The Role of $V_{sx}$ in the Development of Cone Bipolar Cells in Mouse Retina

by

Zhiwei Shi
M.Sc., Nankai University, 2004
B.Sc., Shandong University, 2001

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

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in the Department of Biology

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Supervisory Committee

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Supervisory Committee

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Abstract

Visual system homeobox 1 (Vsx1) is a paired-like:CVC homeodomain transcription factor that is expressed in a subset of retinal bipolar cells. \textit{Vsx1}-null mice have previously been shown to have defects in bipolar cell terminal differentiation characterized by the reduced expression of four OFF bipolar cell-specific markers and electrophysiological defects in the OFF visual signaling pathway. The availability of recently identified bipolar cell markers enables a further characterization of the \textit{Vsx1}-null mutant. I determined that \textit{Vsx1} is expressed in Type 7 ON bipolar cells and observed the upregulation of three cell markers: Cabp5, Chx10, and \textit{alpha}-gustducin:GFP in this cell type in \textit{Vsx1}-null mice. These data reveal a trend in which \textit{Vsx1} functions as a transcriptional repressor in Type 7 ON bipolar cells and as an activator in Type 2 OFF bipolar cells. Lastly, my data indicate that \textit{Vsx1} is required for the expression of two Type 3a bipolar cell markers, however, the mechanism by which it does so appears to be complex, as I was unable to detect Vsx1 protein or reporter gene expression in this cell type.
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List of Abbreviations

AMPA receptor – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

BC – bipolar cell

β-gal – β-galactosidase

bHLH – basic helix-loop-helix

Cabp5/CaB5 – Calcium binding protein 5

CMZ – ciliary margin zone

CNS – central nervous system

CVC – Chx10, vsx1/vsx2, ceh-10 (domain)

ERG – electroretinogram

FGF – fibroblast growth factor

GCL – ganglion cell layer

GFP – green fluorescent protein

HCN4 – hyperpolarization-activated and cyclic nucleotide-gated ion channel 4

HD – homeodomain

INL – inner nuclear layer

IPL – inner plexiform layer

mGluR6 – metabotropic glutamate receptor 6

miRNA – microRNA

NES – nuclear export signal
Neto1 – neuropilin (NRP) and tolloid (TLL)-like 1

NK3R – neurokinin-3 receptor

NLS – nuclear localization signal

NMDA receptor – N-methyl-D-aspartate receptor

ONL – outer nuclear layer

OOA – outer optic anlage

OrJ mouse – ocular retardation J mouse

PKARIIβ – the regulatory subunit RIIβ of the enzyme PKA

PPCD – posterior polymorphous corneal dystrophy

Prd-L:CVC TFs – paired-like:CVC transcription factors

PTD – protein transduction domain

RPC – retinal progenitor cells

RPE – retinal pigmented epithelium

SEM – standard error of the mean

Shh – Sonic hedgehog

Syt2 – synaptotagmin 2

TGFβ/BMP – transforming growth factor β/ bone morphogenetic protein

UTR – untranslated regions

VEGF – vascular endothelial growth factor

Vsx1 – Visual system homeobox 1

Wnt – Wingless
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Chapter 1 Introduction

1.1 Research Objectives

The broad goal of my M.Sc. thesis is to further characterize the molecular phenotype of the \( Vsx1 \)-null mutant mouse. Visual system homeobox 1 (\( Vsx1 \)) belongs to the homeodomain transcription factor family (Levine et al., 1994; Passini et al., 1997). In \( Vsx1 \)-deficient mice, although retinal bipolar cell specification is grossly normal, the terminal differentiation of the OFF-cone bipolar cells is incomplete (Chow et al., 2004). This incomplete differentiation was marked by a substantial reduction in the expression of four bipolar cell type specific markers: NK3R, recoverin and Neto1 in Type 2 bipolar cells, and Cabp5/CaB5 in Type 3 OFF cone bipolar cells (Chow et al., 2004). In addition, \( Vsx1 \)-null mice were shown to have defects in OFF visual signal pathway (Chow et al., 2004; Ohtoshi et al., 2004). Together, these observations indicate that \( Vsx1 \) gene function is required for the activation of some terminal gene expression in OFF bipolar cells and that these defects in gene expression disrupt OFF bipolar cell activity.

In addition to OFF cone bipolar cells, \( Vsx1 \) is also expressed in a subset of ON cone bipolar cells (Chow et al., 2004). However, due to the lack of specific ON bipolar cell markers, little is known about the role of \( Vsx1 \) in these cell types. There are clues, however, that \( Vsx1 \) function is involved in the development and function of ON bipolar cells. For example, Cabp5 immunostaining in the ON sublamina of the inner plexiform layer is defective in \( Vsx1 \)-null mice characterized by the loss of a characteristic “gap” between the axonal terminals of ON Type 5 bipolar cells and rod bipolar cells (Chow et al., 2004). Also, humans harboring a H244R \( VSX1 \) mutation associated with a dominant posterior polymorphous corneal dystrophy (PPCD) have mild defects in ON cone visual signaling but not in cone OFF or in rod ON bipolar cells (Valleix et al., 2006).

Given that \( Vsx1 \) plays a role in OFF bipolar cell development, I hypothesize that \( Vsx1 \) also plays similar roles in the late development of ON bipolar cells. This hypothesis forms the basis for two of the central objectives of my work: (i) to determine which of the 5 ON cone bipolar cell types express \( Vsx1 \) and (ii) to determine whether there are any
ON bipolar cell defects in Vsx1-null mice? Another objective of my work is to further characterize the subset of OFF bipolar cell types that express Vsx1. To date, the only bipolar cell type that is definitively known to express Vsx1 is the Type 2 OFF bipolar cell. The availability of recently identified bipolar cell markers has enabled me to examine whether Vsx1 is also expressed in other OFF bipolar cell types and to determine whether the development of these cells is affected by the loss of Vsx1 function.

Below, I will introduce the relevant background information for my research.

1.2 Structure and physiology of vertebrate retina

1.2.1 Structure and visual pathways in retina

The retina is a light-sensitive neuronal tissue lining the back of the eye where visual signaling is initiated. It is derived from the anterior neural tube as an out-pouching of the future brain; therefore, it shares common features with other parts of the central nervous system (CNS). The vertebrate retina is a laminated structure, consisting of three well-defined nuclear layers that contain five major classes of neurons (Fig. 1) and one class of glial cells, the Müller glia, whose cell bodies are located in the inner nuclear layer (INL) and have extensions that span vertically across the retina. The sensory photoreceptor cells: rods and cones, are located in the outer nuclear layer (ONL). These cells respond to light and initiate the phototransduction cascade and visual signaling. Bipolar cells are interneurons located in the inner nuclear layer (INL) that mediate the vertical transmission of visual signals from photoreceptors to ganglion cells, which are the projection neurons in the innermost ganglion cell layer (GCL). Horizontal cells located in the outer part of the inner nuclear layer modulate the signaling between photoreceptors and bipolar cells, while another class of neurons in the inner nuclear layer, the amacrine cells, modulates signaling between bipolar cells and ganglion cells (Wässle and Boycott, 1991; Kolb, 1994).

1.2.2 Retinal cell type diversity

A tremendous diversity in neuronal cell types present in CNS underlies its remarkable capacity to process information. Like other parts of CNS, the vertebrate retina
Figure 1. Organization of retinal structure.
The vertebrate retina is a laminated structure, consisting of three well-defined nuclear layers that contain five major classes of neurons and one class of glial cells, the Muller glia (which aren’t shown here). The two types of sensory photoreceptor cells (PC), rods and cones, are located in the outer nuclear layer (ONL); they respond to light and initiate the phototransduction cascade and visual signaling. Bipolar cells (BC) are interneurons located in the inner nuclear layer (INL) and mediate the vertical transmission of visual signals from photoreceptors to ganglion cells (GC), the projection neurons in the innermost ganglion cell layer (GCL). Horizontal cells (HC) located in the outer part of the inner nuclear layer (INL) modulate the signaling between photoreceptors and bipolar cells, while another class of neurons in the inner nuclear layer, the amacrine cells (AC), modulate signaling between bipolar cells and ganglion cells. Abbreviations: Outer plexiform layer (OPL), inner plexiform layer (IPL). (From Dr. R. L. Chow)
is also a much more complex tissue than the simple one described in the previous section. It is comprised of numerous neuronal cell types: there are more than 65 and probably about 100, distinct cell types in mouse retina (Sanes and Zipursky, 2010). Each of the five major retinal neuronal cell classes consists of multiple types that are distinguished by morphological, physiological, and molecular properties (Sanes and Zipursky, 2010). Amongst the photoreceptor cell class, there is one type of rod and several types of cones (two in mice and three in humans; depending on types of visual pigments) (Sanes and Zipursky, 2010). There are 11 types of bipolar cells found in mice (Ghosh et al., 2004; Mataruga et al., 2007; Wässle et al., 2009). Two types of horizontal cells are identified in most mammals, but mice and rats have only one type (Masland, 2001). At least 30 types of amacrine cells and 20 types of ganglion cells have been identified in mammals (Sanes and Zipursky, 2010).

While it is thought that the major cell populations have been identified, a growing number of cell-specific markers have allowed additional cell types to be discovered. For example, two newly characterized cell markers, HCN4 (hyperpolarization-activated and cyclic nucleotide-gated ion channel 4) and PKARIIβ (the regulatory subunit RIIβ of the enzyme PKA), divide the morphologically-defined Type 3 bipolar cells into Type 3a and 3b bipolar cells in mouse retina, respectively (Masland, 2001; Ghosh et al., 2004; Mataruga et al., 2007; Wässle et al., 2009; Sanes and Zipursky, 2010). Different retinal cell types function within distinct functional circuitries that form multiple parallel pathways within the retina (Gollisch and Meister, 2010). These parallel pathways provide a rapid mechanism to process variations of light intensity, contrast, color, shape, orientation and motion. Thus, the diversity of retinal cell types underlies the multiple visual signaling mechanisms that are needed to reflect the complexity of the visual world.

1.2.3 Classification of retinal bipolar cells

Retinal bipolar cells form a class of heterogeneous interneurons required for the visual signal processing and transmission from the sensation photoreceptors to the projection ganglion cells in the retina. At least eleven types of morphologically distinct bipolar cells have been distinguished in mice according to their dendritic branching
pattern, cell body shape and the stratification of their axon terminals within different levels of the inner plexiform layer (IPL) of retina (Ghosh et al., 2004; Wässle et al., 2009). This classification of bipolar cells in mice shares remarkable similarity to that in other mammalian retinas (Ghosh et al., 2004).

Rod bipolar cells and cone bipolar cells are the two basic types present in mammalian retina defined by their synapses to rod and cone photoreceptors, respectively (Fig. 2). There is one type of rod bipolar cell and ten types of cone bipolar cells in mice. In addition, bipolar cells can also be divided into two major classes based on their responses to increases in light intensity: OFF bipolar cells (Types 1-4 cone bipolar cells) that hyperpolarize following an increase in light intensity, and ON bipolar cells (Types 5-9 cone and rod bipolar cells) that depolarize following an increase in light intensity (Fig. 2). These opposite responses are due to the expression of different glutamate receptors on these two subclasses of bipolar cells. There are three groups of ionotropic glutamate receptors [N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5- methyl-4-isoxazolepropionic acid (AMPA) receptor, and kainite receptors] present in OFF bipolar cells (Thoreson and Witkovsky, 1999), and one type of metabotropic glutamate receptor, mGluR6, expressed in ON bipolar cells (Gerber, 2003). The existence of distinct OFF and ON pathways is thought to maximize the efficiency of visual transmission from both increases and decreases in light intensity, and thus forms the basis of contrast vision (Kolb, 1994; Schiller, 1995).

The major standard for the classification of bipolar cells is cell morphology, based mostly on the stratification of axonal terminals within inner plexiform layer (IPL). The retinal inner plexiform layer can be subdivided into five distinct horizontal sublaminae of equal thickness (Ghosh et al., 2004). The five sublaminae are defined by calretinin immunolabeling within the inner plexiform layer. Calretinin is a calcium-binding protein expressed in amacrine cells, displaced amacrine cells, and nearly all ganglion cells. Calretinin immunolabeling of amacrine projections, labels three characteristic bands within the inner plexiform layer that demarcate the sublaminar levels (Haverkamp and Wässle, 2000). The outer two sublaminae (i.e. residing closest to the inner nuclear layer) define sublaminar region “a”, which is where OFF cone bipolar cell axons terminate. The innermost three sublaminae define sublamina “b”, which is where the axonal terminals
There are at least 11 types of morphologically distinct bipolar cells in mice that are characterized by their dendrite branching pattern, cell body shape, and stratification of their axon terminals in different sublamina (1-5) of the inner plexiform layer (IPL) of retina. Recently, Type 3 bipolar cells have been shown to consist of 2 distinct cell types in mouse: Type 3a and 3b (Mataruga et al., 2007). Two basic bipolar cell types: rod bipolar cells ("ROD", RB) and cone bipolar cells ("CONE") are characterized by their synaptic connectivity to rod and cone photoreceptors, respectively. In addition, bipolar cells can be classified according to their response to changes in light intensity: ON bipolar cells (including RB) depolarize in response to increments in light intensity and have their axonal termini within region "b" of the inner plexiform layer (sublamina 3-5). OFF bipolar cells depolarize with the offset of light and their axonal termini reside in region "a" of the inner plexiform layer (sublamina 1-2). (Adapted from Dr. R.L.Chow)
of ON (both cone and rod) bipolar cells terminate (Kolb, 1994).

Cell-specific markers can also be used to identify specific cell types by immunohistochemistry (Adler, 2005). Some of these markers have even enabled us to identify new bipolar cell types (e.g., Types 3a and 3b bipolar cells as discussed above). However, the immunohistochemical characteristics of some cell markers are variable between different species, and the same marker may either fail to label the corresponding cell types or display different labeling pattern in different species. For example, recoverin, a calcium-binding protein, labels Type 2 OFF bipolar cells in mouse retina and the homologous OFF midget bipolar cells in monkey retina, whereas Type 2 OFF and Type 8 ON bipolar cells in rat retina (Ghosh et al., 2004). Therefore, validation of immunohistological characters is needed when using the same marker across different species (Haverkamp and Wässle, 2000; Haverkamp et al., 2003; Ghosh et al., 2004).

Transgenic mouse technology also provides a powerful approach for labeling a population of cells and for examining gene function in vivo. Transgenic mice have been engineered in which reporter genes [e.g. β-galactosidase (β-gal) and green fluorescent protein (GFP)] are expressed in specific retinal bipolar cell types under the control of cell-specific promoters (Wässle et al., 2009). For example, all ON bipolar cells are labeled by β-gal in a transgenic mouse line in which its expression is driven by metabotropic glutamate receptor 6 (mGluR6) promoter (Ueda et al., 1997). Type 7 bipolar cells are labeled with GFP in a reporter transgenic mouse, which utilizes the α-gustducin promoter (Huang et al., 2003). In addition, a reporter transgenic mouse line expressing, Clomeleon (Clm), a ratiometric fluorescent indicator for chloride ions, is present specifically in blue cone bipolar cells under the control of Thy-1 promoter (Haverkamp et al., 2005).

1.3 Retinal cell determination and differentiation

1.3.1 Overview of retinal development

During development, retinal cell types are thought to derive directly from one common population of multipotent retinal progenitor cells (RPCs). Retinal progenitor
cells have the capacity to generate all retinal cell types, including the five classes of neurons and Müller glial cells (Agathocleous and Harris, 2009). Development of the retina in mice begins at embryonic day 9.5 (E9.5), when retinal progenitor cells become specified and begin to proliferate. Retinal progenitor cell proliferation continues in mice until postnatal day 8 (P8) (Young, 1985; Cepko et al., 1996). Throughout retinal development, progenitor cells exit the cell cycle and begin to differentiate into one of the retinal neuronal or glial cell types. This process of cell specification and differentiation spreads across the retina in a central-to-peripheral wave-like manner (Agathocleous and Harris, 2009).

Although a homogeneous population of retinal progenitor cells is believed to be present in early retinal development, it is thought to transit toward a lineage-restricted heterogeneous population of retinal progenitor cells through the constant integration of both extrinsic and intrinsic signals (Agathocleous and Harris, 2009). These signals trigger the subsequent differentiation and generation of the major retinal cell types in a sequential yet overlapping order (Fig. 3). Remarkably, this time course progression is conserved across vertebrate species such that ganglion cells are born first, followed by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and the last cell type, Müller glial cells (Ohsawa and Kageyama, 2008; Agathocleous and Harris, 2009). Newly generated post-mitotic cells migrate to their final destination where they differentiate into distinct cell types and are organized into properly functioning circuits (Marquardt, 2003). As is common in nervous system, more cells are born than present in the mature retina; therefore, programmed cell death occurs (between postnatal day 8 and 27) to eliminate excess cells and inappropriate connections (Vecino et al., 2004).

The ability of retinal progenitor cells to give rise to mature retinal neurons and glia is thought to change over developmental time. For example, it has been shown that progenitors can only generate appropriate subpopulations of retinal cells at specific developmental stages (Cayouette et al., 2006). Moreover, intrinsic competence of stage-fixed retinal progenitor cells does not seem to change when exposed to new environments (Cayouette et al., 2006). Rat retinal progenitor cells show similar characteristics in the dividing times and generation of cell types between the cultures with and without serum
Figure 3. Temporal ordering of cell birth in the mouse retina.
The development of the retina in mice begins at embryonic day 9.5 (E9.5) and retinal progenitor cells are present and proliferate up to postnatal day 8 (P8) (Young, 1985; Cepko et al., 1996). In the developing retina, the subsequent differentiation and generation of the major retinal cell types followed a sequential yet overlapping order over time: ganglion cells are born at first, followed by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and the last cell type, Müller glial cells. (From Ohsawa and Kageyama, 2008)
(Cayouette et al., 2003). Early embryonic retinal progenitor cells do not adopt late-born cell fates when they are cultured with postnatal retinal cells (Austin et al., 1995). Similarly, postnatal retinal progenitor cells are not respecified into early-born cells when they’re cultured with an excess of embryonic cells (Belliveau et al., 2000).

In summary, during retinal development, uncommitted retinal progenitor cells exit the cell cycle, are specified and establish the appropriate composition of diverse retinal cell classes and types. Part of the developmental process includes the assembly of these cells into the complex circuits that function within the retina. Precise coordination of the multiple steps in retinal development is critical to generate the correct proportion of cell types, proper organization and function of the retina.

1.3.2 Extrinsic and intrinsic signaling in neural development

A major focus of neurodevelopmental biology is to elucidate how progenitor cells arise, divide, and ultimately give rise to the numerous cell types that compose a well-organized tissue. Although many neural progenitor cells express similar genes in their early development, they ultimately express unique combinations of genes that contribute to the acquisition of distinct cellular identities. A large body of work has shown that there are several determinative extrinsic cues (e.g., secreted or transmembrane signals in the local extracellular environment) and a multitude of intrinsic regulators (e.g., transcriptional regulation factors within the cell) that function at different stages of development (Edlund and Jessell, 1999; Yang, 2004; Silver and Rebay, 2005; Esteve and Bovolenta, 2006; Guillemot, 2007).

