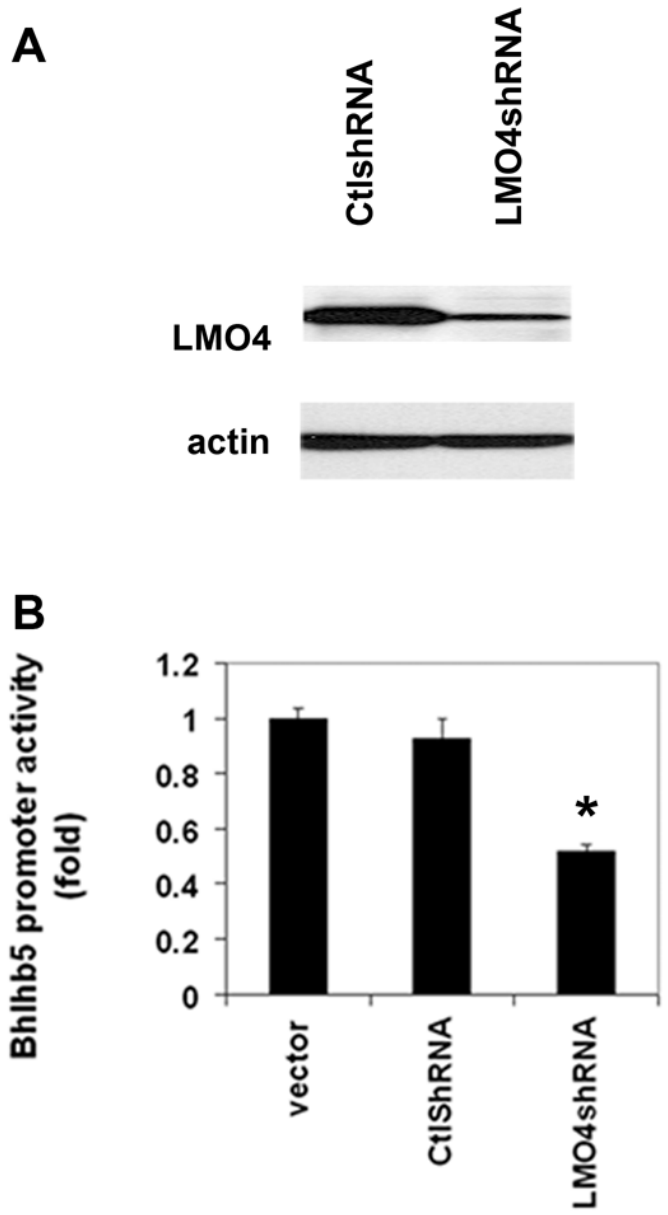


Figure 16: Knockdown of LMO4 reduced Bhlhb5 promoter activity.
(A) Western blot immunostained for anti-Flag antibody shows the efficacy of LMO4shRNA to knockdown LMO4 expression in transiently transected F11 cells expressing exogenous Flag-tagged LMO4. (B). LMO4-specific silencing shRNA (LMO4shRNA) reduced the Bhlhb5 promoter-dependent luciferase activity in F11 neuronal cells. In contrast, the non-silencing control shRNA (CtlshRNA) had no effect. Empty vector only (vector) was also used as a control for shRNA. Mean luciferase activities, normalized to a cotransfected beta-gal reporter, are shown with standard error of mean (n=3 independent experiments, each with 3 replications. *, $p<0.05$).

when LMO4 was knocked down by shRNA, and this mechanism may contribute to reduced numbers of Bhlhb5+ neurons in LMO4 cko retinas.

3.6 Impaired retinal response in α-Cre/LMO4flox mice.

To test whether the reduction of Bhlhb5+ GABAergic amacrine cells in *LMO4* cko mice affects retinal function, we performed electroretinography (ERG) on dark-adapted adult *LMO4* cko or littermate control mice. ERG measures the electrical response of the various components of the retina to light stimulation. The response normally comprises of a hyperpolarization "a-wave" and a depolarization "b-wave" that measure photoreceptor and inner nuclear layer function, respectively. ERG recording revealed a significant reduction in the amplitude of the b-wave (Figure 17A), consistent with the loss of Bhlhb5 amacrine cells. Both the amplitude and latency of the a-wave (Figure 17B, C) were not affected (Figure 17C) consistent with similar photoreceptor cell numbers in *LMO4* cko and littermate controls.