Pattern formation in the developing neural tube has been studied in great detail and provides an elegant model of combinatorial extrinsic and intrinsic controls (Briscoe and Ericson, 1999; Briscoe, 2009). The dorsal to ventral diffusion of the secreted morphogen, Sonic Hedgehog (Shh), establishes a gradient from its tissues of origin: the ventrally located notochord and neural tube floor plate. A series of specific homeobox transcription factors are expressed within the neural tube in response to different concentrations of Shh along the morphogen gradient. Cross repression of neighboring
transcription factors from adjacent regions is a major mechanism that contributes to the
delineation of boundaries of different expression domains, within which populations of
specific progenitors dominate (Briscoe and Ericson, 1999). Thus, the concentration of the
Shh gradient is translated to an array of distinct progenitor populations in defined regions
of the ventral neural tube by the activity of an array of transcription factors (Briscoe and
Ericson, 1999; Dessaud et al., 2008).

1.3.3 Extrinsic signaling in retinal development

In this section, I’ll discuss some of the extrinsic signaling pathways that are
involved in retinal development and explain how they are deployed to regulate this
process.

Retinal progenitor cells are exposed to different microenvironments and extrinsic
signals, which are thought to be encoded into cell intrinsic programs. The intrinsic
programs, in turn, affect the internal expression or the activity level of receptors, the
multiple components of transduction pathways, and/or the transcription factors, which
determine neuronal cell fate. The responding cells behave not only passively, but also
actively to modulate the extracellular graded signal, and contribute to information
processing. For example, in neural tube patterning, Shh activates expression of the
receptor Patched 1 (Ptc1) by the intracellular downstream Gli transcription factors; and
then Ptc1 represses Shh signaling. Therefore, extrinsic signaling control and cellular
internal responses interact, feedback with each other, orchestrating a spatiotemporally
dynamic process (Jaeger et al., 2008; Kutejova et al., 2009).

The major extrinsic signal transduction pathways involved in neuronal
development/determination include Notch/Delta (Perron and Harris, 2000), Hedgehog
(Hh) (Esteve and Bovolenta, 2006; Wallace, 2008), Wingless (Wnt) (Agathocleous and
Harris, 2009), TGFβ/BMP (transforming growth factor β/ bone morphogenetic protein)
(Close et al., 2005; Duenker, 2005; Ma et al., 2007) and FGF (fibroblast growth factor)
(Chow and Lang, 2001; Rowan et al., 2004; Yang et al., 2004). These signaling pathways
are modular and repeatedly used in all embryonic developing tissues and processes to
control the proliferation of progenitor cells, specification and differentiation (Esteve and Bovolenta, 2006).

**Notch.** Notch signaling has multiple roles in retinal development. One of its major roles is to maintain the progenitor pool and negatively regulate neurogenesis. The basic helix-loop-helix (bHLH) transcription factors, such as *Hes1* and *Hes5* (homologues of *Drosophila hairy* and *Enhancer of split* genes), are canonical effectors of Notch signaling and are expressed in retinal progenitor cells. These factors act as transcriptional repressors, recruiting the co-repressor protein Groucho to influence the expression of the key proneural genes in cell-fate specification of progenitors, generally inhibiting cell differentiation (Perron and Harris, 2000; Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008). In retina, Notch1 along with its effectors, Hes1 and Hes5, is also able to promote Müller glial cell fate (Dorsky et al., 1995; Furukawa et al., 2000; Gaiano and Fishell, 2002; Hojo et al., 2000; Scheer et al., 2001). Due to the early embryonic lethality in the Notch double mutant, a Cre/loxP approach was applied to generate mice with conditional Notch1-inactivation in retinal progenitor cells (Jadhav et al., 2006; Yaron et al., 2006). The proper retinal morphology and size are disrupted, and retinal cell numbers are reduced; however, photoreceptor cell fate is strongly favored (Jadhav et al., 2006; Yaron et al., 2006). Notch1 depletion results in downregulation of Hes1 and Hes5, as well as the upregulation of several proneural transcription factors (Yaron et al., 2006). Dependent on different driving promoters, either cone fate or a dual-photoreceptor fate (early depletion of Notch1 produces cones, and later depletion produces rods) is dramatically enhanced at the expense of other retinal cell types. Both of the two studies show that Notch signaling is crucial to inhibit the photoreceptor cell fate in retinal development (Jadhav et al., 2006; Yaron et al., 2006). A model of Notch activity has been developed as follows: at a certain high level Notch tends to guide the retinal progenitor cells to maintain the progenitor state; at a very low level of Notch (e.g. the conditional inactivation status), progenitors are biased to photoreceptor cell fate; and at the intermediate level, Notch1 facilitates the production of diverse retinal cell types through combination with some intrinsic transcription factors (Jadhav et al., 2006).

**Sonic hedgehog.** Sonic hedgehog (Shh) signaling also plays many different roles in retinal development. It stimulates retinal progenitor proliferation, regulates ganglion
cell genesis, and is required for proper retinal organization (Wang et al., 2005; Esteve and Bovolenta, 2006; Wallace, 2008). In *Xenopus*, Shh promotes retinal progenitor cell proliferation by regulating the components of cell cycle, such as Cyclins D1, A2, B1, and Cdc25, accelerating the cell cycle of the precursors by shortening the length of G1 and G2 phases (Locker et al., 2006). In conditional *Shh*−/− mouse, the depletion of retinal progenitor cells is due to the earlier exit of cell cycle and the expression level of both *cyclin D* and *Hes1* is decreased (Wang et al., 2005). In the early stage of zebrafish retinal development, Shh secreted by newly differentiated ganglion cells promotes the adjacent retinal progenitor cells into ganglion cell fate, leading to a wave of neurogenesis across the retina (Neumann & Nuesslein-Volhard, 2000). On the contrary, in the chick retina, Shh impedes ganglion cell production (Zhang and Yang, 2001). In the developing mouse retina, the conditional *Shh* knockout leads to an overproduction of ganglion cells owing to retinal progenitor cells being biased towards a ganglion cell fate associated with a decline of bipolar and Müller glia, the late born retinal cell types (Wang et al., 2005). It will be interesting to determine why there is a discrepancy among different species with respect to the role of Shh in the development of retinal ganglion cells (Esteve and Bovolenta, 2006). However, it is clear that Shh function is involved in the timing and balancing of the retinal progenitor cell proliferation and in the specification of ganglion cells.

*Wnt.* The Wnt pathway has been shown to activate proliferation in developing retina (Agathocleous and Harris, 2009). In amphibians, Wnt signaling inhibits differentiation in a peripheral region of the retina known as the ciliary margin zone (CMZ), and maintains a population of progenitor cells or retinal stem cells in peripheral retinal region of the adult (Agathocleous and Harris, 2009). Wnt is thought to inhibit neuronal differentiation by inhibiting the expression of multiple proneural bHLH genes (Kubo et al., 2005). Hairy1, is both necessary and sufficient for the molecular characteristics of the chick CMZ in response to Wnt signaling. Although *Hairy1* is a canonical Notch signaling effector in other developmental processes, its expression is activated in this specific context by multiple Wnt-responsive transcription factors (Kubo and Nakagawa, 2009). Interestingly, Wnt can also regulate some factors that ultimately drive progenitors to differentiate (Agathocleous and Harris, 2009).

*TGFβ.* Members of TGFβ superfamily play a critical role in inhibiting retinal
progenitor cell proliferation and mediate programmed cell death in the developing vertebrate retina (Close et al., 2005; Duenker, 2005). The TGFβ/BMP signal pathway can trigger apoptosis of ganglion cells (Beier et al., 2006; Franke et al., 2006). Through negative feedback, TGFβII also represses the cell number of rods and amacrine cells in cell autonomous and non-autonomous manners, respectively. Zac1, a zinc finger transcription factor, is required to regulate cell number negatively during this TGFβII-mediated retinal developmental process (Ma et al., 2007).

FGF. FGF signaling is involved in the partitioning of the bipotential optic vesicle neuroepithelium into two domains: neural retina and retinal pigment epithelium. It can induce presumptive retinal pigment epithelium to transdifferentiate into neural retina by suppressing genes such as Mitf, which are required for retinal pigment epithelium determination through RTK/Ras/MAPK transduction pathway (Chow and Lang, 2001; Rowan et al., 2004; Yang et al., 2004). Mitf, a basic helix-loop-helix leucine zipper transcription factor, is initially expressed throughout the optic vesicle and is later restricted into presumptive retinal pigment epithelium, by antagonizing with transcription factor Chx10, which is involved in the maintenance of retinal progenitor cells (Nguyen and Arnheiter, 2000; Rowan et al., 2004; Horsford et al., 2005).

In summary, multiple extrinsic signals are deployed and orchestrated in specific cell microenvironments at particular times during retinal development. These pathways should not necessarily be thought of as acting independently of each other. For example, the conditional ablation of Shh results in a decrease of retinal progenitor cells (RPCs) and a downregulation of Hes1, one of the Notch effectors in mouse retina (Wang et al., 2005). In response to either Shh or vascular endothelial growth factor (VEGF) signals, the enhanced Hes1 activity promotes the proliferation of retinal progenitor cells and suppresses the specification of retinal ganglion cells in dissociated chick retinal cells. It is proposed that Hes1 functions as a common downstream target of Shh/VEGF, a convergent node to integrate inputs from multiple extracellular signals. However, this process is believed to be independent of Notch signaling (Hashimoto et al., 2006). Another example is that Wnt signaling is employed in concert with Notch signaling during retinal progenitor cell proliferation and differentiation (Das et al., 2008). One challenge is how to decipher the interactions, cross-talk of multiple cell-extrinsic cues.
It’s even more complicated to elucidate these mechanisms when we consider other factors, such as the constantly changing neighboring cell types, extracellular matrix and even neurotransmitter release, interplay to regulate retinal development.

1.3.4 Retinal cell fate determination by specific interactions between homeodomain and basic helix-loop-helix transcription factors

Transcription factors play a central role in directing the genetic programs that underlie the generation of cell diversity in the retina. Their expression in a unique spatiotemporal pattern is thought to establish a combinatorial code to control this developmental process (Guillemot, 2007; Ohsawa and Kageyama, 2008). Several classes of transcription factors have been shown to control various steps in the proliferation and specification of progenitor cells in retinal development. Among these, homeodomain (HD) protein and the basic helix-loop-helix (bHLH) protein families of transcription factors are major determinants that contribute to the generation of different kinds of retinal neurons and glial cells (Marquardt, 2003; Zaghloul et al., 2005). HD transcription factors have been established as having a major role in developmental processes such as pattern formation, cell fate determination and specification, differentiation in central nervous system (Akin and Nazarali, 2005). Many members of bHLH family genes are well known to be necessary for neurogenesis, as coordinators of cell cycle exit and neural differentiation (Ohnuma et al., 2001; Vetter and Brown, 2001; Bertrand et al., 2002). During retinal development, these two classes of transcription factors are thought to interact and participate in overlapping processes. For instance, both the HD transcription factor Chx10 and the bHLH transcription factors Mash1/Math3 are required for the specification of retinal bipolar cells (Burmeister, 1996; Hatakeyama et al., 2001).

Several bHLH proteins have been characterized as “proneural” transcription factors based on their role in neurodevelopment and have been functionally divided into repressors and activators (Hatakeyama and Kageyama, 2004). The bHLH repressors, such as Hes1 and Hes5 (homologues of Drosophila hairy and Enhancer of split genes), are expressed in common progenitors, as canonical effectors of Notch/Delta signaling to inhibit neuronal differentiation and promote maintenance of progenitors. When Hes1 and
Hes5 are downregulated, bHLH activators such as Mash1 and Math5 (homologues of the Drosophila proneural genes acheate-scute and atonal) promote neuronal differentiation (Hatakeyama and Kageyama, 2004). Thus the bHLH transcription factors are major determinants of both retinal progenitor maintenance and neuronal differentiation.

**Homeodomain transcription factors.** Homeodomain proteins contain a highly conserved domain called homeodomain (HD), which is involved in the transcriptional regulation of target genes through its binding to a specific DNA consensus sequence. There are 227 members of HD transcription factors in mouse, thus making it the second largest transcription factor family (Gray et al., 2004). The dispersed homeobox gene superfamily can be classified into at least 16 different classes according to: (i) the diversity of primary sequence/structure homology of the homeodomain, (ii) the additional conserved region(s) lying outside the homeodomain and (iii) the characteristics of association with other sequence motifs (Gehring et al., 1994).

The homeodomain (HD) has typically 60 amino acids in length and the homeodomain peptide folds into a characteristic structure composed of three α-helices, and an unstructured N-terminal arm (Svingen and Tonissen, 2006; Noyes, et al., 2008). Helix II and Helix III of the homeodomain form as a “helix-turn-helix” structure (Svingen and Tonissen, 2006; Noyes, et al., 2008). The core 4-base pair sequence, TAAT, is a high-affinity consensus binding site conserved for nearly all homeodomains (Fig. 4) (Svingen and Tonissen, 2006; Joshi et al., 2007; Berger, et al., 2008; Noyes, et al., 2008). High-throughput studies on HD-DNA interactions have shown that the recognition of a 5-8 bp consensus DNA binding sequence is provided by Helix III of the homeodomain oriented in the DNA major groove with additional contact mediated by the N-terminal arm positioned in the DNA minor groove (Ades and Sauer, 1995; Svingen and Tonissen, 2006; Joshi et al., 2007; Noyes, et al., 2008).

Although the homeodomain is highly conserved and exhibits similar DNA binding characters in vitro, most HD transcription factors have unique target recognition sequences in vivo (Chariot et al., 1999). This raises the question of how DNA target recognition specificity is achieved. Evidence suggests that the flanking base pairs abutting the core binding site contribute the protein-DNA recognition (Berger et al., 2008; Noyes et al., 2008). Two base pairs immediately adjacent to the 3’ of the 4-bp
Figure 4. Homeodomain-DNA recognition.

(legend on next page) (From Noyes et al., 2008)
Figure 4. Homeodomain-DNA recognition.

The homeodomain is a highly conservative domain of 60 amino acids, encoded by a 180-bp homeobox DNA sequence. The homeodomain peptide folds into a characteristic structure composed of three $\alpha$-helices [Helix I and Helix II are green helices, and Helix III is in yellow in (A)], and an unstructured N-terminal arm. Helix II and Helix III of the homeodomain form as a “helix-turn-helix” structure, and Helix III is the recognition helix (A, B). The homeodomain-DNA interactions are represented by the structure of Msx-1 binding to DNA (A, B). The two amino acid residues of recognition contacts (A, B - red) at positions 2 and 5 of the N-terminal arm (A, B - orange) interact with DNA base structure in the minor groove; and the residues at positions 47, 50, 51, and 54 from the recognition helix (A,B - yellow) contact the major groove. (C) Top: Sequence logos represent the diversity of amino acid sequence from 84 homeodomains in *D. melanogaster*. Bottom: The schematic diagram shows homeodomain-DNA interactions in the black window. The core 4-base pair sequence, TAAT, is a high-affinity consensus binding site conserved for nearly all homeodomains. The amino acid diversity in DNA-recognition regions is blown-up in windows showing the N-terminal arm (red) and recognition helix (yellow). The key recognition positions are labeled with asterisks. (From Noyes et al., 2008)
core motif have been shown to confer strong binding preference (Ades and Sauer, 1995). It has recently been shown that longer 8-mer DNA sequences built around the TAAT core have distinct binding profiles that can explain the complex and diverse patterns of sequence specificity (Berger et al., 2008).

HD transcription factor activity is mediated by more than just DNA binding. They are known to take part in protein-protein interactions with other HD transcription factors, non-HD transcription factors, and other cofactors (Mikkola et al., 2001; Akin and Nazarali, 2005; Svingen and Tonissen, 2006; Joshi et al., 2007). It has been shown that they are able to function as monomers, homo-/hetero-dimers or heteromultimers. The differences in HD flanking amino acid sequences are thought to mediate this protein-protein interaction specificity (Joshi et al., 2007). Through these interactions, it is believed that a limited number of HD and other transcription factors can generate a vast number of different transcriptional activities. This “transcription factor coding” also provides a mechanistic explanation for how a single transcription factor may function distinctively in different cells types (Wilson et al., 1993; Wolberger, 1999; Akin and Nazarali, 2005; Silver and Rebay, 2005).

In addition to protein-protein interactions, other mechanisms function to regulate the activities of HD transcription factors such as phosphorylation, acetylation, chromatin structure, and subcellular trafficking (Chariot et al., 1999; Reményi et al., 2004; Akin and Nazarali, 2005). Furthermore, multiple copies of a particular HD-DNA binding site (both low and high affinity) likely exist in the regulatory regions of a gene, which will have an effect on the activity of a transcription factor (Dorval et al., 2005). Therefore, the manner in which a homeodomain transcription factor participates in transcriptional regulation is not necessarily easy to predict as there are many variables that can impact its activity.

1.3.5 Transcription factors in pan-bipolar cell development

A further precise investigation shows that retinal bipolar cells are generated postnatally in the mouse between P2 and P8 (Dr. E. N. Star, unpublished data, Chow lab). The generation of bipolar cells, as a class and with respect to individual subtypes, is
dependent on the activity of both HD and bHLH transcription factors (Marquardt, 2003; Hatakeyama and Kageyama, 2004; Zaghloul et al., 2005; Ohsawa and Kageyama, 2008). In this section I will discuss how these transcription factors are involved in the development of retinal bipolar cells.

**Chx10/Mash1/Math3.** The HD gene *Chx10* and the bHLH genes *Mash1* and *Math3* are key regulators of pan-bipolar genesis in the mammalian retina. All three of these transcription factors are initially expressed in retinal progenitor cells and then become restricted to mature bipolar cells (Hatakeyama et al., 2001). A role for Chx10 in retinal development has been demonstrated in the naturally occurring mouse mutant *ocular retardation J (orJ)* (Burmeister et al., 1996). *OrJ* mice possess a premature stop codon in the *Chx10* coding region just before the homeodomain. *OrJ* homozygous mutants are characterized by a profound decrease of retinal progenitor cell proliferation, and a complete loss of bipolar cell specification. Despite defects in retinal lamination, other retinal cell types are specified (Burmeister et al., 1996).

Two bHLH proteins Mash1 and Math3 (also called Neurod4, neurogenic differentiation 4) are both expressed in putative differentiating bipolar cells. In *Mash1*-null mouse retinal explants, the differentiation of bipolar cells is delayed and the bipolar cell number is dramatically reduced (Tomita et al., 1996). Interestingly, *Math3* mutants alone do not have any apparent bipolar cell abnormalities (Tomita et al., 2000). In *Mash1* and *Math3* double mutants, however, bipolar cells are completely missing and the number of Müller glial cells is increased (Tomita et al., 2000). This is consistent with the hypothesis that *Mash1* and *Math3* normally function as proneural genes and that glial fates arise in their absence (Tomita et al., 2000). In contrast, gliogenesis is not promoted in the *Chx10* mutant (Tomita et al., 2000), suggesting *Chx10* and *Mash1/Math3* have distinct functions in bipolar cells specification. In gain-of-function experiments, misexpression of *Chx10* leads to an increase of cells in the retinal INL, including Müller glia, but fails to rescue the production of bipolar cells in *Mash1/Math3* mutants. Misexpression of Mash1 or Math3 alone fails to generate bipolar cells in the wild type retina, but produces photoreceptors at the expense of Müller glia. However, misexpression of Chx10 along with Mash1 or Math3 promotes the generation of bipolar cells by inhibiting gliogenesis in the retinal INL (Hatakeyama et al., 2001). These studies
reveal a selective combinatorial interaction between HD and bHLH transcription factors in directing bipolar cell fates whereby Chx10 is thought to regulate the laminar identity of INL cells, whereas Mash1 and Math3 are involved in specification of the related neuronal classes, and in determination of neuronal versus glial fates (Hatakeyama et al., 2001).

*Otx2.* Otx2 is expressed in bipolar cells, and is able to promote bipolar cell specification in *Xenopus* and mouse retinas (Viczian et al., 2003; Koike et al., 2007). Disruption of Otx2 in mouse retinal progenitors significantly decreases bipolar cell genesis, and conditional knockout Otx2 in mouse shows impaired immunocytochemical and electrophysiological evidence of mature bipolar cells (Koike et al., 2007). These data suggest that Otx2 is required in both early development, and terminal differentiation of retinal bipolar cells (Viczian et al., 2003; Koike et al., 2007).

### 1.3.6 Transcriptional control of bipolar cell type diversity

Compared to the transcription regulation in retinal neuronal cell class specification, much less is known about the transcriptional programs that control the retinal neuronal subtype diversity (Silver and Rebay, 2005; Sanes and Zipursky, 2010). As discussed in the previous section, Chx10, Mash1, Math3 and Otx2 are required for the specification of all bipolar cells, as a class. *How then are the 11 distinct subtypes of retinal bipolar cells generated?* Recent work has demonstrated roles for several transcription factor candidates in this process, including work on the transcription factors such as Isl1, Vsx1, Irx5, Bhlhb4, and Bhlhb5. Unlike Chx10 and Math3/Mash1, these transcription factors function post-specification, during bipolar cell differentiation and are restricted in their expression to a subpopulation of bipolar interneurons.

*Isl1.* Isl1 (Islet-1) is a highly conserved transcription factor, belonging to the LIM-HD subclass. It plays an essential role in CNS differentiation (Pfaff et al., 1996). Its retinal expression starts at P5 and is restricted to amacrine cells, ganglion cells, and ON bipolar cells in the mature mouse retina. Isl1 is present at different expression levels in different ON bipolar cells in both neonatal and adult mouse retinas and is present at much higher levels in rod bipolar cells than in cone bipolar cells. The co-expression pattern of Isl1 with Chx10 (a pan bipolar cell marker) appears before the clear organization of
differentiated bipolar cells and persists in the mature retina (Elshatory et al., 2007a). Retinal bipolar cells are normally born in *Isl1*-null mice, but differentiation is significantly affected with cell number profoundly reduced. The expression of Vsx1 and Bhlhb4, two bipolar subtype-specific transcription factors (see below), are disrupted in *Isl1*-null mice. In addition, the b-wave of electroretinogram (ERG) is diminished in *Isl1*-null mice, demonstrating a severe loss of retinal interneuron (i.e. bipolar cell) function (Elshatory et al., 2007b).

**Irx5.** *Irx5*, an *Iroquois* homeobox gene family member, is expressed in Types 2 and 3 bipolar cells in the developing and mature retina (Cheng et al., 2005). In *Irx5*-deficient mice, gross retinal development appears normal, but Type 2 and Type 3 OFF cone bipolar cells have reduced levels of the OFF bipolar cell markers of recoverin, PMCA1 and Cabp5. These data suggest *Irx5* plays a role in the terminal differentiation of cone bipolar cells (Cheng et al., 2005).

**Bhlhb4.** *Bhlhb4*, a member of the Olig family of bHLH transcription factors, has been shown to play a specific role in rod bipolar cell development. In *Bhlhb4*/*-/-* mice, rod bipolar cells are born in normal numbers, but significantly decline, through apoptosis, by P8. The corresponding disruption of rod signaling is exhibited by the loss of the scotopic electroretinogram (ERG) b-wave. These data indicate that *Bhlhb4* is essential for maturation of rod bipolar cells (Bramblett et al., 2004).

**Bhlhb5.** *Bhlhb5* (also called Beta3) is detected in retina early at E11.5 in the neuroblast layer of the central retina, gradually expands to the whole retina, and is finally restricted to GABAergic amacrine cells and Type 2 OFF bipolar cells. The depletion of *Bhlhb5* shows selective loss of these two retinal cell types. A significant reduction in the number of Type 2 bipolar cells labeled by Vsx1 and recoverin antibodies, indicates that *Bhlhb5* is indispensable in development of major Type 2 bipolar cells. Furthermore, these findings suggest that Bhlhb5 works upstream of the transcription factor Vsx1 in this cell type. In *Bhlhb5*-null mouse retinas, there is no noticeable change of Mash1 and Math3 expression. Since Mash1/Math3 are required for the specification of bipolar cells, it is likely that *Bhlhb5* functions downstream of Mash1/Math3 (Feng et al., 2006). Although Bhlhb5 does not appear to bind DNA directly, it has been shown to mediate a strong transcriptional repressor response through its interaction with other bHLH proteins.
1.4 The Vsx family of homeodomain transcription factors

1.4.1 Structural and functional characteristics of Vsx transcription factors

Vsx1 (Visual system homeobox 1) encodes a paired-like homeodomain and was first identified from an adult goldfish retinal cDNA library screen (Levine et al., 1994; Passini et al., 1997; Galliot et al., 1999). In addition to goldfish, Vsx1 homologues have been identified in *C. elegans, Drosophila, zebrafish, frog, chicken, bovine, mouse and human* (Svendsen and McGhee, 1995; Erclik et al., 2008; Passini et al., 1998a; D'Autilia et al., 2006; Chen and Cepko, 2000; Chow et al, 2001; Ohtoshi et al., 2001; Hayashi et al., 2000; Semina et al., 2000).

Paired-like homeodomain transcription factors are part of the paired class of homeodomain transcription factors, which is named after the *Drosophila* gene *paired* (*prd*). Paired class homeodomain proteins, such as Pax6, Pax2, Hesx1, Crx, Rx and Chx10/Vsx2, have been shown to function as major determinants in eye formation of both vertebrates and invertebrates (Prosser and van Heyningen, 1998; Sanyanusin et al., 1995; Dattani et al., 1998; Freund et al., 1997; Mathers et al., 1997; Burmeister et al., 1996). Paired class homeodomains are characterized by the presence of six invariant amino acid residues within the homeodomain, and can be subdivided on the basis of the amino acid residue at position 50 of the homeodomain. This amino acid can be a glutamine (Q50) as in the Aristaless-type homeodomain (which includes Vsx1), a serine (S50) in the Pax-type, or a lysine (K50) in the Bicoid-type (Frigerio et al., 1986; Galliot et al., 1999). Similar to other members of the Q50 paired-like homeodomain sub-class, Vsx1 lacks a second DNA binding domain called the paired domain, which is found in the S50 Pax-type homeodomain proteins (Frigerio et al., 1986; Passini et al., 1997; Galliot et al., 1999).

In addition to the homeodomain, Vsx1 encodes a conserved amino acid region known as the CVC domain (Fig. 5). The CVC domain is named after the four Vsx genes initially described in *C. elegans* (*geh-10*) (Svendsen and McGhee, 1995), goldfish...
Figure 5. Mouse Vsx1 gene structure and mRNA sequence.

Exons 1-5 are represented by the number boxed regions in the top schematic. Black shaded regions indicate open reading frame and white regions show untranslated regions. The bottom cartoon shows the spliced Vsx1 mRNA with conserved domains indicated: OP- octapeptide; HD- homeodomain; CVC- Chx10, vsx1/vsx2, ceh-10 domain; RV-RINX/Vsx1 homology region; UTR- untranslated region; A- poly-adenylation sequence. (From Chow et al., 2001)
(vsx1/vsx2) (Levine et al., 1994; Levine et al., 1997) and mouse (Chx10) (Liu et al., 1994). These genes form a distinct sub-family of paired-like transcription factors known as the paired-like:CVC (Prd-L:CVC) transcription factors (Levine et al., 1997). The CVC domain is approximately 50–60 amino acid residues long (the variation depends on different genes/species), and is located immediately adjacent to the C-terminus of the homeodomain. The function of the CVC domain is unknown; however, studies have suggested that it is involved in modulating DNA binding or protein-protein interactions (Svendsen and McGhee, 1995; Passini et al., 1998a). The CVC domain is also thought to be involved in poly-ubiquitin-dependent proteolysis of Vsx1 (Kurtzman et al., 2000). Although initial ubiquitination of Vsx1 is not affected in the Vsx1 deletion constructs that lack CVC domain, the ubiquitin chain elongation (or polyubiquitinated) is reduced, as demonstrated by the biochemical experiments (Kurtzman et al., 2000).

All Prd-L:CVC proteins can be classified into two groups according to their sequence similarity to either Vsx1 or Vsx2/Chx10 (Chow et al., 2001). There are five group-specific amino acid residues within the homeodomain/CVC domains between these two Vsx groups. Alternatively spliced Vsx2 orthologues found in both chick and zebrafish (also called Alx) have a conserved insertion of 21 amino acid residues within CVC domain, in which 13 out of the 21 amino acids are identical (Chen and Cepko, 2000; Barabino et al., 1997). The function of this 21 amino acid insertion is not known. In addition, Vsx2 Prd-L:CVC proteins (but not Vsx1) contain a region known as the OAR domain (named after orthopedia/aristaless/Rax) (Furukawa et al., 1997). The OAR domain is present in several paired class homedomain proteins (Galliot et al., 1999) and is thought to function as a transactivator (Simeone et al. 1994). While the OAR domain is absent in Vsx1 orthologues, it is replaced by a region of unknown function called the RV(RINX/VSX1) domain (Hayashi et al., 2000). The RV domain is highly conserved among Vsx1 transcription factors and, like the OAR domain in Vsx2, is located close to the C-terminus (Chow et al., 2001; Hayashi et al., 2000; Liang and Sandell, 2008). Human VSX1 contains two additional domains that do not exist in Vsx1 proteins from other species: a proline-rich domain and an acidic domain similar to the transactivation domains observed in other transcription factors (Hayashi et al., 2000).
In addition to the paired-like homeodomain, the CVC domain and several other conserved domains, all the Vsx family transcription factors contain both a nuclear export signal (NES) and a nuclear localization signal (NLS) (Kurtzman and Schechter, 2001; Knauer et al., 2005) that enable themselves to shuttle between nucleus and cytoplasm (Fig. 6) (Knauer et al., 2005). A highly conserved leucine-rich region near the 5’ end of the protein, described as the octapeptide motif (OP) (Fig. 5), functions as an NES. *In vitro* studies suggest that the octapeptide interacts with Crm1, an export receptor for leucine-rich NES’s, to mediate nuclear export (Knauer et al., 2005). Deletion of the NES prevents export of Vsx proteins from nucleus, and leads to an increase in the transactivation of a Chx10-promoter driven luciferase reporter construct (Knauer et al., 2005). A highly conserved NLS is located at the N-terminus of the homeodomain (Kurtzman and Schechter, 2001; Knauer et al., 2005). Ubiquitin-like-conjugating enzyme 9 (Ubc9) has been shown to bind to the NLS and is required for the shuttling of Vsx1 to the nucleus (Kurtzman and Schechter, 2001). In SW13 AK1 cells, that express low levels of Ubc9, Vsx1 accumulates in a perinuclear ring in the endoplasmic reticulum (Kurtzman and Schechter, 2001). Interestingly, the Vsx NLS amino acid sequence is highly homologous to the sequence that encodes the protein transduction domain (PTD). The PTD domain has the ability to enable proteins to translocate across plasma membrane by an as of yet, undetermined extracellular secretion mechanism (Nickel, 2003). Thus, NLS appears to function dually for both nuclear import and intercellular trafficking (Knauer et al., 2005; Vivès et al., 2003). In summary, shuttling between the nucleus and the cytoplasm is a dynamic process thought to regulate Vsx transcriptional activity, and can be further modulated by mechanisms such as proteasomal degradation (Fig. 6) (Kurtzman and Schechter, 2001; Knauer et al., 2005). It is important to note, however, that the studies describing these processes have all been performed *in vitro*, using cell culture models and it remains to be determined what role these processes play, *in vivo*, in the retina.

1.4.2 Vsx gene expression and function in invertebrates

*ceh-10* is the only Prd-L:CVC gene found in *C. elegans*. It is more closely related to *Vsx2/Chx10* than it is to *Vsx1* in terms of the sequence similarity to the HD and the
Subcellular localization of paired-like:CVC (Prd-L:CVC) homeodomain proteins (such as Vsx1) is thought to involve nuclear import-export transport mechanisms mediated by the dual activities of NLS and NES. The proteasomal degradation pathway is involved in the nuclear export pathway, allowing the Prd-L:CVC homeodomain protein levels in cytoplasm to be regulated. Another way in which Prd-L:CVC cellular localization is regulated is through an unconventional extracellular secretion process. Intercellular transport and transactivation may be mediated by the protein transduction domain (PTD)/NLS (the Vsx NLS amino acid sequence is highly homologous to the sequence that encodes the PTD). Abbreviations: HD- homeodomain; CVC- Chx10, vsx1/vsx2, ceh-10 domain; NES- nuclear export signal; NLS- nuclear localization signal; PTD- protein transduction domain). (From Knauer et al., 2005)
CVC domains (Svendsen and McGhee, 1995). The similarity between CEH-10 and human CHX10 protein is 77% within these two domains (Erclik et al., 2008). This suggests that the function of CEH-10 and Vsx2/Chx10 is fairly conserved between these two remarkably different species. In Drosophila, dVsx1 and dVsx2, two homologues of Chx10 were identified from screens of embryonic and adult head cDNA libraries. Both share 81% amino acid identity with human CHX10 within the HD and CVC domains (Erclik et al., 2008). dVsx1 does not contain the OAR domain as other Vsx2/Chx10 proteins do (Erclik et al., 2008).

The invertebrate Vsx proteins: C. elegans CEH-10 and fly dVsx1 and dVsx2, are all more closely related to vertebrate Vsx2/Chx10 than to Vsx1. This suggests that the Vsx2/Chx10 subgroup is more ancestrally ancient. The duplication of a single Prd-L:CVC gene likely occurred after the segregation of chordates, nematodes, and arthropods. It is hypothesized that after the separation of the chordates from nematodes and arthropods, there was a gene duplication of an ancestral Vsx2/Chx10 in a common vertebrate ancestor that gave rise to Vsx1 (Svendsen and McGhee, 1995; Passini et al., 1998a; Chow et al., 2001; Erclik et al., 2008).

C. elegans does not possess a retina. However, the expression of ceh-10 is detected in a small number of sensory interneurons called AIY interneurons. AIY interneurons form synapses with thermosensitive AFD sensory neurons in the C. elegans amphid, a sensory structure located at the anterior end of the nematode. Interestingly, the AFD neuron is similar to a photoreceptor neuron located in a similar position in the amphids of a marine nematode species (Svendsen and McGhee, 1995). It has been suggested that ceh-10 plays a role in the sensory interneurons of nematodes similar to the role of Vsx2 and/or Vsx1 in vertebrate retinal bipolar cells (Svendsen and McGhee, 1995).

ceh-10 is required for the proper differentiation of AIY interneurons in C. elegans (Altun-Gultekin et al., 2001). In C. elegans, CEH-10 interacts with LIM homeodomain protein TTX-3 to regulate AIY development and function (Altun-Gultekin et al., 2001). In either ceh-10 or ttx-3 mutants, AIY interneurons are generated with expression of pan-neuronal genes; however, the specific subtype identity of AIY is disturbed, which means the differentiation of AIY is incomplete (Altun-Gultekin et al., 2001). Interestingly,
although *ceh-10* is more similar to *Vsx2/Chx10* subgroup in sequence, its function in worms more closely resembles the function of *Vsx1* in mouse. In mouse, *Vsx1*, like *ceh-10*, is required for the terminal differentiation of bipolar interneuron but is not required for their specification (Chow et al., 2004; Ohtoshi et al., 2004), whereas *Vsx2/Chx10* is required for bipolar cell specification (Burmeister et al., 1996).

In *Drosophila*, the expression of *dVsx1* is observed in a group of multipotent outer optic anlage (OOA) progenitor cells in the optic lobe at embryogenesis stage (Erclik et al., 2008). In contrast to vertebrate eyes, flies have compound eyes and their retina only consists of photoreceptor cells, which directly project to either the first or the second ganglion cells sited respectively in the lamina or the medulla of the optic lobe. Similar to the expression pattern of vertebrate *Chx10/Vsx2*, when OOA progenitors become neuroblasts, *dVsx1* expression is downregulated and subsequently upregulated in the medulla (Erclik et al., 2008). Mutations in *dVsx1* lead to hypocellular, smaller, and disorganized optic lobes by impeding the proliferation of the progenitor cells (Erclik et al., 2008). One difference between flies and vertebrates is that in fly, *dVsx1* expression is confined to a subset of OOA progenitors, whereas *Vsx2/Chx10* is expressed in all retinal progenitor cells. *dVsx1*, along with *dVsx2*, are also co-expressed in a subpopulation of neurons in the developing and mature medulla. In the medulla, *dVsx1/2* are expressed in transmedullary neurons, which receive input directly from photoreceptors. *dVsx* genes are also expressed in medullar local neurons, different from the vertebrate *Vsx* genes which are not found in other retinal interneurons such as horizontal cells and amacrine cells (Erclik et al., 2008).

**1.4.3 *Vsx2/Chx10* in vertebrate retinal development**

*Vsx2/Chx10* is initially expressed in proliferating retinal neuroepithelial cells during optic cup formation as early as E9.5 in mouse embryos, closely followed by the onset of competent retinal progenitor cell proliferation at E10 (Liu et al., 1994). It is one of the most specific and earliest markers of retinal progenitor cells during eye development (Liu et al., 1994; Burmeister et al., 1996; Levine and Green, 2004). *Vsx2/Chx10* is downregulated as the progression of retinal progenitor cells differentiating
to various retinal cells. It remains expressed, however, in bipolar cells in both developmental and in the adult stages (Liu et al., 1994; Levine et al., 1997; Passini et al., 1997). It is also expressed in Müller glial cells in mice and zebrafish in the mature retina (Rowan and Cepko, 2004; Vitorino et al., 2009). The retinal margin of postnatal chickens retains proliferating progenitor cells, resembling the frog ciliary margin zone (CMZ) and expressing PCNA, Pax6 and Chx10 (Fischer and Reh, 2000). Vsx2/Chx10 is found in the proliferative CMZ of adult amphibians, which maintains neural progenitors or stem cells (Lamba et al., 2008). In goldfish and zebrafish retinas that keep on growing persistently during their adulthood, Vsx2/Chx10 is strongly expressed in the mitotically active progenitors of the germinal zone at the retinal peripheral margin and iris epithelium (Levine et al., 1997; Passini et al., 1997; Raymond et al., 2006; Vitorino et al., 2009).

Chx10 mutations can lead to microphthalmia (or small eyes) a form of congenital blindness, marked by a cataractctous lens, a poorly developed retina, and a severely abnormal iris in both humans and mice (Burmeister et al. 1996; Ferda Percin et al., 2000). Ocular retardation J (orJ) mice carry a natural nonsense mutation within the homeodomain of Chx10, characterized by a profound reduction in the proliferation of retinal progenitor cells during retinogenesis and a specific loss of differentiated bipolar cells in the lamination-disturbed, smaller-sized retina (Burmeister et al. 1996). Injection of Alx1 (orthologue of Chx10 in zebrafish) antisense oligonucleotides into zebrafish embryos causes small eye malformations, similar to the phenotype of the orJ mice (Barabino et al., 1997). Thus, Vsx2/Chx10 plays two distinct roles in eye development: it is required for retinal progenitor cell proliferation and for the specification of retinal bipolar cells (Burmeister et al. 1996; Belecky-Adams et al., 1997).