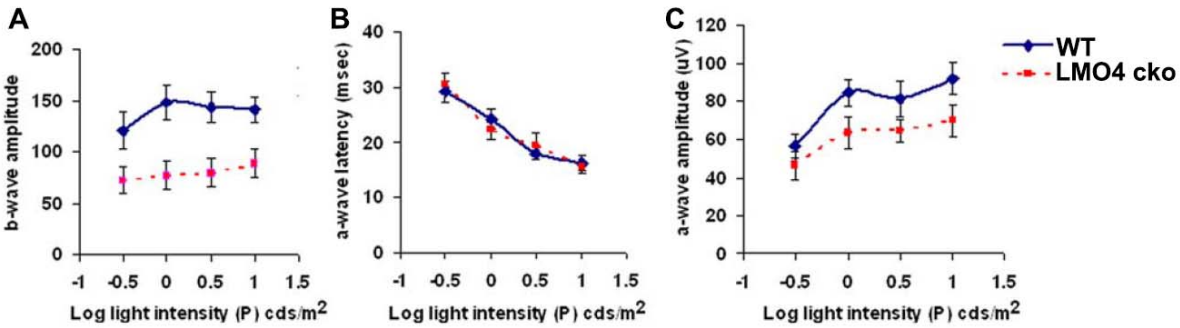


Figure 17: Electroretinography reveals a deficit in the b-wave in LMO4 cko mice. ERGs were recorded from 8 week old littermate control (diamonds and solid line) and LMO4 cko (squares and dashed line) mice. (A) b-wave average amplitude was significantly reduced at all light intensities. (B) a-wave latency was not different between control and LMO4 cko mice. (C) a-wave amplitude tended to be lower in LMO4 cko mice, but was not significantly different. All measures are means ± SEM for n=13 control (WT) and n=12 LMO4 cko mice.

4. Discussion

4.1 Summary

Germline ablations of essential transcription regulatory factors that cause embryonic lethality preclude the analysis of their function at later developmental stages. This issue is especially problematic for the study of perinatal differentiation. In particular, the development of the retina is not complete until two weeks after birth. Here, we selectively ablated *LMO4* in peripheral retinal progenitors and found that LMO4 is required for visual function and specifically, the development of GABAergic amacrine cells and type 2 OFF-cone bipolar cells (Figure 18).

4.2 LMO4 in eye development

Our observation of coloboma in *LMO4* germline deleted mice, reported here for the first time, lead us to explore the function of LMO4 in retinal development. We found that coloboma was not always linked to exencephaly, suggesting that the impaired mechanisms that underlie these phenotypes might be separable or only partially penetrant. In contrast to the increased eye size observed in zebrafish by morpholino knockdown of the *LMO4b* gene [102], we often observed smaller eyes in *LMO4* null mice. It should be noted that the sequence of the zebrafish LMO4a gene, rather than *LMO4b*, is more similar to *LMO4* of mammals. Whether the different phenotype in zebrafish represents a species-specific difference or the partial redundancy of the two *LMO4* duplicated genes of zebrafish is not known.

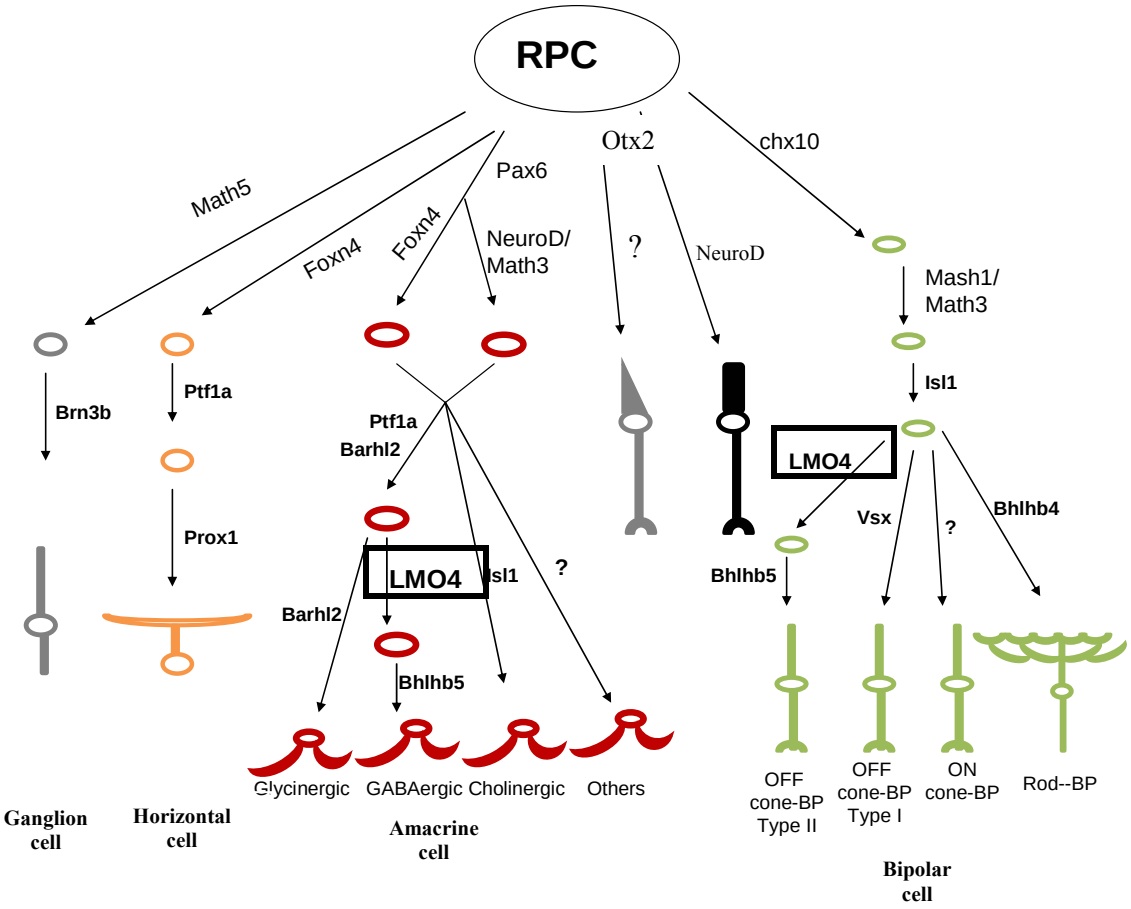


Figure 18: LMO4 and neurogenesis in the retina. With these findings, LMO4 can now be incorporated within the transcriptional program involved in neurogenesis. Specifically, LMO4 is necessary for Bhlhb5 expression is thus depicted as upsteam of Bhlhb5 for GABAergic amacrine and OFF cone-bipolar cell-type II specification.

4.3 LMO4 in amacrine cell development

Amacrine cells in the retina constitute an assortment of different subtypes of interneurons that perform highly complex information processing. The transcription factors that control the differentiation of amacrine cells are gradually being revealed from genetic models. Here, we showed that LMO4 is co-expressed with Pax6, a pan amacrine cell marker. Unfortunately, we could not detect LMO4 protein using our antibody within the progenitor population since the expression is lower than the threshold detectable by this particular antibody during embryogenesis. We can only infer by our *in situ* hybridization results that lower level expression within a different population of cells was present throughout development in RPCs. Given the lack of background signal in our sense control, it is unlikely that this low-signal represents background artifacts.

4.4 Selective loss of Bhlhb5+ cells in α-Cre/LMO4flox retina

By selective ablation of LMO4 in retinal progenitor cells we demonstrated that LMO4 is required for Bhlhb5+ amacrine cell development. The expression of Bhlhb5 is restricted to GABAergic amacrine cells and Type 2 OFF-CB subtypes. Targeted disruption of Bhlhb5 causes the selective loss of GABAergic amacrine and Type 2 OFF-cone bipolar cells. We confirmed that loss of LMO4 lead to a reduction of GABAergic amacrine and OFF-cone bipolar cells in the mature retina at postnatal day 15.

4.5 Cell death in α-Cre/LMO4flox retina

Although we did not detect any difference in caspase 3 expression at P0 between wild-type and LMO4 cko; we cannot rule out the possibility that some dead cells went

undetected at P0. A tunnel assay, which labels the terminal end of fragmented DNA in dying cells could have confirmed this. In addition, an increase in cell death in *LMO4* cko could have taken place at another developmental stage and went undetected. Further studies will determine exactly what happens to these cells that fail to express Bhlhb5 to a detectable level in *LMO4* cko.

4.5 LMO4-dependent *Bhlhb5* promoter activation

Our study shows that the *Bhlhb5* promoter is dependent upon LMO4 for expression in neuronal cells in culture. However, since LMO4 is not known to bind DNA directly, the effects of LMO4 on *Bhlhb5* promoter activity are likely to be indirect. Future studies will be required to determine whether LMO4 interacts with these bHLH transcription factors to activate *Bhlhb5* expression in the retina. It will be interesting to investigate if LMO4 can activate the *Bhlhb5* promoter in dissociated retinal progenitors or in retinal explants *in vitro.*

4.6 Possible cell fate change in α-Cre/LMO4flox retina

Ptf1a is necessary for the differentiation of GABAergic interneurons in the spinal cord[113]. The absence of Ptf1a in the spinal cord causes a transdifferentiation into glutamatergic neurons. LMO4 is also necessary for the balance between excitatory and inhibitory neurons in the spinal cord by promoting an inhibitory GABAergic fate since loss of LMO4 in the spinal cord causes a switch from inhibitory GABAergic to excitatory glutamatergic[93]. Ptf1a is also expressed in postmitotic amacrine precursors but is absent

in terminally differentiated amacrines[48, 54]. Inactivation of the *Ptf1a* gene in retinal explants led to a complete loss of glycinergic and GABAergic amacrine cells[54]. In addition, a good proportion of Ptf1a-deficient cells transdifferentiated into ganglion cells[54]. To detect a possible cell fate change in *LMO4* deficient retinas, Cre recombinase could be used to replace the *LMO4* allele and then cross these mice with mice carrying a reporter such as GFP under the control of a housekeeping gene. Upstream of this housekeeping gene would be a stop codon floxed by two LoxP sites, preventing transcription. Upon activation of the *LMO4* promoter, *Cre* would be activated, induced the recombination of the stop codon and allow transcription of the GFP gene in recombinant cells and their progeny. Examination of GFP+ cells in the mature retina and their colocalization with subtype-specific markers would allow for a thorough analysis of the different cell types produced from wild-type progenitors versus progenitors lacking *LMO4*. In addition, a look at Müller glial cells and quantification would have made it possible to eliminate the possibility of some bipolar cells being mispecicified into Müller cells, which are produced at the same time.