One possible mechanism has been suggested for how Vsx2/Chx10 is involved in the regulation of retinal progenitor cell proliferation. In the Chx10-null mice, the cell cycle of retinal progenitor cells is thought to be prolonged in G1 phase by accumulation of the cyclin-dependent kinase inhibitor (CDKI) protein p27kip1 (Green et al., 2003). In Chx10 and p27kip1 double knockout mice, the hypocellularity is alleviated in the retina and proper retinal lamination is partially restored. However, the elimination of p27kip1 is insufficient to rescue bipolar cells (Green et al., 2003). This indicates that distinct mechanisms regulate retinal progenitor cell proliferation and bipolar cell specification,
and also indicates that other gene(s) are involved in Chx10 regulation of retinal progenitor cell proliferation besides the p27Kip1-dependent way. It is interesting to note that the wild type p27Kip1 protein is only present in post-mitotic retinal cells, whereas p27Kip1 mRNA coexists with both Chx10 mRNA and protein in retinal progenitor cells. Therefore, Chx10 is suggested to regulate p27Kip1 post-transcriptionally (Green et al., 2003).

Chx10 might regulate retinal progenitor cell proliferation through activating Shh signaling in presumptive retina (Sigulinsky et al., 2008). The conditional Shh knockout mouse shows a depletion of retinal progenitor cell pool (Wang et al., 2005). In the orJ Chx10 mouse mutant, both the expression and activity of Shh is impeded and correspondingly, the differentiation of ganglion cells is delayed during retinal development. When pre-processed recombinant Shh ligand (Shh-N) is applied in explant culture, the restricted orJ retinal progenitor cells expand their proliferation and some of the repressed Hh target genes are expressed (Sigulinsky et al., 2008).

The depletion of retinal bipolar cells in Chx10-null mice is not caused by retinal progenitor cell proliferation defects because Müller glia are born later than bipolar cells and are present in the absence of Chx10 (Burmeister et al. 1996). Also, as bipolar cells are not rescued in Chx10 and p27Kip1 double mutants, it suggests Chx10 is involved in specification and/or the differentiation of bipolar cells (Green et al., 2003). It has been suggested that Chx10 promotes bipolar cells by inhibiting rod photoreceptor differentiation (Livne-Bar et al., 2006). Although misexpression of Chx10 is not sufficient for maturation of bipolar cells, misexpression of Chx10 along with Mash1/Math3 can promote the generation of bipolar cells by inhibiting gliogenesis in the retinal INL (Hatakeyama et al., 2001).

1.4.4 Vsx1 genes in vertebrate retinal development

1.4.4.1 Gene structure

The mouse Vsx1 gene was screened and cloned from a strain129 mouse genomic library and an adult retina cDNA library (Chow et al., 2001). The Vsx1 gene maps to chromosome 2 between coloboma (78 cM) and blind sterile (83 cM) in mouse, and is
syntenic to VSX1-mapped region in human chromosome 20p11.2 (Chow et al., 2001; Hayashi et al., 2000). The Vsx1 genomic DNA spans a region of approximately 6.3 kb, containing 5 exons in mouse (Fig. 5). The mRNA coding region of mouse Vsx1 is 1089 bp in length and encodes 363 amino acid residues (Chow et al., 2001; Ohtoshi et al., 2001). The human genomic orthologue VSX1/RINX spans a 6.6 kb region, and encodes a 365-amino acid protein within 5 exons. The human VSXI cDNA was isolated from both a human embryonic craniofacial cDNA library and an adult retinal cDNA library by two research groups (Semina et al., 2000; Hayashi et al., 2000). RT-PCR analysis revealed two major classes (L and S) of human VSXI mRNA transcripts expressed in retina, which are derived from alternate transcription termination signals (Hayashi et al., 2000). The major L1 transcript encodes a complete 365-amino acid protein, whereas the major S1 transcript lacks the encoding region of Helix III of homeodomain, CVC domain and RV domain (Hayashi et al., 2000). Alternate splicing is thought to generate other four minor human VSXI transcripts: L2, L3, S2 and S3, all of which encode truncated forms of VSX1 (Hayashi et al., 2000). Interestingly, some of these truncated VSX1 proteins have an extra region that is encoded by an intronic sequence. Not all of the truncated human VSX1 sequences are predicted to be able to bind to DNA due to their lack of a complete homeodomain. However, the proline-rich and acidic domains for transcriptional activation are all maintained in these 4 transcripts, which may indicate that they can modulate transcriptional activity by competing as cofactors to the full length VSX1 protein or other transcription factors (Hayashi et al., 2000).

In 2008, two novel downstream exons (exon 6 - 108bp in length, and exon 7 - 535 bp in length) were identified in human VSXI (Hosseini et al., 2008). These two exons contain non-coding region and are alternatively spliced (Hosseini et al., 2008). How these alternatively spliced versions of human VSXI function are generated and whether similar splice variants are observed in mouse is currently unknown. It is quite common that a single piece of DNA can generate a diversity of mRNAs, and that the resulting proteins possess distinct functions in different tissues or under different physiological conditions. Very recently Barash et al. (2010) revealed “the second genetic code” of alternative splicing regulation to determine the sites of mRNA processing based on the complexity of RNA features through a computational approach. The prediction was markedly
successful in some trials. Splicing regulation is both tissue-specific and species-specific, and the generation of truncated proteins is commonly applied in gene expression control by skipping the exon(s) during the transition from embryonic to adult stages. There is only about 20% conservation of the regulation between humans and mice, indicating that alternative splicing regulation is highly variable between species (Barash et al., 2010).

1.4.4.2 Translational control of Vsx1 expression

RT-PCR studies have shown that low levels of Vsx1 mRNA are detected in mice as early as embryonic day 6.5 (E6.5), in the embryonic eye and retina (Ohtoshi et al., 2001; Chow et al., 2001). It is that Vsx1 translation is repressed in proliferating progenitor cells and activated much later in bipolar cells (Ohtoshi et al., 2001; Chow et al., 2001). What mechanism might underlie the developmental regulation of Vsx1 translation? In other species, a distinct timing gap also exists between the transcription and translation of certain genes. Research with Xenopus laevis shows that cell cycle progression is required to activate the translation of Xvsx1, Xotx2 and Xotx5b mRNAs transcripts (that are already present in early retinal progenitors) in differentiated neurons. The timing of translation of Xvsx1 and Xotx2 coincides with the generation of bipolar cells and the timing of Xotx5b coincides with the generation of photoreceptors (Decembrini et al., 2006). Furthermore, the 3’ untranslated regions (UTRs) of these three genes have been shown to block their own translation in early progenitor cells (Decembrini et al., 2006). Similarly, in goldfish embryos, Vsx1 3’ UTR has been shown to mediate translation specifically in retinal bipolar cell at late developmental stage (Chen et al., 2009). It has been suggested that the sequential translational activation of homeodomain proteins mediated by a “cellular clock” determines cell birth dates and identities of the distinctive retinal cell types over developmental time (Decembrini et al., 2006).

Four cell cycle-related microRNAs (mir-129, mir-155, mir-214, and mir-222) have been found to participate in the translational inhibition of Xotx2 and Xvsx1 in early progenitors (Decembrini et al., 2009). These microRNAs (miRNAs) are abundantly
expressed in the early embryo. The four miRNAs act downstream of cell cycle machinery; and when the cell cycle slows down in late progenitor cells, their expression is downregulated. In vivo inactivation of these miRNAs de-represses the translation of Xotx2 and Xvsx1, and biases early progenitors towards bipolar cell fate specifically by being unable to target 3’UTR of Xotx2 and Xvsx1 (Decembrini et al., 2009). Thus, translational control by miRNAs appears to be critical for retinal bipolar cell development in Xenopus by providing a mechanism to temporally regulate the translation of genes involved in bipolar cell fate determination and differentiation.

1.4.4.3 Vsx1 expression in vertebrate eye development

In multiple vertebrates, a common feature of Prd-L:CVC genes is their high expression level in the inner nuclear layer (INL) of both embryonic and adult retinas, indicating that the Vsx gene family plays an important and evolutionarily conserved role in the development of retinal interneurons. Vsx genes are also expressed in the interneurons in the hindbrain and spinal cord (Liu et al., 1994; Levine et al., 1994 and 1997; Burmeister et al., 1996; Barabino et al., 1997; Passini et al. 1997, 1998a and 1998b; Chen and Cepko, 2000; D'Autilia et al., 2006). In mice, Vsx1 expression is first detected by in situ hybridization and immunostaining in the inner nuclear layer region of central developing retina at postnatal day 5 (P5), two days after the peak of bipolar cell birth, which suggests that Vsx1 functions post-mitotically during bipolar cell differentiation. Vsx1 expression expands in a central-to-peripheral retina pattern (following the normal pattern of retinal cell differentiation) and by P12, its expression spans the entire length of the INL. In the adult retina, Vsx1 is maintained and restricted to a subpopulation of OFF and ON cone bipolar cells, suggesting that it is also required for the maintenance of these interneurons (Chow et al., 2001 and 2004). This subset of bipolar cells in which Vsx1 is expressed comprises approximately 35% of all bipolar cells in adult mouse retina (Jeon et al., 1998; Rowan and Cepko, 2004; Chow et al., 2004).

1.4.4.4 Is Vsx1 expressed in other ocular tissues?
In addition to expression in some types of bipolar cells in the retina, \( Vsx1 \) expression has also been observed in other ocular tissues, despite the discrepancies among these findings. \( VSX1 \) expression has been documented in human embryonic craniofacial tissue, and in both the cornea and retina of adult humans (Semina et al. in 2000). In contrast, this result is different from other studies, which find that \( VSX1/Vsx1 \) expression is undetectable in both human and mouse cornea and lens, only present in retinal tissues (Héon et al., 2002; Chow et al., 2001).

Expression of \( VSX1 \) in the human cornea is of interest, because \( VSX1 \) has been associated with the two clinically and genetically heterogeneous corneal dystrophies: keratoconus and posterior polymorphous corneal dystrophy (PPCD) (Héon et al., 2002; Mintz-Hittner et al., 2004; Bisceglia et al., 2005; Valleix et al., 2006; Mok et al., 2008). Interestingly, the H244R \( VSX1 \) mutant associated with a dominant PPCD shows defects in cone ON visual signaling, providing an example of a selective heritable retinal defect in humans (Valleix et al., 2006). However, the relationship \( VSX1 \) and corneal dystrophies is controversial and other studies fail to support the involvement of \( VSX1 \) in keratoconus and PPCD1 (Aldave at al., 2006; Tang et al., 2008; Hosseini et al., 2008).

Recently Hosseini et al. (2008) reported that the expression of \( VSX1 \) is present in the neonatal human cornea but absent in the adult cornea; however, the corneal expression in this study is at near threshold levels of detection. Another study has shown that \( VSX1 \) is dramatically upregulated in the human and mouse cornea in response to the cornea wounding (Barbaro et al., 2006). Upregulation of \( Vsx1 \) has also been observed in retinal bipolar cells during retinal regeneration in goldfish following retinal injury (Levine et al., 1994). In the cornea, it has been hypothesized that \( VSX1 \) plays a role in the corneal wound response mechanism (Barbaro et al., 2006). This hypothesis thus provides a possible mechanism to explain the association of \( VSX1 \) mutations with human corneal dystrophies keratoconus and posterior polymorphism dystrophy despite the absence of \( Vsx1 \) expression in the normal cornea.

1.4.4.5 \( Vsx1 \) is essential for cone bipolar cell differentiation and visual signaling
Studies on knockout mice with \textit{Vsx1} loss-of-function mutations have shown that \textit{Vsx1} is essential for bipolar cell terminal differentiation (Chow et al., 2004; Ohtoshi et al., 2004). The gross survival and specification of cone bipolar cells appear normal in the retina of \textit{Vsx1} knockout mice as the expression of Chx10 and Ret-B1, two pan-bipolar cell markers are unaffected (Chow et al., 2004). However, the terminal differentiation of Type 2 OFF bipolar cells is incomplete and is accompanied by a substantial reduction in the expression of three Type 2 bipolar cell markers: recoverin, NK3R (neurokinin-3 receptor) and Neto1 [neuropilin (NRP) and tolloid (TLL)-like 1] (Chow et al., 2004). In addition, a reduction of Cabp5 (Calcium binding protein 5) is observed in the axonal terminal region of Type 3 OFF bipolar cells. The immunohistological defects in \textit{Vsx1}-null mice are accompanied by defects in visual signaling: bipolar cell dysfunction is inferred by a mildly reduced electroretinography (ERG) b-wave (Chow et al., 2004; Ohtoshi et al., 2004), and reduced OFF ganglion cell responses are determined by \textit{in vitro} ganglion cell single unit recording (Chow et al., 2004). These findings indicate that \textit{Vsx1} is essential for the differentiation of OFF cone bipolar cells and the proper function of OFF cone visual signaling.

\section*{1.4.5 Interaction of \textit{Vsx1} and \textit{Chx10}/\textit{Vsx2}}

Homologous genes display similar structure, extensive expression patterns and function in many cases. Therefore, it is quite common that homologous genes have redundancy and overlapping in genetics and function, as well as inter-regulation between them (Kafri et al., 2006 and 2009). \textit{Vsx1} and \textit{Vsx2}/\textit{Chx10} are the only two known paralogues in vertebrates, which contain the common structure domains: paired-like homeodomain and CVC domain. From what we already know (as discussed in Sections 1.3.3 and 1.3.4.5), \textit{Vsx1} expression is restricted to a subset of retinal bipolar cells and is essential for the terminal differentiation and function of OFF bipolar cells, while \textit{Vsx2}/\textit{Chx10} plays an indispensable role in retinal progenitor cell proliferation and in the specification and maintenance of all bipolar cells. \textit{In situ} hybridization shows that initially \textit{Vsx2} is highly expressed in the mitotically active retinal progenitor cells during retinal development of zebrafish and goldfish. When \textit{Vsx2} is downregulated in the
differentiating INL of the fish retina, \( Vsx1 \) is coincidently upregulated in differentiating presumptive bipolar cells (Levine et al., 1994; Passini et al., 1997). This inverse expression pattern is also observed in bipolar cells of the normal, mature mouse retina by immunohistochemistry (Clark et al., 2008). It has been suggested that \( Vsx1 \) and \( Vsx2/Chx10 \) may negatively regulate each other’s expression (Clark et al., 2008).

Both Chx10 and Vsx1 can function as transcriptional repressors in \textit{in vitro} transcriptional transactivation assays (Dorval et al., 2005). In contrast, Chx10 is able to weakly activate transcription of some genes in chick retinal cultures (Dorval et al., 2005). In addition, the substantial reduction of three cell markers (recoverin, NK3R and Neto1) in \( Vsx1 \)-null mice (Chow et al., 2004), suggests, although only in an indirect way, that Vsx1 may function as a transcription activator. The possibility that Chx10 can negatively regulate Vsx1 by directly binding to the \( Vsx1 \) promoter is supported by the ability of Chx10 to repress luciferase transcriptional activity of the \( Vsx1 \) promoter (Clark et al., 2008). The distinct phenotypes in the \( Vsx1 \) mutant and the \( Chx10 \) mutant indicate these paralogues have different roles. They are unable to compensate each other in absence of the other gene, possibly because they regulate different downstream target genes, and are physically interacting with different transcription factors, even though they have similar structure and DNA binding/recognizing sites (Clark et al., 2008).
Chapter 2 Materials and Methods

2.1 Mouse strains and genotyping

Mouse strain. Vsx1: τLacZ-Vsx1 mice maintained in a 129S1 genetic background (The Jackson Laboratory) were crossed with α-gustducin:GFP transgenic mice (Huang et al., 1999 and 2003). Vsxl^τLacZ knock-in mice were previously generated by replacement of the first 86 base pairs of the exon 1 of Vsx1 with the τ-LacZ reporter gene by homologous recombination (Fig. 7) (Chow et al., 2004). The expression of α-gustducin:GFP in mouse retina is strong in a subset of Type 7 bipolar cells and weak in rod bipolar cells (Huang et al., 2003). mGluR6:NLS-LacZ mice were generated by cloning the 9.5 kb upstream region of the mGluR6 encoding gene (Grm6) (Ueda et al. 1997) with 5’ of a NLS (nuclear localization signal) -LacZ reporter gene (Dr. R. L. Chow, unpublished data). mGluR6:NLS-LacZ transgenic mice were crossed to mice containing α-gustducin:GFP transgenic reporter. All mouse work was done with approval by the University of Victoria Animal Care Committee, in accordance with the Canadian Council for Animal Care. The mouse strains I used in my research are listed in Table 1.

Genotyping. Biopsies obtained from ear notching of mice were denatured in 75 μl of 50 mM NaOH at 95 °C for 10 minutes, followed by the addition of 25 μl of 0.5 M Tris pH 8.0. Samples were then shaken vigorously by hand and spun down briefly. 1-2 μl of ear notch DNA was used for genotyping.

PCR reaction. In general PCR reactions were prepared as listed in Table 2. PCR amplification was applied using T3 Thermocycler (Biometra), by denaturation at 97 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C (for Vsxl^τLacZ) and 68 °C (for α-gustducin:GFP) for 30 s, elongation at 72 °C for 1 min; and elongation at 72 °C for 5 min. 10× PCR buffer contained 500mM KCl, 100mM Tris (pH8.3), 15mM MgCl₂ and 0.1% gelatin. A list of genotyping primers used is presented in Table 3.
Figure 7. The generation of Vsx1:τLacZ knock-in mouse.

Vsx1:τLacZ knock-in mice were generated by replacement of the first 86 base pairs of the exon 1 of Vsx1 gene with tau-LacZ reporter gene by homologous recombination, including the replacement of Vsx1 start codon, ATG, by the start codon of τLacZ reporter gene. p and p’ indicate the Southern blot probes used to identify the wild type and mutant Vsx1 alleles, respectively. (From Chow et al., 2004)
Table 1. List of mouse strains used in my thesis.

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-gustducin:GFP</td>
<td>- transgene insertion utilizing 8.4 kb upstream region of α-gustducin to direct GFP expression  - strong GFP expression in Type 7 bipolar cells, weak expression of GFP in rod bipolar cells</td>
<td>Huang et al., 2003</td>
</tr>
<tr>
<td>Vsx1τLacZ</td>
<td>- Vsx1 knock-in null allele  - tau-LacZ reporter gene is inserted into exon 1 of the Vsx1 gene</td>
<td>Chow et al., 2004</td>
</tr>
<tr>
<td>mGluR6:NLS-LacZ</td>
<td>- transgene insertion utilizing 9 kb upstream region of Grm6 to direct nuclear localized β-galactosidase expression  - all ON bipolar cells</td>
<td>Generated by R.L. Chow using 9.5 kb Grm6 upstream region (Ueda et al., 1997)</td>
</tr>
</tbody>
</table>

Table 2. The assembly of genotyping PCR reactions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× PCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTPs (4mM each)</td>
<td>1</td>
</tr>
<tr>
<td>Primer mix (10µM each)</td>
<td>1 or 0.5</td>
</tr>
<tr>
<td>Homemade Taq</td>
<td>0.2</td>
</tr>
<tr>
<td>60% sucrose/cresol red</td>
<td>3</td>
</tr>
<tr>
<td>DNA</td>
<td>1 or 2</td>
</tr>
<tr>
<td>H₂O</td>
<td>to total of 25µl</td>
</tr>
</tbody>
</table>
Table 3. List of genotyping primers

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Primer - Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-gustducin:GFP</td>
<td>5' SC - CCGGGCCCCCTCTGCTAACC 3' GFP - GGTAGAGCTTTCCGTATGTCG</td>
<td>Huang et al., 2003</td>
</tr>
<tr>
<td>Vsx1τLacZ</td>
<td>5' common - TTCTAGGGCTGTCTAGGTCTC 3' wild-type - TGATGGCAAGCTTCTGAAGG 3' τLacZ - TTGCCTTTACTGACCATGC</td>
<td>Chow et al., 2004</td>
</tr>
<tr>
<td>mGluR6:NLS-LacZ</td>
<td>5' Nuc_Lac_F - TCTTTTGGCAATGTGAGGGC 3' Nuc_Lac_R - TGAATACGCTTGAGGAGGC</td>
<td>R. L. Chow - unpublished</td>
</tr>
</tbody>
</table>

2.2 Tissue preparation, Immunocytochemistry, Confocal imaging and image analysis

*Tissue preparation.* Animals were sacrificed by CO₂ followed by cervical dislocation. The eyes were removed, quickly enucleated, and transferred to a petri dish containing ice-cold phosphate buffer (PBS, pH 7.4). The cornea, lens and vitreous body were rapidly removed and the eyecups were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Cat. # 157-8; Hatfield, PA) in PBS either at room temperature for 20 minutes or on ice for 1 hour. After fixation, the eyecups were rinsed for three times in PBS and cryoprotected in graded series of sucrose/PBS solutions (10% w/v for one hour, 20% for 3 hours and 30% overnight). The eyecups were embedded and frozen in Tissue-Tek O.C.T. compound (Sakura Finetek, Cat. No. 4583; Torrance, CA). Sections were cut at 12~14 µm or 30 µm thickness with a cryostat (Leica CM1850UV; Germany), and collected on silane-treated slides (Newcomer, MA) and stored at -20 °C.

*Immunocytochemistry.* Immunostaining was performed by the indirect fluorescence method. Retinal sections were rinsed in PBS to wash off the O.C.T. compound, permeabilized in 1% Triton X-100/phosphate-buffered saline (PBS, pH 7.4) for 30 minutes and blocked in 10% horse serum (Sigma, H0146; St. Louis, MO) /PBS at
room temperature for one hour. Sections were incubated with the mixture of primary antibodies diluted (listed in Table 4) in 1% horse serum/PBS overnight at 4°C. After washing in PBS for 3~5 times, the sections were then incubated with the diluted secondary antibodies (see below) for 1 hour at room temperature. Finally, sections were mounted with a drop of ImmnoMount (Thermo, Cat. No. 9990402; Pittsburgh, PA).

Secondary antibodies: Alexa Fluor® 488, 543 or 633 (Molecular Probes, Eugene, OR; working dilution of 1:200) or Cy™-2, 3 or 5 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; working dilution of 1:100).

Confocal imaging and image analysis. Fluorescence-stained sections were visualized and confocal micrographs were taken using Nikon confocal laser scanning microscope (Nikon Eclipse TE-2000U, Japan) equipped with a 488 argon (Ar) laser, and two helium-neon (HeNe) 543 and 633nm lasers. The following objectives were used: S Fluor 40×/NA1.30, 60×/NA1.49, or 100×/NA1.49 oil immersion objectives (Nikon) at a resolution of 1024 or 2048 pixels. The acquired images were optimized for brightness and contrast using Adobe Photoshop CS (Adobe System, San Jose, CA, USA).

2.3 Quantitation of Chx10 levels in Type 7 and Type 2 retinal cone bipolar cells

Retinal sections were triple-stained and examined for immunofluorescence using sheep anti-Chx10, chicken anti-GFP, rabbit anti-PKCα and rabbit anti-NK3R antibodies. Images were taken with the 60× objective as stacks of 4~5 optical sections both above and below the brightest focal plane of Chx10 intensity with a step size of 0.45 μm. The brightest level of Chx10 fluorescence was acquired by setting up the high enough gain, but well below its saturation for each individual z-stack. For the evaluation of Chx10 level in each cell of a-Gustducin:GFP-positive (which is presumed to be a Type 7 bipolar cells), PKCα-positive (rod bipolar cells), or NK3R-positive (Type1/2 bipolar cells), the maximum value of Chx10 intensity within the stack was measured. All the images were acquired using the same microscopic conditions. It was assumed that in Vsx1 wild type and null retinas, the Chx10 levels in rod bipolar cells (labeled out by PKCα staining)
### Table 4. List of primary antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Target</th>
<th>Dilution/Species</th>
<th>Remarks</th>
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<td>ICN, 55976</td>
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<td>Zebrarfish International Resource Center, Univ. of Oregon, Eugene, OR</td>
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were not changed, and these values were used for normalization. Student’s t-test was applied to compare Chx10 levels between *Vsx1* wild type and null retinas.

### 2.4 Flow cytometry analysis of α-gustducin:GFP fluorescence in Type 7 bipolar cells

*Retinal dissection.* Mice were euthanised by carbon dioxide and cervical dislocation, and eyes were enucleated. After removal of cornea, lens and vitreous body, retinas were rapidly dissected and separated from retinal pigmented epithelium in cold PBS. Retinal tissue was rinsed twice in PBS and then cut into 1- to 2-mm² pieces.

*Retinal Dissociation.* The papain dissociation mix was prepared as Table 5. Four mL of papain (20U/mL, Worthington Biochemical corporation, Lakewood, NJ, Cat # LS003126) was first activated in Hank's balanced salt solution (HBSS, Gibco, Invitrogen, Cat # 14175-095) at 37°C for 20 minutes or until the papain was completely dissolved and the solution appeared clear (incubation was to insure full solubility and activity), and then the solution was filtered with a 0.22 micron membrane. 2 µl of DNaseI (200 U/µl, Invitrogen, 18047-019) was added to a final concentration of 100 U/mL.

1). Retinal pieces were incubated in 4 mL papain/HBSS for 12~14 min in a 5 mL falcon tube at 37°C water bath with gentle agitation until the pieces looked lighter and smaller.

2). The enzymatic reaction was stopped by adding 4 mL (an equal volume) of prewarmed 10% FCS (fetal calf serum, Gibco, Invitrogen, Cat # 10437036) /HBSS, and changed to a 15-mL tube. Waited for 2~5 mintues until the retinal tissue fell down to the bottom of the tube, and then removed the solution by pipette.

3). 3 mL pre-warmed 1% FCS/HBSS containing 100U/mL DNase I was added. The mixture was triturated gently 8~10 times using 5 mL pipette or until the tissue “dissolved”.

4). The sample was passed through a 70µm cell strainer (BD Biosciences, BD Falcon, REF352350, USA) to collect the single cell suspension.

5). The concentration of the acquired cells was determined with a Bright-Line Hematometer (Hausser Scientific). 10 µl of cell solution was used to estimate the cell concentration. Usually there were 1.6×10⁷ cells/ 4 retinas.
6). Cell suspension was kept on ice until used.

*Cell Analysis/sorting by flow cytometry.* α-gustducin:GFP +’ve cells from dissociated retinas were detected and sorted by the high-speed cell sorter FACS Vantage Diva (Becton Dickinson) at Biomedical Research Centre of University of British Columbia. The data were analyzed using the CellQuest Pro 5.2 software (Becton Dickinson).

**Table 5. The preparation of papain dissociation mix.**

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<th>Stock Conc.</th>
<th>Volume</th>
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<td>Varies</td>
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<tr>
<td>L-cysteine (Fluka, 30086),</td>
<td>5.5 mM</td>
<td>1M (200×)</td>
<td>20 µl</td>
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<tr>
<td>β-mercaptoethanol (Sigma, M3148)</td>
<td>0.067 mM</td>
<td>13.9M (2×10e5)</td>
<td>Trace amount</td>
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<tr>
<td>EDTA</td>
<td>1.1 mM</td>
<td>0.5M (500×)</td>
<td>8 µl</td>
</tr>
<tr>
<td>DNase I</td>
<td>100 U/µl</td>
<td>200 U/µl</td>
<td>2 µl</td>
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<tr>
<td>HBSS (No Ca2+ and Mg2+)</td>
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<td>to 4 mL</td>
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Chapter 3 Results

3.1 Vsx1 is expressed in Type 7 ON bipolar cell types

As discussed above, previously published data examining τLacZ knock-in reporter gene expression in heterozygous Vsx1\(^{LacZ/+}\) mice indicate that a subset of Vsx1-expressing cells are ON cone bipolar cells (Fig. 8A) (Chow et al., 2004). The data from this study, however, did not determine which of the five ON cone bipolar cell types expresses the Vsx1 reporter. In order to address this issue, I took advantage of the well-defined cellular morphology of τ-β-galactosidase immunolabelled ON bipolar cells in Vsx1\(^{LacZ/+}\) heterozygous mice. This approach for identifying Vsx1-expressing cell types has the advantage that the β-galactosidase reporter is synthesized as a τ-fusion protein which enables it to efficiently label axons, thus making it ideal for examining neuronal morphology (Callahan and Thomas, 1994). Vsx1\(^{LacZ/+}\) heterozygous mice were useful for this approach because they only express the τLacZ knock-in reporter gene in a subset (about 30%) of Vsx1-expressing cells, and thus allow one to observe individual cells (Chow et al., 2004). Calretinin immunolabeling reveals three distinct bands in the inner plexiform layer that define distinct sublaminae, which are used to characterize different bipolar cell types (Ghosh et al. 2004). I examined the Vsx1: τ-β-galactosidase reporter immunolabelling relative to calretinin immunolabelling in the inner plexiform layer of the retina, as bipolar cell types can be classified, in part, by the location of their axonal terminals relative to 3 stereotypical calretinin immunolabelled bands that subdivide this layer into 5 sublaminae (Fig. 8B) (Ghosh et al., 2004). In all cases, the Vsx1: τ-β-galactosidase immunolabelled axonal projections of ON cone bipolar cell terminated in sublamina 4 of the inner plexiform layer, narrowly stratified, just below the innermost (i.e. closest to the ganglion cell layer) caleretinin labeled band (Fig. 8C). This pattern of Vsx1: τ-β-galactosidase reporter immunolabelling is most similar to that observed for Type 7 ON cone bipolar cells, which also project to sublamina 4 of the inner plexiform layer. Reporter immunolabelling was not observed in sublamina 3 or 5, which are two additional sublamina where ON bipolar cells project (Fig. 8B). Thus, in Vsx1\(^{LacZ/+}\)
Figure 8. Morphological identification of Vsx1-expressing ON cone bipolar cell types from Vsx1\(^{\tau LacZ/+}\) mouse retina.

(A) Two types of bipolar cells are shown by Vsx1:\(\tau\)-\(\beta\)-galactosidase immunostaining (Chow et al., 2004). The arrowhead indicates a putative ON bipolar cell type; the arrow indicates an OFF bipolar cell. (B) Schematic diagram showing the five types of ON cone bipolar cells in mouse retina (Ghosh et al., 2004). The right side of this panel show the relative position of the three calretinin immunolabelled bands that are used to define different inner plexiform sublaminae. (C) Morphological determination of the Vsx1:\(\tau\)-\(\beta\)-galactosidase labelled ON bipolar cell types. All of these cells project their axonal terminals to sublamina 4 of the inner plexiform layer just below the innermost calretinin-labelled band (colored in cyan), in a manner that is most similar to that observed for Type 7 ON cone bipolar cells. Scale bar in C (iv) = 14 \(\mu\)m. (Data from Z. Shi and D. Jervis)
heterozygous mice, the *Vsx1*: τ-β-galactosidase knock-in reporter labels cells that resemble Type 7 ON cone bipolar cells.

As no Type 7 bipolar cell specific immunohistological markers have been identified, further validation that the *Vsx1*:τ-β-galactosidase immunolabelled ON cone bipolar cells were in fact Type 7 cells required an alternative approach. My approach utilized the *α-gustducin*:GFP transgenic reporter mouse line in which GFP reporter expression is directed by an 8.4 kb upstream promoter region of the *α-gustducin* gene (Huang et al., 1999). The *α-gustducin* gene was initially identified as the alpha subunit of a heterotrimeric G-protein complex involved in taste cell receptor transduction, but fortuitously the *α-gustducin*:GFP transgene has also been shown to be specifically expressed only in Type 7 cone bipolar cells and rod bipolar cells in the retina (Huang et al., 1999 and 2003; Wong et al., 1999). In *α-gustducin*:GFP mice Type 7 ON bipolar cells are strongly labeled by GFP, while rod bipolar cells are weakly labeled (Huang et al., 2003; Ghosh et al., 2004; Wässle et al., 2009). In retinal sections from mature *α-gustducin*:GFP mice, *Vsx1* immunostaining was present in putative Type 7 ON bipolar cells expressing high levels of GFP (Fig. 9) but not in putative rod bipolar cells expressing low levels of GFP (Fig. 9). This observation is consistent with previous observations that *Vsx1* is not expressed in rod bipolar cells (Chow et al., 2001), and indicates that *Vsx1* is expressed in Type 7 bipolar cells.

To determine whether all of the *Vsx1*-expressing ON bipolar cells are Type 7 bipolar cells, *α-gustducin*:GFP mice were crossed to a transgenic mouse generated in the Chow lab that expresses nuclear localized version of β-galactosidase (NLS-β-galactosidase) in all ON bipolar cell types, by virtue of the upstream regulatory region of the *mGluR6* gene (i.e. *GRM6*) that has been shown to drive gene expression in all ON bipolar cell types (Ueda et al., 1997) (this experiment was performed by Dr. E.N. Star, Chow lab). Co-localization of *Vsx1*, *α-gustducin*:GFP and *mGluR6*:β-galactosidase immunolabelling in these double transgenic reporter mice was observed (Fig. 10, arrowheads). Not all *Vsx1*; *mGluR6*:NLS-β-galactosidase double-labeled ON bipolar cells, however, were co-labeled with *α-gustducin*:GFP (Fig. 10, arrows). It is possible that the *α-gustducin*:GFP reporter is not fully penetrant in all Type 7 bipolar cells (Huang
Figure 9. Colocalization of Vsx1 and the α-gustducin:GFP reporter in putative Type 7 ON cone bipolar cells.

In retinal sections from mature α-gustducin:GFP mice, Vsx1 immunolabelling is present in putative Type 7 ON bipolar cells with high levels of GFP immunolabelling (arrowhead), but not in putative rod bipolar cells expressing low levels of GFP (arrow). The dashed lines indicate the boundary of the inner nuclear layer. Scale bar in A = 30 μm.
Figure 10. Colocalization of Vsx1, α-gustducin:GFP and mGluR6:NLS-β-galactosidase immunostaining in α-gustducin:GFP; mGluR6:NLS-LacZ mice.

In reporter transgenic mice carrying both the α-gustducin:GFP and the mGluR6:NLS-β-galactosidase transgenes, colocalization of Vsx1, α-gustducin:GFP and mGluR6:NLS-β-galactosidase immunostaining was observed (white arrowheads). Not all Vsx1 + mGluR6:NLS-β-galactosidase co-labeled ON bipolar cells expressed α-gustducin:GFP (arrows). Scale bar in C = 20 μm. (From Dr. E. N. Star, Chow lab)
Figure 11. Colocalization of Vsx1:τ-β-galactosidase and α-gustducin:GFP in putative Type 7 bipolar cell in Vsx1τLacZ/τLacZ mice.

In Vsx1τLacZ/τLacZ mice, Vsx1:τ-β-galactosidase and α-gustducin:GFP co-immunolabelling is observed in putative Type 7 bipolar cell (arrows in D-F). Some Vsx1:τ-β-galactosidase cells (white arrowhead) are not labelled by α-gustducin:GFP, while some weakly immunolabelled α-gustducin:GFP cells do not express the Vsx1:τ-β-galactosidase reporter (open arrowheads). The boxed region in C is magnified in panels D-F. Scale bar in B: A-C = 40 µm; C-E = 10 µm.
et al., 2003). Alternatively, Vsx1 might be expressed in other ON cone bipolar cell type(s), in addition to Type 7 bipolar cells, that are not labeled by the \( Vsx1: \tau \text{LacZ} \) knock-in reporter in \( Vsx1^{\tau \text{LacZ}+} \) heterozygotes.

I next crossed mice carrying the \( \alpha \text{-gustducin}: \text{GFP} \) transgenic reporter to mice harbouring the \( Vsx1: \tau \text{LacZ} \) knock-in reporter gene, in order to determine whether the expression of the \( \alpha \text{-gustducin}: \text{GFP} \) in Type 7 ON bipolar cells was affected by the loss of \( Vsx1 \) gene function. In both \( Vsx1^{\tau \text{LacZ}+} \) and \( Vsx1^{\tau \text{LacZ}\Delta \text{LacZ}} \) mice, \( \alpha \text{-gustducin}: \text{GFP} \) was still observed and co-labelled with \( Vsx1: \tau \beta \)-galactosidase in putative Type 7 bipolar cells (Fig. 11 and data not shown in \( Vsx1^{\tau \text{LacZ}+} \)). The axonal terminals of \( \alpha \text{-gustducin}: \text{GFP} \) Type 7 cells in \( Vsx1^{\tau \text{LacZ}\Delta \text{LacZ}} \) mice were indistinguishable from wild type and \( Vsx1^{\tau \text{LacZ}+} \) heterozygotes, indicating that \( Vsx1 \) is not required for the specification and gross morphogenesis of Type 7 bipolar cells. Because the \( \alpha \text{-gustducin}: \text{GFP} \) reporter gene is functional in both wild type and \( Vsx1 \)-null mice, it represents a useful genetic tool to examine the role of \( Vsx1 \) in Type 7 ON bipolar cells.

### 3.2 Cabp5 is ectopically expressed in Type 7 bipolar cells in \( Vsx1 \) null mice

As my observations in Section 3.1 of this chapter indicated that \( Vsx1 \) function is not necessary for the formation of Type 7 bipolar cells, I next asked whether, \( Vsx1 \) played a role in the regulation of gene expression in Type 7 bipolar cells, similar to the role it plays in Type 2 bipolar cells. I first examined whether the expression of calcium binding protein 5 (Cabp5 or CaB5) was affected by the loss of \( Vsx1 \) function in Type 7 bipolar cells (Chow et al., 2004). Cabp5 is a calmodulin-like calcium-binding protein that is thought to function as a modulator of voltage-gated calcium channels (Haeseleer et al., 2000). In the wild type retina, Cabp5 is absent from Type 7 bipolar cells (Fig. 12 A-I) and is expressed only in a subset of bipolar cells (Type 3 OFF, Type 5 ON and rod bipolar cells) where it labels the soma, dendrites and axonal projections (Haeseleer et al., 2000; Haverkamp et al., 2003; Ghosh et al., 2004). Previous work has shown that in Cabp5-immunolabelled retinal sections of \( Vsx1 \)-null mice, the characteristic “gap” (i.e. absence of Cabp5 labelling) that is normally observed between the axonal terminals of Type 5 and rod bipolar cells in the inner plexiform layer is no longer evident (Fig. 12,
compare open arrowhead in B with bracketed region in K) (Chow et al., 2004). As the “gap” between the axonal terminals of Type 5 and rod bipolar cells lies in a region of the inner plexiform layer where one would normally find the axonal terminals of Type 7 bipolar cells (Fig. 12 H, arrows), we hypothesized that Cabp5 was upregulated in Type 7 bipolar cells in the Vsx1-null retina. Using α-gustducin:GFP reporter expression as a tool to identify Type 7 bipolar cells, we saw that Cabp5 immunolabelling was upregulated in Type 7 cells (Fig. 12 M-R). In the Vsx1-null mutant, Cabp5 immunolabelling in GFP-expressing Type 7 bipolar cells could be detected in both the soma (Fig. 12 M-O) and axonal terminals (Fig. 12 P-R). The ectopic expression of Cabp5 in Type 7 bipolar cells in Vsx1-null mice, therefore, provides an explanation for the previously described Cabp5 immunostaining defects of “gap” loss in Vsx1-null mice (Chow et al., 2004) and also indicates that Vsx1 is required for the repression of Cabp5 expression in Type 7 bipolar cells.