4.7 LMO4 as a possible cofactor of Ptf1a and Barhl2

Another gene that was downregulated in *Ptf1a* deficient retinas was *Barhl2*[54]. A recent study showed that in *Barhl2* null retinas, there was a reduction in GABAergic and glycinergic amacrines[46]. Again, it would be interesting to see if Ptf1a and/or Barhl2 can directly activate the Bhlhb5 promoter and if LMO4 acts as a cofactor for one of these transcription factors.

4.8 Bhlhb5 regulation by LMO4 as a potential general mechanism during CNS development

Similar to its requirement for inhibitory amacrine interneuron development in the eye, Bhlhb5 is also required for the survival of a subtype of inhibitory interneurons in the spinal cord that regulates pruritis; loss of inhibitory synaptic input in the *Bhlhb5* null mice results in an excessive abnormal itch.[114] In contrast, in the cerebral cortex Bhlhb5 specifically regulates the differentiation of excitatory glutamatergic projection neurons of neocortical layers II–V and is required for structural organization in the somatosensory and caudal motor cortices[115]. *LMO4* is also expressed in the layer II/III and V cortical neurons[116, 117] and is critical for patterning thalamocortical connections during development [94]. In light of our finding that LMO4 is required for Bhlhb5+ retinal neuron development, the similar spatial and temporal expression patterns of LMO4 and Bhlhb5 make it plausible that LMO4 regulates *Bhlhb5* expression in the developing cortex and participates in the development of glutamatergic projection neurons.

4.9 No change in a-wave in α-Cre/LMO4flox mice

Our study found no deficit in photoreceptor cell number and function as reflected by a normal a-wave amplitude and latency. Consistent with our finding, the Bhlhb5-null mutation does not affect photoreceptor cell development [45]. The reduction in b-wave amplitude seen in *LMO4* cko indicated a reduced bipolar cell activity and is consistent with the reduction in bipolar cells in *LMO4* cko.

4.10 LMO4 in bipolar cell development

Another important finding of our study is that LMO4 contributes to the differentiation of Bhlhb5+ type 2 OFF-cone bipolar cells, but not the rod bipolar cells whose development, on the other hand, depends on Bhlhb4[73] (Figure 15). Although this result would argue against a role for LMO4 in cone bipolar cell function, alternatively, there is loss in both ON- and Off- bipolar cells, as reflected in 30% reduction in the number of Chx10+ bipolar cells. Several commercially available bipolar subtype markers could have been used to discriminate subpopulations of bipolar cells missing in our α-Cre/LMO4flox mice in addition to the Bhlhb5+ OFF-cone bipolar, type II. For example, ON-bipolar cells express the G-protein subunit Goá, CABP5 labels subsets of ON- and OFF-cone bipolars as well as rod bipolar cells, Vsx1 is restricted to OFF-cone bipolar cells while recoverin labels OFF-cone bipolar cells type II[43].

4.11 Potential spinal cord transcriptional networks conserved in the retina

The basic helix-loop-helix transcription factors Math3+ and NeuroD+ are important for amacrine cell development, whereas the Math3+ and Mash1+ are important for the type 2 OFF-cone bipolar cells [45]. Previous studies showed that LMO4 forms a complex with the basic helix-loop-helix transcription factor SCL and GATA2 to regulate interneuron differentiation in the spinal cord[93]. Future studies will be required to determine whether LMO4 interacts with Math3, Mash1 and/or NeuroD to activate *Bhlhb5* expression in the retina. Also in the spinal cord, the coordination of LIM-homeodomain transcription factors and LIM domain-containing cofactors such as Ldb1 and LMO4

participate in motor neuron and interneuron diversification[95]. A recent study has shown that these same players are expressed in the retina at the time of bipolar cell genesis[56]. More specifically, *Isl1*, *Lhx3*, *Lhx4*, and *Ldb1* have been shown to be expressed at around postnatal day 7 (developmetal stage when bipolar cells are born) in the region corresponding to bipolar cells. Interestingly, Ldb1, another LIM domain transcription cofactor with which LMO4 has a strong physical interaction[118-120] is not only expressed within developing bipolar cells but also in the region corresponding to amacrine cells[56]. Thus, the transcriptional network of LIM-containing genes that has been well documented during spinal cord development might very well be conserved in the retina.

Inhibitory GABAergic interneurons play a critical function in controlling retinal image processing, and are important for neural networks in the central nervous system. Our finding of an essential postnatal function of LMO4 in the differentiation of Bhlhb5-expressing inhibitory interneurons in the retina may be a general mechanism whereby LMO4 controls the production of inhibitory interneurons in the nervous system.

REFERENCES

1. Kolb, H. & ScienceDirect (Online service). Concepts and challenges in retinal biology a tribute to John E. Dowling (Elsevier, Amsterdam; London, 2001).
2. Kandel, E. R., Schwartz, J. H., Kandel, E. R. & Schwartz, J. H. Principles of neural science (Appleton & Lange, Stamford, Conn., 2000).
3. Masland, R. H. The fundamental plan of the retina. Nat Neurosci 4, 877-86 (2001).
4. Cook, J. E. & Chalupa, L. M. Retinal mosaics: new insights into an old concept. Trends Neurosci 23, 26-34 (2000).
5. Kolb, H., Nelson, R., Ahnelt, P. & Cuenca, N. Cellular organization of the vertebrate retina. Prog Brain Res 131, 3-26 (2001).
6. Neitz, M. & Neitz, J. The uncommon retina of the common house mouse. Trends Neurosci 24, 248-50 (2001).
7. Ohsawa, R. & Kageyama, R. Regulation of retinal cell fate specification by multiple transcription factors. Brain Res 1192, 90-8 (2008).
8. MacNeil, M. A., Heussy, J. K., Dacheux, R. F., Raviola, E. & Masland, R. H. The shapes and numbers of amacrine cells: matching of photofilled with Golgi-stained cells in the rabbit retina and comparison with other mammalian species. J Comp Neurol 413, 305-26 (1999).
9. Westheimer, G. The ON-OFF dichotomy in visual processing: from receptors to perception. Prog Retin Eye Res 26, 636-48 (2007).
10. Snellman, J., Kaur, T., Shen, Y. & Nawy, S. Regulation of ON bipolar cell activity. Prog Retin Eye Res 27, 450-63 (2008).
11. Bloomfield, S. A. & Dacheux, R. F. Rod vision: pathways and processing in the mammalian retina. Prog Retin Eye Res 20, 351-84 (2001).
12. Kolb, H., Ripps, H., Wu, S. M.-s. & Dowling, J. E. Concepts and challenges in retinal biology: a tribute to John E. Dowling (Elsevier, Amsterdam; New York, 2001).
13. MacNeil, M. A., Heussy, J. K., Dacheux, R. F., Raviola, E. & Masland, R. H. The population of bipolar cells in the rabbit retina. J Comp Neurol 472, 73-86 (2004).
14. Chalupa, L. M. & Gunhan, E. Development of On and Off retinal pathways and retinogeniculate projections. Prog Retin Eye Res 23, 31-51 (2004).
15. Shen, W. & Jiang, Z. Characterization of glycinergic synapses in vertebrate retinas. J Biomed Sci 14, 5-13 (2007).
16. Contini, M. et al. Synaptic input of ON-bipolar cells onto the dopaminergic neurons of the mouse retina. J Comp Neurol 518, 2035-50.
17. Schmidt, M. GABA(C) receptors in retina and brain. Results Probl Cell Differ 44, 49-67 (2008).
18. Bringmann, A. et al. Muller cells in the healthy and diseased retina. Prog Retin Eye Res 25, 397-424 (2006).
19. Audo, I., Robson, A. G., Holder, G. E. & Moore, A. T. The negative ERG: clinical phenotypes and disease mechanisms of inner retinal dysfunction. Surv Ophthalmol 53, 16-40 (2008).

20. McCall, M. A. & Gregg, R. G. Comparisons of structural and functional abnormalities in mouse b-wave mutants. J Physiol 586, 4385-92 (2008).
21. Peachey, N. S. & Ball, S. L. Electrophysiological analysis of visual function in mutant mice. Doc Ophthalmol 107, 13-36 (2003).
22. Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M. & Ezzeddine, D. Cell fate determination in the vertebrate retina. Proc Natl Acad Sci U S A 93, 589-95 (1996).
23. Cayouette, M., Barres, B. A. & Raff, M. Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron 40, 897-904 (2003).
24. Harris, W. A. Cellular diversification in the vertebrate retina. Curr Opin Genet Dev 7, 651-8 (1997).
25. Young, R. W. Cell differentiation in the retina of the mouse. Anat Rec 212, 199-205 (1985).
26. Marquardt, T. & Gruss, P. Generating neuronal diversity in the retina: one for nearly all. Trends Neurosci 25, 32-8 (2002).
27. Agathocleous, M. & Harris, W. A. From progenitors to differentiated cells in the vertebrate retina. Annu Rev Cell Dev Biol 25, 45-69 (2009).
28. Zuber, M. E., Gestri, G., Viczian, A. S., Barsacchi, G. & Harris, W. A. Specification of the vertebrate eye by a network of eye field transcription factors. Development 130, 5155-67 (2003).
29. Hatakeyama, J. & Kageyama, R. Retinal cell fate determination and bHLH factors. Semin Cell Dev Biol 15, 83-9 (2004).
30. Akagi, T. et al. Requirement of multiple basic helix-loop-helix genes for retinal neuronal subtype specification. J Biol Chem 279, 28492-8 (2004).
31. Livesey, R. & Cepko, C. Neurobiology. Developing order. Nature 413, 471, 473 (2001).
32. Martinez-Morales, J. R. et al. Differentiation of the vertebrate retina is coordinated by an FGF signaling center. Dev Cell 8, 565-74 (2005).
33. Wallace, V. A. Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina. Brain Res 1192, 61-75 (2008).
34. Neumann, C. J. & Nuesslein-Volhard, C. Patterning of the zebrafish retina by a wave of sonic hedgehog activity. Science 289, 2137-9 (2000).
35. Perron, M. & Harris, W. A. Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. Cell Mol Life Sci 57, 215-23 (2000).
36. Kageyama, R., Ohtsuka, T., Shimojo, H. & Imayoshi, I. Dynamic Notch signaling in neural progenitor cells and a revised view of lateral inhibition. Nat Neurosci 11, 1247-51 (2008).
37. Rasmussen, J. T. et al. Regulation of eye development by frizzled signaling in Xenopus. Proc Natl Acad Sci U S A 98, 3861-6 (2001).
38. Hashimoto, T., Zhang, X. M., Chen, B. Y. & Yang, X. J. VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation. Development 133, 2201-10 (2006).
39. Ma, L. et al. Zac1 functions through TGFbetaII to negatively regulate cell number in the developing retina. Neural Dev 2, 11 (2007).