3.3 Upregulation of Chx10 in Type 7 bipolar cells in Vsx1 null mice

Having established the presence of a Type 7 bipolar cell gene expression defect in Vsx1-null mice, I continued to utilize the α-gustducin:GFP reporter to determine whether there were other Type 7 bipolar cell gene expression defects. The next gene I examined was the Vsx1 parologue, Chx10. In the wild type retina, Chx10 is expressed in all retinal bipolar cells (Burmeister et al., 1996). Previous studies have shown that the relationship between Vsx1 and Chx10 co-expression in bipolar cells tends to be inverse, such that cells expressing high levels of Chx10 have low levels of Vsx1 and cells with high levels of Vsx1 have low levels of Chx10 (Passinin et al., 1997; Clark et al., 2008). Studies on the Chx10 ocular retardation J loss-of-function mutant have shown that Chx10 negatively regulates Vsx1 expression (Clark et al., 2008). I therefore hypothesized that Vsx1 negatively regulates Chx10 expression and examined this hypothesis by comparing the levels of Chx10 immunofluorescence in wild type and Vsx1-null bipolar cells.

Quantitation of Chx10 immunofluorescence posed several challenges. A major
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**Figure 12.** Ectopic expression of Cabp5 in Type 7 bipolar cells in $Vsx1^{\text{LacZ/LacZ}}$ null mice. (legend on next page)
Figure 12. Ectopic expression of Cabp5 in Type 7 bipolar cells in Vsx1<sup>ΔLacZ/ΔLacZ</sup> null mice.

In the wild type retina, Calcium binding protein 5 (Cabp5 or CaB5) immunolabelling is not detected in Type 7 bipolar cells (A-I, GFP-expressing soma outlined in red dashed lines in E), but instead is expressed in a subset of bipolar cells (Type 3 OFF, Type 5 ON and rod bipolar cells) (Haverkamp et al., 2003). In the IPL region, it shows two obvious gaps in the axonal projections between Types 3 and 5 bipolar cells (below the asterisk in B), and Types 5 and rod bipolar cells (open arrowhead in B). The open arrowhead in G-I indicates the axonal terminals of rod bipolar cells that are co-labelled with Cabp5. In the Vsx1-null mutant, the gap between the Cabp5-immunolabelled axonal terminals of Type 5 and rod bipolar cells is not evident (compare the open arrowhead region in B with the bracketed region in K) (Chow et al., 2004). Instead Cabp5 immunolabelling is detected in the IPL region where the axonal terminals of Type 7 bipolar cells are normally located (arrows in P-R, outlined in red dashed lines in Q). Colocalization of Cabp5 immunolabelling with α-gustducin:GFP reporter is also observed in the cell bodies of putative Type 7 cells (M-O, outlined in red dashed lines in N). The boxed regions in C and L are magnified in panels (G-I) and (P-R) respectively. Abbreviations: IPL- inner plexiform layer; INL, inner nuclear layer; GC, ganglion cell layer. Scale bar: A-C, J-K = 40 µm; D-G, M-R = 20 µm.
concern was the fact that the immunofluorescence intensity level for any given antigen depends on many factors (e.g. fixation conditions, length of primary/secondary antibody incubation). As a consequence, fluorescence intensity can vary from section to section, even between sections taken from the same slide series. To address this issue, I developed an approach whereby the levels of Chx10 immunofluorescence in Type 7 bipolar cells were normalized to the levels of Chx10 in rod bipolar cells co-labelled for the rod bipolar cell specific marker, protein kinase C α (PKCα) within the same section (Fig. 13A-D, I). Normalizing my data in this manner required that I assumed the levels of Chx10 protein in rod bipolar cells were unaltered in Vsx1 null mice. This assumption was based on the observation that Vsx1 is not expressed in rod bipolar cells and that no rod bipolar cell specific defects have been observed in Vsx1 mutant mice (Chow et al., 2001 and 2004). However, as I cannot rule out the possibility that there are non-cell autonomous defects in rod bipolar cells in Vsx1-null mice, this assumption presents a caveat to my experimental approach that should be taken into consideration when evaluating the data.

In both the wild type and Vsx1 null mice, the highest Chx10 expression level was always found in PKCα +ve rod bipolar cells. Therefore, the relative Chx10 immunofluorescence level is always less than 1 by comparison. Using the above approach to quantify Chx10 immunofluorescence, I observed that Chx10 was upregulated in Type 7 bipolar cells in the Vsx1-null retina almost 3 fold compared to Chx10 levels that observed in the wild-type retina (0.610 ± 0.018 in the Vsx1 null compared to 0.202 ± 0.018 in wild type, mean ± SEM, n=3) (Fig. 13I). I next examined whether Chx10 protein levels in the Vsx1 null mutant were also altered in Type 1/2 bipolar cells, as Vsx1 is also expressed in these cell types in wild type mice (Fig. 13E-I). The same approach as described above was used, except that instead of quantifying Chx10 immunofluorescence levels in α-gustducin:GFP-positive Type 7 bipolar cells, I compared the levels of Chx10 in NK3R-immunolabelled Type 1 and 2 OFF bipolar cells (again normalizing to the level of Chx10 in rod bipolar cells). In contrast to Type 7 bipolar cells, the level of Chx10 immunofluorescence in NK3R immunolabelled OFF bipolar cells did not change much in the Vsx1-null retina (0.338 ± 0.011 in the Vsx1 null compared to 0.342 ± 0.010 in the wild-type, Mean ± SEM, n=3) (Fig. 13I). Together, these results show that Vsx1
Figure 13. Upregulation of Chx10 in Type 7 bipolar cells in $Vsx1^{lacZ/lacZ}$ null mutants.

(legend on next page)
Figure 13. Upregulation of Chx10 in Type 7 bipolar cells in $Vsx1^{\tau\text{LacZ}/\tau\text{LacZ}}$ null mutants.

Immunolabelling of adult retinal sections for Chx10, PKC$\alpha$ and the $\alpha$-gustducin:GFP reporter (A-D) and Chx10, PKC$\alpha$ and NK3R (E-H). Chx10 immunofluorescence levels were normalized in Type 7 bipolar cells (outlined in dashed green lines as “7” in B and D) to the levels of Chx10 in PKC$\alpha$-positive rod bipolar cells (outlined in dashed blue lines as “R” in B and D) within the same section. The same approach was used to compare the levels of Chx10 in NK3R-immunolabelled Type 1 and 2 OFF bipolar cells (outlined in dashed green lines as “N” in F and H) between wild type and $Vsx1$ null mice (E-H). Chx10 is upregulated in Type 7 bipolar cells in the $Vsx1$-null retina (I) to almost three times of that observed in the wild-type retina ($0.610 \pm 0.018$ in the $Vsx1$-null compared to $0.202 \pm 0.018$ in the wild type, mean ± SEM, n=3). The level of Chx10 immunofluorescence does not change significantly in NK3R immunolabelled OFF bipolar cells in the $Vsx1$-null versus the wild type retina (I) ($0.338 \pm 0.011$ in the $Vsx1$ null compared to $0.342 \pm 0.010$ in the wild-type, mean ± SEM, n=3). The asterisk in (I) indicates a significant difference by Student’s t-test ($p < 0.05$). Scale bar in D = 14 $\mu$m for A-H.
function is necessary for the repression of Chx10 expression in Type 7 ON bipolar cells but not in Type 2 OFF bipolar cells.

3.4 Upregulation of α-Gustducin:GFP in Type 7 bipolar cells in Vsx1 null mice

Through the course of my studies utilizing the α-Gustducin:GFP reporter gene, my visual observations suggested that the level of GFP fluorescence was more pronounced in the Vsx1-null retina than in the wild type (Fig. 14A,B). Given that the results in Sections 3.4 and 3.5 suggest that Vsx1 functions as a repressor of Cabp5 and Chx10 in Type 7 bipolar cells, I examined the possibility that the α-Gustducin:GFP reporter gene, itself, was negatively regulated by Vsx1.

Western blotting was used initially to compare the level of GFP in wild type and Vsx1-null mice (Fig. 14C) (experiment performed by Dr. M. Zhu, Chow lab). The results of these experiments revealed an increase of GFP protein levels in the Vsx1-null mice (n=3) with up to a 76% increase in the total amount of GFP signal. One problem with interpreting this western blot data, however, is that it does not distinguish between increases in cell number and/or cell intrinsic expression levels. As an alternative approach to address these issues, I used flow cytometry to quantitate and compare the α-Gustducin:GFP-expressing cell populations in wild type and Vsx1-null mice (Fig. 15). Flow cytometry has the advantage that it allows for the quantitation of both the number of fluorescing cells and the relative mean fluorescence level per cell. Papain-dissociated retinas from 2-5 month old wild type and Vsx1-null mice were analyzed on the BD FACS flow cytometer. Forward and side scatter analysis was used to identify a cell population that best encompassed single retinal cell population (Fig. 15B). A population of total GFP-fluorescing cells was gated for both wild type and mutant (“R3” in Fig. 15 C,D). A 56% increase in mean GFP fluorescence intensity was observed for the total GFP-positive cell population (i.e. R3 gate) in Vsx1-null mice [Fig. 15E; 411±39 in wild type, 643 ± 36 in Vsx1LacZ/LacZ (arbitrary units measuring fluorescence intensity); n=3, P< 0.05], which corroborates our earlier Western blot data. Interestingly, there was a significant increase in the proportion of cells with high levels of GFP fluorescence (R4/R2) in Fig. 15 C, D, and F (0.11 ± 0.005 in wild type, 0.21 ± 0.03 in Vsx1LacZ/LacZ,
Figure 14. $\alpha$-Gustducin:GFP expression in wild type and $Vsx1^{tlacZ/tlacZ}$ null mutant mice.

GFP immunolabelling of the $\alpha$-Gustducin:GFP reporter in the wild type (A) and $Vsx1$-null (B) retina. (C) Western blot showing GFP protein levels from total retinal lysates of wild type (column 1) and $Vsx1$-null (column 2) mice (experiment performed by Dr. M. Zhu, Chow lab). $\beta$-tubulin was used as a loading control to normalize data. A cell line transfected with a GFP-expressing plasmid was used as a positive control for GFP detection (column 3). Abbreviations: IPL- inner plexiform layer; INL, inner nuclear layer. Scale bar in B = 35 $\mu$m.
Figure 15. Upregulation of α-Gustducin:GFP in the Vsx1<sup>LacZ/LacZ</sup> null mutant mice.

(legend on next page)
Figure 15. Upregulation of α-Gustducin:GFP in the Vsx1\textsuperscript{LacZ/\textlacinZ} retina.

Flow cytometry data was obtained from papain-dissociated retinas from wild type and Vsx1\textsuperscript{LacZ/\textlacinZ} mice carrying the α-Gustducin:GFP transgene. Representative examples showing the forward and side scatter of cell populations are shown in (A) and (B); the R2-gated cell population in (B) is derived from the R1-gated cells (A) and represents the gated cell population examined in (C, D). GFP fluorescing cells in (C, D) are plotted along the x-axis. The R3 gated cells (C, D) indicate GFP-expressing cells and was determined by comparing fluorescence profiles of retinal cells not carrying the α-Gustducin:GFP transgene (data not shown). The R4 gated cells indicate a population of GFP-fluorescing cells that consistently form a cluster of highly fluorescing cells in both wild type and Vsx1\textsuperscript{LacZ/\textlacinZ} mice. (E) Relative mean GFP fluorescence in the R3 cell population (i.e. all of the GFP-fluorescing cells) was significantly higher in Vsx1\textsuperscript{LacZ/\textlacinZ} mice (wild type = 411 ± 39; Vsx1\textsuperscript{LacZ/\textlacinZ} = 643 ± 36; n=3, Student’s t-test P< 0.05). Within the R4 cell population (E), the mean fluorescence intensity was not significantly increased suggesting that cell that were already highly-fluorescing in the wild type retina were not getting brighter in Vsx1\textsuperscript{LacZ/\textlacinZ} retina. (F) The total number of GFP-fluorescing cells (R3/R2) was unchanged in the Vsx1\textsuperscript{LacZ/\textlacinZ} retina, however, a significant increase in the number of cells with high GFP-fluorescence (i.e. R4/R2) was observed (asterisks in E and F indicate P< 0.05 by Student’s t-test).
n=3, P< 0.05). Since there was no change in the overall number of GFP expressing cells (i.e., R3/R2; Fig. 15F), my data suggests that the increased level of GFP in the Vsx1-null retina is due to elevated GFP expression per cell as opposed to an increase in the total number of cells expressing GFP. Thus, these data are consistent with the idea that Vsx1 is required for the repression of α-gustducin:GFP transgene expression in Type 7 bipolar cells.

3.5 Is Vsx1 expression in Type 6 ON cone bipolar cells?

In retinal sections from Vsx1LacZlLacZ; α-Gustducin:GFP mice I observed β-galactosidase-positive ON bipolar cells (i.e. cells with axonal terminals that projected to the ON sublamina of the inner plexiform layer) that did not colocalize with the GFP-positive axonal terminals of Type 7 bipolar cells (Fig. 16, arrows in A-C). In contrast to the narrowly stratified α-Gustducin:GFP Type 7 bipolar cells, these axonal terminals had loose delicate axonal terminals that stretched out from the sublamina 3 and descended to form multiple branches within sublamina 4. The axonal and dendritic morphology of these cells (Fig. 16, bracketed region in A-C) closely resembled that of the Type 6 ON bipolar cell (Fig. 16D). To determine if these were in fact Type 6 bipolar cells, I co-labeled β-galactosidase in Vsx1LacZlLacZ retinal sections with antibodies to synaptotagmin 2 (Syt2). Syt2 is a membrane-associated protein that, in the retina, is expressed specifically in ON Type 6 bipolar cells as well as in OFF Types 2 and 3 bipolar cells (Fox and Sanes, 2007; Wässle et al., 2009). In the ON sublamina of the inner plexiform layer, Syt2 immunolabelling colocalized with Vsx1:β-galactosidase reporter immunolabelling in the axonal terminals of putative Type 6 bipolar cells (Fig. 16E-G, arrow). To examine whether Vsx1 protein normally colocalizes with Syt2 in Type 6 bipolar cells, I triple-immunolabelled retinal sections from wild type mice carrying the mGluR6:NLS-β-LacZ transgene with antibodies to Vsx1, Syt2 and β-galactosidase. β-galactosidase was used in this context to discriminate the Vsx1-ON versus Vsx1-OFF subpopulation. Although Syt2 membrane labelling of OFF bipolar cell somata were detected (i.e. negative for mGluR6:NLS-β-galactosidase), the Syt2 cell body labelling in mGluR6:NLS-β-galactosidase-positive ON bipolar cells was not detected (data not shown). Several
Figure 16. Vsx1:β-galactosidase reporter expression in Type 6 ON cone bipolar cells of Vsx1\(^{\text{rLacZ/rLacZ}}\) retina.

In the Vsx1\(^{\text{rLacZ/rLacZ}}\) retina, some Vsx1:β-galactosidase-expressing ON bipolar cells in the IPL region do not colocalize with the GFP-positive axonal termini of Type 7 bipolar cells (arrows in A-C). The axonal and dendritic (see bracketed region in C) morphology of these cells closely resembles that of the Type 6 ON bipolar cell (D). The open arrowhead in A-C indicates colocalization of Vsx1:β-galactosidase and α-Gustducin:GFP of Type 7 bipolar cells. In the ON sublamina of the IPL, synaptotagmin 2 (Syt2) is colocalized with Vsx1:β-galactosidase reporter immunolabelling in the axonal terminals of putative Type 6 bipolar cells (open arrowheads in E-G). The arrow in (E-G - also outlined in yellow dashed lines) points to Vsx1:β-galactosidase-positive axonal terminals of Type 7 ON bipolar cells and do not colocalize with Syt2 immunolabelling. Abbreviations: IPL-inner plexiform layer; INL-inner nuclear layer. Scale bar in G: A-C = 25 μm, E-G = 10 μm.
different fixation and immunostaining conditions were used, but Syt2-labelling in mGluR6:NLS-β-galactosidase-positive ON bipolar cell somata was never observed. Thus, although the Vsx1 knock-in β-galactosidase reporter colocalized with Syt2 in Type 6 bipolar cells in Vsx1-null mice, I was unable to determine conclusively whether Vsx1 is expressed in Type 6 bipolar cells in the wild type retina.

Although Syt2 immunolabelling could still be detected in Vsx1-null mice, it was abnormal compared to wild type controls. Notably, most of the Syt2 immunolabelling of Type 2 OFF bipolar cells was lost in Vsx1-null mice as indicated by the lack of immunolabelling in the outermost sublaminar region of the inner plexiform layer (Fig. 17). All of the few Syt2-immunolabelled Type 2 OFF bipolar cells that remained in the Vsx1-null retina were co-labeled with the Vsx1 knock-in β-galactosidase reporter (Fig. 17C-E, arrow) indicating that other factors can regulate Syt2 expression in this cell type. In contrast to Type 2 bipolar cells, Syt2 immunolabelling in the axonal terminals of Type 3 and Type 6 bipolar cells was more intact. A careful analysis, however, would be required to determine whether there were significant changes in Syt2 expression and in the number of cells expressing Syt2 in these cells types.

3.6 Role of Vsx1 in Types 3 and 4 OFF bipolar cells

In addition to the presence of Type 2 OFF bipolar cell defects in Vsx1-null mutants, Type 3 OFF bipolar cell defects are also observed (Chow et al., 2004). Specifically, there is a reduction in Cabp5 immunolabelling in Type 3 bipolar cell axonal terminals in Vsx1-null mice (Chow et al. 2004). In contrast, the labelling of Type 3 bipolar cell terminals by another marker of this cell type, PMCA1, was not affected by the loss of Vsx1, indicating that Type 3 bipolar cells are still specified in Vsx1-null mice (Chow et al., 2004). To examine whether Vsx1 is expressed in Type 3 bipolar cells, Vsx1 was co-immunolabelled retinal sections using antibodies specific to Cabp5. As Cabp5 is expressed in both OFF (Type 3) and ON (Type 5 and rod) bipolar cells, this immunolabeling experiment was performed in mice carrying mGluR6:NLS-LacZ transgene (experiment was performed by Dr. R. L. Chow). Since this transgene is only expressed in ON bipolar cells, it enabled us to distinguish the Cabp5-positive Type 3
Figure 17. Syt2 expression defects in Vsx1\textsuperscript{\textlactZ/\textlactZ} null mice.

Reduced Syt2 immunolabelling is observed for Type 2 bipolar cells (open arrowheads in A-E) in Vsx1-null mice (B) compared to wild type mice (A). The few Type 2 OFF bipolar cells that remain immunolabelled for Syt2 in the Vsx1-null mouse (arrow in C-E), are co-labelled with the Vsx1:\(\beta\)-galactosidase reporter. The closed arrowhead in (A-B) indicates the relative position of Type 3 OFF bipolar cell axonal terminals, and the bracketed region in (A, B) indicates the axonal terminals of Type 6 ON bipolar cells. The dashed outlines and open arrowheads in (C-E) indicate Vsx1:\(\beta\)-galactosidase-positive on Type 2 or 3 bipolar cells. Scale bar in B: A,B = 40 \(\mu\)m, C-E = 30 \(\mu\)m.
bipolar cells from the ON Cabp5-positive subset. Although Vsx1 immunolabelling was detected in both ON (Fig. 18, Cell 2) and OFF (Fig. 18, Cell 3) bipolar cell types, it did not colocalize with Cabp5 in either the OFF-Cabp5 (Type 3) (Fig. 18, Cell 1) or the ON-Cabp5 (Fig. 18, Cell 4) immunolabelled cells.

Since the double immunolabeling protocol used to detect Vsx1 and Cabp5 (both of which were generated in rabbits) was done sequentially and required an intermediate neutralizing step, which slightly attenuated the Vsx1 and Cabp5 signals, I also examined other Type 3 bipolar cell markers to see if they co-expressed with Vsx1. I examined the following two Type 3 bipolar cell markers: hyperpolarization-activated and cyclic nucleotide-gated channel 4 (HCN4) and the regulatory subunit RIIβ of protein kinase A (PKARIIβ) (Mataruga et al., 2007). Within the bipolar cell population, both of these markers are expressed specifically in Type 3 bipolar cells and are expressed in a mutually exclusive manner to define two distinct cell types: “3a” (HCN4-positive), and “3b” (PKARIIβ-positive) (Mataruga et al. 2007). In double immunolabelling experiments with Vsx1, neither HCN4- (Fig. 19A-C) nor PKARIIβ- (Fig. 19D-F) expressing bipolar cells co-immunolabeled with Vsx1. These results further demonstrate the absence of detectable levels of Vsx1 in Type 3 bipolar cells.

I further investigated whether V sx1 is expressed in Type 3 bipolar cells by examining the cell types labelled with the Vsx1:τLacZ knock-in reporter (Chow et al. 2004). The τ-β-galactosidase reporter efficiently labels axons, and is ideal for examining Vsx1-expressing neuronal morphology (Callahan and Thomas, 1994). Furthermore, since ubiquitin-dependent proteolysis has been shown to act on Vsx1 (Kurtzman et al. 2000), the β-galactosidase reporter has the added advantage that it may reveal cells in which Vsx1 expression is tightly regulated post-translationally. In order to discriminate between different OFF bipolar cell types, β-galactosidase immunolabelling was performed in retinal sections co-labeled for calretinin (Fig. 20), just as the examination of Vsx1 ON cell types (Fig. 8). Calretinin immunolabeling reveals three distinct bands in the inner plexiform layer to define distinct sublamina, which are used to characterize different bipolar cell types (Ghosh et al. 2004). Cell types with axonal terminals residing in sublamina 1 were observed that resembled Type 1 (Fig. 20A) and Type 2 (Fig. 20B)
**Figure 18. Absence of Vsx1 and Cabp5 co-immunolabelling in retinal bipolar cells.**

Triple immunolabelling for Vsx1, Cabp5 and β-galactosidase of a retinal section taken from a 2 month old mouse carrying the *mGluR6:NLS-LacZ* transgene (experiment performed by Dr. R. L. Chow). Immunolabelling reveals several cell types defined overlapping and non-overlapping expression patterns (Cell 1-4). “Cell 1” is labeled only by Cabp5, “Cell 2” is labeled by Vsx1 and β-galactosidase, “Cell 3” is labelled only by Vsx1, and “Cell 4” is labelled by both Cabp5 and β-galactosidase. Scale bar in bottom panel = 14 µm.
Figure 19. Vsx1 is not detected in Type 3a and Type 3b bipolar cells.

Immunolabelling of 2-month-old retinal sections for Vsx1 and HCN4 (A-C) and Vsx1 and PKARIIβ (D-E). Dashed cell outlines and arrowheads indicate HCN4 (B) and PKARIIβ (E) immunolabelled cell bodies that are not co-labelled with Vsx1. The dashed lines in C and F indicate the inner nuclear layer boundaries with the photoreceptor cells positioned above the top line and the inner plexiform layer below the bottom line. Scale bar in D = 14 µm.
Figure 20. OFF bipolar cell types identified with the $Vsx1$:β-galactosidase knock-in reporter in $Vsx1^{\tau\text{LacZ}/+}$ retina.

Sections from 2-month-old $Vsx1^{\tau\text{LacZ}/+}$ heterozygous mice were immunolabelled for β-galactosidase and calretinin. The bracketed regions in C,D,G and H indicate the presence of axonal terminals within sublamina 2 of the inner plexiform. The dashed line indicates the boundary between the inner nuclear layer (above) and the inner plexiform layer (below). Numbers 1 through 4 indicate the location of inner plexiform sublaminae defined by calretinin immunolabelling. Scale bar in H = 10 µm.
bipolar cells. Although Types 1 and 2 cells both project to sublamina 1 of the inner plexiform layer, Type 2 bipolar cells have a bushier axon terminal and exhibits a dense plexus of varicosities than Type 1 bipolar cells (Ghosh et al., 2004). In contrast to observing putative Types 1 and 2 bipolar cells, I did not observe cells with axonal terminals residing only within sublamina 2, which is a defining feature of Type 3 bipolar cells (Ghosh et al., 2004).

Despite the lack of Vsx1 immunolabelling in either Type 3a or 3b bipolar cells, I examined whether Vsx1 is required for the expression HCN4 or PKARIIβ given our previous observation that Cabp5 is downregulated in Type 3 OFF bipolar cells in Vsx1-null mice (Chow et al. 2004). HCN4 normally labels axons, dendrites and cell bodies of Type 3a bipolar cells (Fig. 21A), and all of these regions had reduced immunolabelling in Vsx1-null mice (Fig. 21B). HCN4 immunolabelling of Type 3a bipolar cells was not completely lost as faint labelling of axonal terminals was observed (Fig. 21B, open arrowhead) as well as occasional cell body labelling (Fig. 21A, arrowhead). These observations indicate that Type 3a bipolar cell specification is not abolished in the absence of Vsx1. In contrast to HCN4, PKARIIβ immunolabelling in Type 3b bipolar cells was unchanged in the Vsx1-null retina (Fig. 21C,D). Combined with previous Cabp5-immunolabelling defects in the Vsx1-null retina, these observations point to a role for Vsx1 in regulating gene expression in terminally differentiated Type 3a bipolar cells.

In my examination of β-galactosidase in Vsx1\textsuperscript{LacZ/+} heterozygous mice I occasionally observed cells with axonal terminals that appeared to be present in both sublamina 1 and 2 (Fig. 20C,D). As this pattern of axonal stratification is a characteristic feature of OFF Type 4 bipolar cells, I checked whether Vsx1 was co-expressed in cells expressing calsenilin, a recently identified Type 4 bipolar cell specific marker (Haverkamp et al. 2008). Co-immunolabelling experiments failed to show Vsx1 colabelling in calsenilin-positive Type 4 bipolar cells (Fig. 22A-C). Furthermore, calsenilin immunolabelling was unchanged in Vsx1-null mice (Fig. 23A,B). These results indicate that Vsx1 is not required for Type 4 bipolar cell specification and that at least one Type 4 bipolar specific gene (calsenilin) does not depend on Vsx1 for its expression.
Figure 21. Downregulation of the Type 3a bipolar cell marker, HCN4 in \( Vsx1^{\tau LacZ/\tau LacZ} \) mice.

HCN4 immunolabelling is reduced in \( Vsx1^{\tau LacZ/\tau LacZ} \) mice (B) compared to wild type littermates (A). The white arrowhead in (A) points to an HCN4-immunolabelled cell body. The open arrowheads in (A) and (B) point to HCN4 immunolabelling of axonal terminals in the inner plexiform layer. PKARI\( \beta \) immunolabelling of putative Type 3b bipolar cells is indicated by the white arrowheads in the wild type (C) and \( Vsx1^{\tau LacZ/\tau LacZ} \) (D) retina. The dashed lines in (A) -(D) indicate the inner nuclear layer boundaries. Scale bar in (D) = 30 \( \mu m \).
Figure 22. Calsenilin expression in Type 4 bipolar cells does not co-localize with Vsx1.

The arrowheads in (B) and (C) indicate calsenilin positive cells, whose locations are outlined in white dashed lines in (A). The dashed lines indicate the boundary of the inner nuclear layer. Scale bar in (A) = 14 µm
Figure 23. Calsenilin immunolabelling of Type 4 bipolar cells is unaffected in \( V_{sx1}\text{lacZ/lacZ} \) mice.

The arrowhead indicates calsenilin positive bipolar cells. The dashed lines indicate the boundary of the inner nuclear layer. Scale bar in (B) = 25 µm.
3.7 Conclusions

The results presented in this chapter provide insight into the complex mechanism by which *Vsx1* regulates retinal bipolar cell type-specific gene expression. Below is a summary of these findings:

(1) *Vsx1* is expressed in Type 7 ON bipolar cells and functions genetically as a transcriptional repressor in this cell type: the expression of Cabp5, Chx10 and α-Gustducin:GFP reporter expression is all upregulated in the *Vsx1*-null retina. Contrasting the negative regulatory role of *Vsx1* in Type 7 bipolar cells, in Type 2 OFF bipolar cells the loss of *Vsx1* leads to the downregulation of NK3R, recoverin Neto1, (Chow et al., 2004) and Syt2 (this study). Thus, a trend has emerged from my studies suggesting that *Vsx1* functions either as a repressor or an activator and that this activity is determined by bipolar cell type-specific factors.

(2) Despite a requirement for *Vsx1* in the expression of Type 3 bipolar cell markers Cabp5 (Chow et al., 2004) and HCN4 (this study), *Vsx1* expression in Type 3 bipolar cells, either by immunolabelling for Vsx1 protein or *Vsx1*:β-galactosidase knock-in reporter expression, was not detected. These findings suggest that *Vsx1* is either required in a non-cell autonomous manner for Type 3 bipolar cell gene expression or that Vsx1 is expressed in Type 3 bipolar cells at very low levels.
Chapter 4 Discussion

Transcription factors are key determinants in regulating neuronal cell type diversity. As discussed in Chapter 1, numerous studies have shown that rather than functioning as master regulators, multiple transcription factors function together to generate a unique combinatorial code that drives a cell type specific gene expression program. Thus, the cellular environment in which a transcription factor exerts its activity greatly influences its activity. The findings of my thesis work are summarized in Figure 24 along with previous findings for Vsx1. Combined, these data illustrate how a single transcription factor, Vsx1, can direct the expression of distinct genes in very similar cell types within a single class of retinal interneurons (i.e. bipolar cells). My data reveal a trend in which Vsx1 functions as a repressor in Type 7 ON bipolar cells and as an activator in Type 2 OFF bipolar cells. Lastly, my data indicate that although Vsx1 is required to regulate gene expression in Type 3a bipolar cells, the mechanism by which it does so is complex, as Vsx1 expression is not detected in this cell type.

4.1 α-gustducin:GFP expression in Type 7 bipolar cells

It is worth mentioning that the existence of the α-gustducin:GFP transgenic mice greatly facilitated my study of Vsx1 gene function. α-gustducin:GFP in transgenic mice is specifically expressed in a subset of retinal Type 7 ON cone bipolar cells and rod bipolar cells (Huang et al., 1999 and 2003; Wong et al., 1999). Interestingly, α-gustducin is not expressed in the retina in wild type mice (McLaughlin et al., 1992; Wong et al., 1996). Gustducin is a G-protein specifically involved in gustation, mediating transduction of bitter and sweet perception by mammalian taste receptor cells (McLaughlin et al., 1992; Wong et al., 1996). It is closely related to transducins, the G proteins in rod and cone photoreceptor cells, which function in the phototransduction cascade. It is unclear why the α-gustducin:GFP transgene reporter is expressed in bipolar cells. The positional insertion effect of the α-gustducin:GFP transgene may be one reason. For example, the α-gustducin:GFP construct may have fortuitously integrated into a genomic region that
Figure 24. Summary of Vsx1 expression and gene regulation in retinal bipolar cells.
Vsx1 is expressed in OFF Types 1 and 2 (only Type 2 is shown in diagram) and in Type 7 bipolar cells. The question marker for Type 6 bipolar cell type indicates that although Vsx1 β-galactosidase reporter is detected in Type 6 bipolar cells, we have not determined whether Vsx1 protein is expressed in this cell type in wild type mice. The question mark for Type 3a bipolar cells indicates that Vsx1 expression was not detected in Type 3a bipolar cells. In Vsx1-null mice, gene expression is downregulated in Types 2 and 3a OFF bipolar cells and upregulated in Type 7 bipolar cells. These data suggest that Vsx1 functions as a transcriptional activator in Type 2 bipolar cells and as a repressor in Type 7 bipolar cells. How Vsx1 functions to regulate gene expression in Type 3a bipolar cells is unclear as its expression is not detected in this cell type.
activates the upstream \( \alpha\)-gustducin promoter in a specific population of retinal bipolar cells. Another reason could be the loss of a silencer region from the \( \alpha\)-gustducin transgene that normally inhibits expression in restricted cell type(s). The presence/absence of certain transcription factors, regulatory elements, or even microRNAs from the local microenvironment may account for the specific \( \alpha\)-gustducin:GFP expression in Type 7 bipolar cells (Huang et al., 2003; Matthaei, 2007).

My data showed that the \( \alpha\)-gustducin:GFP reporter transgene is upregulated in the \( Vsx1\)-null retina. Thus it would appear that \( Vsx1\) normally functions to repress \( \alpha\)-gustducin:GFP retinal expression. To determine if \( Vsx1\) could directly regulate the 8.4kb \( \alpha\)-gustducin promoter present in the \( \alpha\)-gustducin:GFP transgene construct, I blasted this sequence and detected 8 putative \( Vsx1\) binding sites. One would therefore predict that Type 7 bipolar cells express activating transcription factors that are sufficient to drive \( \alpha\)-gustducin:GFP transgene expression, and that this activity is partially repressed (but not completely) by \( Vsx1\). The ability of \( Vsx1\) to function as a repressor is consistent with previous in vitro studies showing that it can function as a transcriptional repressor (Dorval et al., 2005). The absence of \( \alpha\)-gustducin:GFP transgene expression in other bipolar \( Vsx1\)-expressing OFF bipolar cell types does not indicate that \( Vsx1\) is functioning as a strong repressor of \( \alpha\)-gustducin:GFP transgene expression, however, because in \( Vsx1\)-null mice \( \alpha\)-gustducin:GFP reporter expression is still not observed. Thus, other factors may be repressing the \( \alpha\)-gustducin:GFP transgene in other bipolar cell types, or the activating transcription factors required for \( \alpha\)-gustducin:GFP transgene expression may be absent from these cells.

4.2. \( Vsx1\) and \( Vsx2/Chx10\) cross-regulate each other inversely in a complementary relationship

\( Vsx1\) and \( Vsx2/Chx10\) are the only two known Prd-L:CVC paralogues identified in vertebrates. In present study, I have shown that \( Vsx1\) represses the expression of Chx10 in Type 7 ON bipolar cells but not in \( Vsx1\)-expressing OFF bipolar cells. Moreover, the level of Chx10 expression is dramatically enhanced, almost three times of that in the wild type, once \( Vsx1\) is deprived in this cell type (Fig. 13). In both fish and
mouse retina, the expression of Vsx1 and Vsx2/Chx10 overlaps in a subset of bipolar cells as shown by in situ hybridization and immunohistochemistry (Levine et al., 1994; Passinin et al., 1997; Clark et al., 2008). Previous studies have shown that Chx10 is able to negatively regulate Vsx1 expression by directly binding to the Vsx1 promoter region (Clark et al., 2008). These results are consistent with the conclusion from in vitro experiments: both Chx10 and Vsx1 can function as repressors (Dorval et al., 2005). This suggests that Vsx1 and Vsx2/Chx10 might cross-regulate each other inversely in a complementary relationship. It is quite common for homeodomain proteins to regulate each other’s expression to establish boundaries between different parts of developing CNS (Akin and Nazarali, 2005). One example is neuronal specification in the developing ventral neural tube: a series of homeobox transcription factors is expressed and the neighboring transcription factors cross-repress each other to delineate the boundaries of different expression domains, where a population of specific progenitors dominate (Dessaud et al., 2008). Another example is the formation of the midbrain-hindbrain boundary (MHB) (Wurst and Bally-Cuif, 2001). Here it appears that Vsx1 and Chx10 cross-regulate each other in an incomplete manner, as some cells, such as Type 2 bipolar cells, express Chx10 in a manner that is not affected by the presence or absence of Vsx1.

The distinct phenotypes in the Vsx1 mutant and the Chx10 mutant indicate the paralogues have different roles and are unable to compensate each other in absence of the other gene (Clark et al., 2008). Perhaps the different combination of Vsx1- and Chx10-expressing cells may play a role in determining different bipolar cell types. Since Chx10 is indispensable for the proliferation of retinal progenitor cells in the early stage of retinal development, a conditional knock-out Chx10 in differentiating bipolar cells would be a useful approach, to further investigate the relationship between Chx10 and Vsx1 in bipolar cell development and cell type diversity.

As a pan bipolar cell marker, Chx10 is expressed in all eleven different bipolar cell types in mature mouse retina. I was able to observe variations of Chx10 expression levels in individual bipolar cells when imaging through the retinal sections, and observed several trends (Fig. 13A,E). In both the wild type and Vsx1 null mice, the highest Chx10 expression level was always found in PKCα +ve rod bipolar cells. The Chx10 level was lower in Type 7 ON bipolar cells (0.202 ± 0.018 in wild type, Mean ± SEM, n=3) than in
OFF bipolar cells (0.342 ± 0.010 in wild type, Mean ± SEM, n=3). This observation may suggest that the differences of relative Chx10 expression levels distinguish the identities of different bipolar cell types. Similarly, the relative intensity of Isl1 immunostaining has been shown to vary in different ON bipolar cell types in both neonatal and adult mouse retinas: again with the trend of being much higher in rod bipolar cells than in cone bipolar cells. Another example is seen with the expression of the homeodomain transcription factor, Irx5, which is expressed at high levels in Type 3 bipolar cells and at very low, threshold levels in Type 2 OFF cone bipolar cells (Cheng et al., 2005). One possible reason for these differences in expression levels is that they could provide a mechanism to allow for the segregation of cells within a particular subgroup of bipolar cells (Elshatory et al., 2007a).