40. Murali, D. et al. Distinct developmental programs require different levels of Bmp signaling during mouse retinal development. Development 132, 913-23 (2005).
41. Watanabe, T. & Raff, M. C. Rod photoreceptor development in vitro: intrinsic properties of proliferating neuroepithelial cells change as development proceeds in the rat retina. Neuron 4, 461-7 (1990).
42. Hatakeyama, J., Tomita, K., Inoue, T. & Kageyama, R. Roles of homeobox and bHLH genes in specification of a retinal cell type. Development 128, 1313-22 (2001).
43. Wang, S. W. et al. Requirement for math5 in the development of retinal ganglion cells. Genes Dev 15, 24-9 (2001).
44. Liu, W., Mo, Z. & Xiang, M. The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development. Proc Natl Acad Sci U S A 98, 1649-54 (2001).
45. Pan, L., Deng, M., Xie, X. & Gan, L. ISL1 and BRN3B co-regulate the differentiation of murine retinal ganglion cells. Development 135, 1981-90 (2008).
46. Ding, Q. et al. BARHL2 differentially regulates the development of retinal amacrine and ganglion neurons. J Neurosci 29, 3992-4003 (2009).
47. Li, S. et al. Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. Neuron 43, 795-807 (2004).
48. Fujitani, Y. et al. Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. Development 133, 4439-50 (2006).
49. Dyer, M. A., Livesey, F. J., Cepko, C. L. & Oliver, G. Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. Nat Genet 34, 53-8 (2003).
50. Inoue, T. et al. Math3 and NeuroD regulate amacrine cell fate specification in the retina. Development 129, 831-42 (2002).
51. Gouge, A., Holt, J., Hardy, A. P., Sowden, J. C. & Smith, H. K. Foxn4--a new member of the forkhead gene family is expressed in the retina. Mech Dev 107, 203-6 (2001).
52. Kawaguchi, Y. et al. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 32, 128-34 (2002).
53. Hoshino, M. et al. Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. Neuron 47, 201-13 (2005).
54. Nakhai, H. et al. Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina. Development 134, 1151-60 (2007).
55. Mizuguchi, R. et al. Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. Nat Neurosci 9, 770-8 (2006).
56. Elshatory, Y. et al. Islet-1 controls the differentiation of retinal bipolar and cholinergic amacrine cells. J Neurosci 27, 12707-20 (2007).
57. Feng, L. et al. Requirement for Bhlhb5 in the specification of amacrine and cone bipolar subtypes in mouse retina. Development 133, 4815-25 (2006).
58. Bramblett, D. E., Copeland, N. G., Jenkins, N. A. & Tsai, M. J. BHLHB4 is a bHLH transcriptional regulator in pancreas and brain that marks the dimesencephalic boundary. Genomics 79, 402-12 (2002).

59. Brunelli, S., Innocenzi, A. & Cossu, G. Bhlhb5 is expressed in the CNS and sensory organs during mouse embryonic development. Gene Expr Patterns 3, 755-9 (2003).
60. McLellan, A. S., Langlands, K. & Kealey, T. Exhaustive identification of human class II basic helix-loop-helix proteins by virtual library screening. Gene Expr Patterns 2, 329-35 (2002).
61. Peyton, M. et al. BETA3, a novel helix-loop-helix protein, can act as a negative regulator of BETA2 and MyoD-responsive genes. Mol Cell Biol 16, 626-33 (1996).
62. Xu, Z. P. et al. Functional and structural characterization of the human gene BHLHB5, encoding a basic helix-loop-helix transcription factor. Genomics 80, 311-8 (2002).
63. Mo, Z., Li, S., Yang, X. & Xiang, M. Role of the Barhl2 homeobox gene in the specification of glycinergic amacrine cells. Development 131, 1607-18 (2004).
64. Nishida, A. et al. Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. Nat Neurosci 6, 1255-63 (2003).
65. Furukawa, T., Morrow, E. M. & Cepko, C. L. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91, 531-41 (1997).
66. Mears, A. J. et al. Nrl is required for rod photoreceptor development. Nat Genet 29, 447-52 (2001).
67. Burmeister, M. et al. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat Genet 12, 376-84 (1996).
68. Ohtoshi, A. et al. Regulation of retinal cone bipolar cell differentiation and photopic vision by the CVC homeobox gene Vsx1. Curr Biol 14, 530-6 (2004).
69. Chow, R. L. et al. Vsx1, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells. Mech Dev 109, 315-22 (2001).
70. Ohtoshi, A., Justice, M. J. & Behringer, R. R. Isolation and characterization of Vsx1, a novel mouse CVC paired-like homeobox gene expressed during embryogenesis and in the retina. Biochem Biophys Res Commun 286, 133-40 (2001).
71. Liu, I. S. et al. Developmental expression of a novel murine homeobox gene (Chx10): evidence for roles in determination of the neuroretina and inner nuclear layer. Neuron 13, 377-93 (1994).
72. Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F. & Kageyama, R. Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. Embo J 19, 5460-72 (2000).
73. Bramblett, D. E., Pennesi, M. E., Wu, S. M. & Tsai, M. J. The transcription factor Bhlhb4 is required for rod bipolar cell maturation. Neuron 43, 779-93 (2004).
74. Willbold, E., Berger, J., Reinicke, M. & Wolburg, H. On the role of Muller glia cells in histogenesis: only retinal spheroids, but not tectal, telencephalic and cerebellar spheroids develop histotypical patterns. J Hirnforsch 38, 383-96 (1997).
75. Fischer, A. J. & Reh, T. A. Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci 4, 247-52 (2001).

76. Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M. & Cepko, C. L. rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron 26, 383-94 (2000).
77. Wolburg, H., Liebner, S., Reichenbach, A. & Gerhardt, H. The pecten oculi of the chicken: a model system for vascular differentiation and barrier maturation. Int Rev Cytol 187, 111-59 (1999).
78. Tout, S., Chan-Ling, T., Hollander, H. & Stone, J. The role of Muller cells in the formation of the blood-retinal barrier. Neuroscience 55, 291-301 (1993).
79. Zheng, Q. & Zhao, Y. The diverse biofunctions of LIM domain proteins: determined by subcellular localization and protein-protein interaction. Biol Cell 99, 489-502 (2007).
80. Bach, I. The LIM domain: regulation by association. Mech Dev 91, 5-17 (2000).
81. Dawid, I. B. LIM protein interactions: Drosophila enters the stage. Trends Genet 14, 480-2 (1998).
82. Rabbitts, T. H. LMO T-cell translocation oncogenes typify genes activated by chromosomal translocations that alter transcription and developmental processes. Genes Dev 12, 2651-7 (1998).
83. Yamada, Y. et al. The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. Proc Natl Acad Sci U S A 95, 3890-5 (1998).
84. Yamada, Y., Pannell, R., Forster, A. & Rabbitts, T. H. The oncogenic LIM-only transcription factor Lmo2 regulates angiogenesis but not vasculogenesis in mice. Proc Natl Acad Sci U S A 97, 320-4 (2000).
85. Shi, J. et al. Identification of dopamine responsive mRNAs in glial cells by suppression subtractive hybridization. Brain Res 910, 29-37 (2001).
86. Aoyama, M. et al. LMO3 interacts with neuronal transcription factor, HEN2, and acts as an oncogene in neuroblastoma. Cancer Res 65, 4587-97 (2005).
87. Grutz, G., Forster, A. & Rabbitts, T. H. Identification of the LMO4 gene encoding an interaction partner of the LIM-binding protein LDB1/NLI1: a candidate for displacement by LMO proteins in T cell acute leukaemia. Oncogene 17, 2799-803 (1998).
88. Kenny, D. A., Jurata, L. W., Saga, Y. & Gill, G. N. Identification and characterization of LMO4, an LMO gene with a novel pattern of expression during embryogenesis. Proc Natl Acad Sci U S A 95, 11257-62 (1998).
89. Chen, H. H., Yip, J. W., Stewart, A. F. & Frank, E. Differential expression of a transcription regulatory factor, the LIM domain only 4 protein Lmo4, in muscle sensory neurons. Development 129, 4879-89 (2002).
90. Visvader, J. E. et al. The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. Proc Natl Acad Sci U S A 98, 14452-7 (2001).
91. Abrahams, B. S. et al. Genome-wide analyses of human perisylvian cerebral cortical patterning. Proc Natl Acad Sci U S A 104, 17849-54 (2007).
92. Huang, Z. et al. Transcription factor Lmo4 defines the shape of functional areas in developing cortices and regulates sensorimotor control. Dev Biol 327, 132-42 (2009).

93. Joshi, K., Lee, S., Lee, B., Lee, J. W. & Lee, S. K. LMO4 controls the balance between excitatory and inhibitory spinal V2 interneurons. Neuron 61, 839-51 (2009).
94. Kashani, A. H. et al. Calcium activation of the LMO4 transcription complex and its role in the patterning of thalamocortical connections. J Neurosci 26, 8398-408 (2006).
95. Lee, S. et al. A regulatory network to segregate the identity of neuronal subtypes. Dev Cell 14, 877-89 (2008).
96. Sun, T. et al. Early asymmetry of gene transcription in embryonic human left and right cerebral cortex. Science 308, 1794-8 (2005).
97. Tse, E. et al. Null mutation of the Lmo4 gene or a combined null mutation of the Lmo1/Lmo3 genes causes perinatal lethality, and Lmo4 controls neural tube development in mice. Mol Cell Biol 24, 2063-73 (2004).
98. Lee, S. K. et al. The LIM domain-only protein LMO4 is required for neural tube closure. Mol Cell Neurosci 28, 205-14 (2005).
99. Hahm, K. et al. Defective neural tube closure and anteroposterior patterning in mice lacking the LIM protein LMO4 or its interacting partner Deaf-1. Mol Cell Biol 24, 2074-82 (2004).
100. Song, M. R. et al. Islet-to-LMO stoichiometries control the function of transcription complexes that specify motor neuron and V2a interneuron identity. Development 136, 2923-32 (2009).
101. Manetopoulos, C., Hansson, A., Karlsson, J., Jonsson, J. I. & Axelson, H. The LIM-only protein LMO4 modulates the transcriptional activity of HEN1. Biochem Biophys Res Commun 307, 891-9 (2003).
102. McCollum, C. W., Amin, S. R., Pauerstein, P. & Lane, M. E. A zebrafish LMO4 ortholog limits the size of the forebrain and eyes through negative regulation of six3b and rx3. Dev Biol 309, 373-85 (2007).
103. Kubo, F. & Nakagawa, S. Hairy1 acts as a node downstream of Wnt signaling to maintain retinal stem cell-like progenitor cells in the chick ciliary marginal zone. Development 136, 1823-33 (2009).
104. Marquardt, T. et al. Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105, 43-55 (2001).
105. Chen, H. H., Xu, J., Safarpour, F. & Stewart, A. F. LMO4 mRNA stability is regulated by extracellular ATP in F11 cells. Biochem Biophys Res Commun 357, 56-61 (2007).
106. Chen, H. H. et al. Extracellular ATP-dependent upregulation of the transcription cofactor LMO4 promotes neuron survival from hypoxia. Exp Cell Res 313, 3106-16 (2007).
107. Schock, S. C. et al. Rescue of neurons from ischemic injury by peroxisome proliferator-activated receptor-gamma requires a novel essential cofactor LMO4. J Neurosci 28, 12433-44 (2008).
108. Wallace, V. A. & Raff, M. C. A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development 126, 2901-9 (1999).
109. Leonard, K. C. et al. XIAP protection of photoreceptors in animal models of retinitis pigmentosa. PLoS One 2, e314 (2007).

110. Medina, C. F. et al. Altered visual function and interneuron survival in Atrx knockout mice: inference for the human syndrome. Hum Mol Genet 18, 966-77 (2009).
111. Gomez-Smith, M., Qin, Z., Zhou, X., Schock, S. C. & Chen, H. H. LIM domain only 4 protein promotes granulocyte colony-stimulating factor-induced signaling in neurons. Cell Mol Life Sci 67, 949-57.
112. Baumer, N. et al. Pax6 is required for establishing naso-temporal and dorsal characteristics of the optic vesicle. Development 129, 4535-45 (2002).
113. Glasgow, S. M., Henke, R. M., Macdonald, R. J., Wright, C. V. & Johnson, J. E. Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. Development 132, 5461-9 (2005).
114. Ross, S. E. et al. Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. Neuron 65, 886-98.
115. Joshi, P. S. et al. Bhlhb5 regulates the postmitotic acquisition of area identities in layers II-V of the developing neocortex. Neuron 60, 258-72 (2008).
116. Arlotta, P. et al. Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. Neuron 45, 207-21 (2005).
117. Bulchand, S., Subramanian, L. & Tole, S. Dynamic spatiotemporal expression of LIM genes and cofactors in the embryonic and postnatal cerebral cortex. Dev Dyn 226, 460-9 (2003).
118. Meier, N. et al. Novel binding partners of Ldb1 are required for haematopoietic development. Development 133, 4913-23 (2006).
119. Deane, J. E. et al. Tandem LIM domains provide synergistic binding in the LMO4:Ldb1 complex. Embo J 23, 3589-98 (2004).
120. Mizunuma, H., Miyazawa, J., Sanada, K. & Imai, K. The LIM-only protein, LMO4, and the LIM domain-binding protein, LDB1, expression in squamous cell carcinomas of the oral cavity. Br J Cancer 88, 1543-8 (2003).

Appendices 1

Contributions by others

Xun Zhou: helped with cloning of the Bhlhb5 promoter

Nida L. Yap: helped with cloning of the Bhlhb5 promoter

Linda Jui: helped with the sectioning of the tissues

Jesse Lu: helped with the cell counts