### 4.3 Differential cell type requirements for Vsx1 as a repressor or as an activator

My work has shown that Vsx1 is required for the repression of Cabp5, Chx10 and α-gustducin:GFP in Type 7 ON bipolar cells. In contrast, in Type 2 bipolar cells, Vsx1 is required for the activation of NK3R, recoverin, Neto1 (Chow et al., 2004) and Syt2 (my thesis findings). These observations suggest that Vsx1 functions as a transcriptional repressor and an activator in different expressing-cell types and leads to the question of what cell type specific factors underlie this difference in activity.

Many Hox proteins can act as both transcriptional repressors and activators (Svingen and Tonissen, 2006). It has been proposed that the interaction with transcriptional cofactors can regulate the switch between repression and activation. A multitude of diverse cofactors could account for the tissue-specific transcriptional regulation of the Hox complex. For example, the conversion of the HOX-PBX heterodimer complex (PBX is another homeodomain protein) from a repressor to an activator is obtained by the interacting balance between a co-repressor complex including histone deacetylases (HDACs) -1 and -3 binding to the N-terminus of PBX and a co-activator complex containing CREB- binding protein (CBP) with the HOX partner. The transcriptional activation is mediated in response to protein kinase A (PKA) signaling or cell aggregation (Saleh et al., 2000).
Enabling a single transcription factor access to either co-repressors or co-activators provides an efficient mechanism to generate a complex response to a variety of signals with a relatively limited number of transcription factors (Wolberger, 1999; Reményi et al., 2004; Silver and Rebay, 2005; Svingen and Tonissen, 2006). Therefore, I propose that Vsx1, like those Hox proteins mentioned above, functions distinctively either as a transcriptional repressor or as an activator in different subpopulations of bipolar cells in a context-specific manner. As such, Vsx1 may adopt different activities based on its interaction with different multiprotein complexes in different microenvironments. It is noteworthy, however, that other regulation mechanisms regulate the activities of transcription factors: such as phosphorylation, acetylations, and the subcellular trafficking between cytoplasm and nucleus (Chariot et al., 1999; Reményi et al., 2004; Akin and Nazarali, 2005).

The homeodomain transcription factors Irx5, Irx6, and the bHLH transcription factor Bhlhb5 are all co-expressed with Vsx1 in Type 2 retinal bipolar cells and are all able to regulate, to some degree, each other’s expression in these cell types (Cheng et al., 2005; Feng et al., 2006; E. N. Star, M. Zhu, and R. L. Chow, unpublished data). These transcription factors (as well as other unknown factors) in Type 2 bipolar cells might provide a specific cell context that combine with Vsx1 to activate downstream gene expression. To investigate the biochemical relationship between these transcription factors, luciferase transcriptional transactivation reporter experiments could be used. As transcription factors are able to function as homo-/hetero-dimers or heteromultimers to trans-regulate gene expression, immunoprecipitation and pull-down assays in vitro can also be conducted to examine their functional relationship. However, since Vsx1, Irx5, Irx6, and Bhlhb5 have overlapping and non-overlapping expression profiles and mutant phenotypes in OFF bipolar cells, this indicates that the interactions among those transcription factors in gene expression regulation is complex. Elucidating the combinatorial interactions of these transcription factors, and identifying new factors that interact with them, will therefore contribute to our understanding of retinal interneuron cell type diversity, as well as serving as a model for the development of other neural tissues.
4.4 How does Vsx1 function in Type 3 bipolar cells?

From my experiments, two cell markers of Cabp5 and HCN4 are downregulated in Type 3 cells in the Vsx1-null mouse retina. However, we do not have any evidence that Vsx1 is expressed in Type 3 bipolar cells. There are several possibilities to account for this discrepancy. Firstly, the failure to detect Vsx1 expression could be due to the fact that expression is transient. If Vsx1 is expressed at a certain developmental stage in Type 3 bipolar cells, but I didn’t capture that time, therefore, I could erroneously come to the “non-expression” conclusion. In my study, I used the completely mature retina for my expression analysis. To explore this possibility, a detailed and careful time course study is required along retinal development, in combination with Type 3 bipolar cell markers.

A second reason for our inability to detect Vsx1 in Type 3 bipolar cells is that the expression level of Vsx1 in this cell type could be very low, and the sensitivity of the immunohistochemistry technique is not high enough to detect either Vsx1 protein itself or the Vsx1 knock-in β-galactosidase reporter. Interestingly, there is a moderate upregulation of Vsx1 immunostaining in HCN4-expressing Type 3a bipolar cells in Irx-6 null mice, suggesting that Vsx1 is normally repressed in this cell type (E. N. Star, M. Zhu, R. L. Chow, unpublished data). Thus, it appears that Vsx1 expression is under a tight control by other factors and the Irx6-null data is consistent with the idea that Vsx1 is at very low levels in Type 3 bipolar cells.

A third reason to explain why Vsx1 expression is not detected in Type 3 bipolar cells is that it might be functioning in a non-cell autonomous manner. In this case, Vsx1 would be predicted to translocate to Type 3 cells from another bipolar cell type. Many homeodomain transcription factors can transfer between cells by distinct processes of secretion and internalization, and have non-cell autonomous activities as signaling proteins (Prochiantz and Joliot, 2003). Secretion and internalization domains exist within the highly conserved homeodomain, allowing the homeodomain protein navigating from one cell to another (Prochiantz and Joliot, 2003; Brunet et al., 2007). As mentioned in Section 1.4.1, the Vsx nuclear localization signal (NLS) is highly homologous to the sequence of protein transduction domain (PTD), which contains the capability to translocate across plasma membranes, enabling the intercellular trafficking (Nickel,
Many modifications impairing secretion are associated with prominent cytoplasmic localization, such as the phosphorylation of the homeoprotein engrailed (Brunet et al., 2007; Prochiantz and Joliot, 2003). In this non-cell autonomous case, it is predicted that the detection of Vsx1 would be undetectable if only small amounts are transferred.

Besides intercellular trafficking, are there other possible explanations for how Vsx1 function influences gene expression in Type 3 OFF bipolar cells in a non-cell autonomous manner? As Vsx1 is expressed in Type 2 OFF and Type 7 ON bipolar cells, the physiological activity of these cells could be coupled to Type 3 bipolar cells by neurotransmitter release or gap junction. In this way, a secondary physiological effect of the Vsx1-null defects in Type 2 and 7 bipolar cells could affect gene expression in Type 3 bipolar cells.

In order to determine whether Vsx1 is expressed in Type 3 bipolar cells, single cell RT-PCR can be used to isolate single Type 3 bipolar cells. Double staining of Cabp5 (labels Type 3 OFF, 5 and Rod ON bipolar cells) and mGluR6: β-galactosidase (all ON bipolar cell types) can distinguish Type 3 bipolar cells from the Cabp5-expressing ON bipolar cells. This approach has the advantage over immunofluorescence in that it is highly sensitive and can yield estimates of transcript copy number.

4.5 Role of Vsx1 in Type 7 ON bipolar cell visual signaling?

Previous work on Vsx1-null mice revealed molecular defects in Type 2 cells that were accompanied by defects in retinal function: the bipolar cell dysfunction revealed by a mildly reduced b-wave of electroretinography (ERG), and reduced OFF ganglion cell spiking activity shown by in vitro ganglion recordings (Chow et al., 2004; Ohtoshi et al., 2004). All these data indicated that Vsx1 is essential for the differentiation of a subset of OFF cone bipolar cells and the proper function of OFF cone visual signaling. However, my work has shown a requirement for Vsx1 in Type 7 ON bipolar cells. This leaves the question of what is the role of Vsx1 in this ON bipolar cell type with respect to visual signaling?
Previous studies on the $Vsx1$-null mice have not revealed any obvious ON signaling defects (Chow et al., 2004; Ohtoshi et al., 2004). One possibility is that a moderate (or even subtle) Type 7 bipolar cell defect is masked or compensated by the other four ON bipolar cell types. Interestingly, in studies that have also utilized the $\alpha$-gustducin:GFP transgenic reporter mice, the laboratory of Dr. G. Awatramani (Department of Anatomy and Neurobiology, Dalhousie University) found that Type 7 ON bipolar cells, by the proximity of their axonal terminals, represent excellent candidates as bipolar cells that send input to directionally-selective retinal ganglion cells (unpublished data). Given that we observe subtle gene expression defects in Type 7 bipolar cells, but that the specification of this cell type is not affected by the loss of $Vsx1$, retinal electrophysiological experiments may provide an appropriate assay to tease out an ON pathway defect in $Vsx1$-null mice. We are currently collaborating with Dr. Awatramani’s lab to address this possibility.

4.6 Conclusions and future experiments

My work has shown that $Vsx1$ is required for repressing gene expression in Type 7 bipolar cells. However, my data do not reveal two important things: (1) whether $Vsx1$ is functioning directly as an repressor or whether it does so indirectly (i.e. by activating the expression of another repressor), (2) whether $Vsx1$ functions strictly as a repressor in Type 7 bipolar cells or whether it activates some genes and represses others (it was just by coincidence that we observed downregulation of some proteins). To address these issues, the $\alpha$-gustducin:GFP transgenic reporter mouse will continue to be a vital tool as it will enable the physical isolation of Type 7 bipolar cells from other retinal cell types in dissociated retinas. A microarray study to examine the transcription profile of $Vsx1$ gene regulation in Type 7 bipolar cells from wild type and $Vsx1$-null mice would enable one to determine if there is a trend to support the role of $Vsx1$ as a global repressor in this cell type (see Appendix for more detail and discussion). A similar approach could also be used for Type 2 bipolar cells if an appropriate reporter mouse is developed.

To address the question of whether $Vsx1$ is directly or indirectly regulating transcription, chromatin immunoprecipitation (ChIP) combined either with sequence
analysis (ChIP-seq) or with micro-array study (ChIP-on-chip) could be used to map Vsx1-DNA binding on a genome-wide basis. Furthermore, conducting this same type of analysis in OFF bipolar cells for Bhlhb5, Irx5 and Irx6, and combining it with protein-protein interaction data allows for the generation of an interaction map which would provide a wealth of data to help us understand the mechanisms of gene regulation in different bipolar cell types.

It is quite common for the transcription of many eukaryotic genes to be controlled by combination of multiple proteins. There are still many questions to answer. How do multiple transcription factors/cofactors join together to exert specific functions? How does the transcriptional complex bind *cis*-acting DNA elements and coordinate structural alterations to regulate gene expression (Wolberger, 1999; Reményi et al., 2004; Silver and Rebay, 2005)? The motivation to disclose the questions helps us to further explore the complexity of gene regulation and cell type diversity.
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Appendix
Development of unbiased approaches to identify Vsx1 downstream genes

Introduction

A lot of progress has been made to determine the function of single genes, especially those encoding transcription factors or signaling pathway components through loss- or gain-of-function experiments (Adler, 2005). The examples I presented in Chapter 1, have expanded our understanding of the regulation role of transcription factors in developmental mechanisms in multiple aspects: molecular level, morphology and physiology. More and more members of the HD and bHLH transcription factor families are being implicated in retinal development; however, little is known about how they actually regulate global gene expression. For instance, there is very limited knowledge about the target genes for the majority of the transcription factors aforementioned. Microarray technology provides a powerful approach to identify the downstream genes that are regulated or influenced by transcription factors. A powerful way to utilize this technology is through the comparison of differential gene expression between wild type and loss- or gain-of-function samples. Limitations of this approach should be considered. For example, this type of approach cannot tell whether a gene that is regulated by a transcription factor is regulated directly or indirectly. Another point to be mentioned is that microarray technology can produce a rather large number of false positive data; therefore, further validation of microarray data by \textit{in situ} hybridization, immunohistochemistry, and other specific expression and/or functional approaches is required.

As a transcription factor, Vsx1 is involved in the regulation of gene expression during the differentiation and maintenance of retinal bipolar cells (Chow et al., 2004 and this study). In order to identify genes whose expression is dependent on \textit{Vsx1}, microarray experiments, comparing wild type versus \textit{Vsx1}-null would provide an unbiased identification of differentially expressed genes. Below are two experimental approaches outlining screens that I have started developing to identify \textit{Vsx1} downstream genes.
Approach 1. Utilization of α-gustducin:GFP transgenic mice to enrich Type 7 ON bipolar cells to investigate Vsx1’s possible role in Type 7 ON bipolar cells. This type of bipolar cell represents about 1% of the total mouse retinal cell population (Jeon et al., 1998; Chow et al., 2004; Wässle et al., 2009).

Approach 2. Examination of whole retina mRNA at postnatal day 6.5. This corresponds temporally to the peak of onset for Vsx1 expression and is predicted to identify immediate early downstream targets of Vsx1 that might be more developmentally relevant than genes identified in the adult retina (Lewin, 2008). This alternative approach is not so technically challenging as sorting Type 7 ON bipolar cells to generate high-quality RNA/cDNA (i.e. amplification of RNA is not required) and will likely yield more reliable data.

Methodology

The Methods described below include steps up to the generation of RNA purification (and RNA amplification, if necessary). Following the generation of RNA is the synthesis cRNA microarray probes that will be used for microarray screening. We are using the Affymetrix microarray platform at the Centre for Applied Genomics and The Hospital for Sick Children (Toronto), which include the probe generation steps as part of their service, so these methods are not included or discussed in detail here.

Fluorescence activated cell sorting of adult Type 7 bipolar cells and RNA preparation

List of Instruments and Materials.
- T3 Theromcycler (Biometra), FACS Vantage Diva (BD) (University of British Columbia)
- Glycogen (Invitrogen, Cat. # 10814-010, 20ug/µl)
- PicoPure RNA isolation Kit (Arcturus, Molecular Devices, Cat. # KIT2004)

Retina dissection and retinal dissociation. As addressed in Section 2.4 of Chapter 2.
Cell Analysis/sorting by flow cytometry. α-gustducin::GFP +’ve cells were sorted by high-speed sorter, the flow cytometer as many cells as it can for one sample.

RNA extraction. The Arcturus PicoPure RNA isolation Kit was used for extraction and purification of total RNA from the small amount of samples. The procedure for these steps are indicated below:

1). Cells are kept on ice after sorting (to say around 150 µl) in a 1.5 mL tube.
2). Spin down cells at 1500g for 5min at 4°C.
3). Carefully dispose the supernatant and leave 10~15 µl of PBS inside of the tube, do not pipette up the cells

[Note: (i) incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of binding of RNA to the column membrane; both effects may reduce RNA yield. (ii) For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. ]

4). Lyse the cells with 100µl of lysis buffer (XB). Resuspend the cell pellet gently by pipetting. DO NOT VORTEX. Prior to use, mix XB thoroughly in case there is precipitation formed.
5). Incubate at 42°C for full 30 min.
6). Centrifuge the sample at 3000g for 2 min.
7). Pipette the supernatant containing the extracted RNA into a new 1.5mL tube, avoiding pick-up of pelleted material.
8). Extract the RNA immediately as the following steps or store the lysate on dry ice and then at -80 °C for no longer than two weeks.
9). Pre-condition the purification columns by a) adding 250 µl of conditioning buffer (CB) on the column filter membrane, b) incubating for 5 min at RT and c) centrifuging the column w/ provided collection tube at 16,000 g for1 min.
10). Add 100 µl (or equal volume used for lysing the sample) of 70% EtOH into the cell
extracts, mix well by gently pipetting.

11). Transfer the mix to the column and centrifuge for 2 min at 100 g and then 30 sec at 16,000g.

12). Add 100 µl of wash buffer 1 (W1) to the column and centrifuge at 8,000g for 1 min.

13). DNase I treatment (optional, not required for 3’IVT experiment)
   - RNase-Free DNase Set (Qiagen, Cat. #79254)
   - Pipette 5µl of DNase I stock soln. to 35µl of Buffer RDD (from RNase-Free DNase Set). Mix by gently inverting.
   - Pipette 40µl of DNase incubation mix directly into the purification column membrane. Incubate at RT for 15min;
   - Pipette 40µl PicoPure RNA Kit Wash Buffer 1 (W1) into the purification.

14). Add 100 µl of wash buffer 2 (W2) to the column and centrifuge at 8,000g for 1 min.

15). Add another 100 µl of W2 to the column and centrifuge at 16,000g for 2 min. If there is any residual wash buffer, re-centrifuge at 16,000g for 1 min to ensure that all W2 has been removed.

16). Transfer the column to a new 0.5 ml tube provided.

17). Add 11 µl of elution buffer (EB) to the membrane of the column (gently touch).

18). Incubate the column for 1 min at RT.

19). Centrifuge for 1 min at 1,000g and then 1 min at 16,000g.

20). Measure RNA absorbance of OD260/OD280, OD260/OD230 with NanoDrop to evaluation the quality and quantity of extracted RNA.

21). Use isolated RNA immediately for the next steps.

RNA amplification. The amount of total RNA that was purified from FACS isolated α-gustducin:GFP expressing cells was very low (1-5 ng) and therefore two rounds of RNA amplification must be applied to the sorted cell samples. A detailed description of the procedure for the amplification of mRNA according to Sugino et al. (2005) is found in this link (http://mouse.bio.brandeis.edu/2005-celltype/protocol.pdf).
Some differences are: (i) instead of “T7-oligo(dT) primer of HPLC purified”, I used the PAGE purified. (ii) Instead of “Pellet Paint”, I used Glycogen (20ug/µl, Invitrogen, Cat. # 10814-010) for nucleic precipitation; 0.5µl (or 10ug) is suitable for one sample. Note: For the 1st round of in vitro transcription (IVT), during the experiment, store other reagents on ice, but keep the 10× Reaction (rmx) Buffer at room temperature while assembling the reaction.

**Microarray studies with whole retina RNA isolated by TRIzol reagent combined with RNaseasy column**

**Instruments and materials.**
- homogenizer: Precellys 24 (Precellys, Bertin Technologies, Cat # 03119.200.RD000)
- TRIzol reagent (Invitrogen, Cat. # 15596-026)
- 75% Ethanol (RNase-free)
- RNase-free H₂O
- RNeasy Mini Kit (Qiagen, Cat. # 74104)
- Precellys Lysing Kit (Precellys, Bertin Technologies, Cat. # 0810-09)

**Experimental procedure.**
1). Pups of postnatal 6.5 (P6.5) were killed by being decapitated and eyes were enucleated.
2). After removal of cornea, lens and vitreous body, the two retinas were rapidly dissected without pigment epithelium in 300 µl of Trizol reagent.
3). Retinas were frozen in Trizol in dry ice and stored at -80 °C until all the tissues needed were collected.
4). Homogenization: Added 700 µl of Trizol to the melt 300 µl of TRIzol with retina inside, and homogenized with power homogenizer at the predefined Program #2.
5). Phase separation: Incubated the homogenized samples for 5 minutes at room temperature. Added 0.2 mL of chloroform per 1 mL of TRIzol Reagent. Shaked tubes vigorously by hand for 15 seconds and incubated them at room temperature for 2 to 3 minutes. Centrifuged the
samples at no more than 12,000g for 15 minutes at 4 °C. RNA remained exclusively in the colorless upper aqueous phase. Transferred the aqueous phase to a fresh tube, for about 60% of the volume of TRIzol Reagent used.

6). Added 1 volume of 70% ethanol drop by drop, mix thoroughly.

7). RNA clean-up protocol by Qiagen RNeasy Mini Column Kit

   i) Apply the mixture to a Qiagen RNeasy mini column, centrifuge the column at 3000g for 30 s. Transfer the flow-through to the same column for maximum binding.
   ii). Spin down the column at > 8000 g for 15 s, discard the flow-through.
   iii). Add 700 µl of buffer RW1 to the column, spin at > 8000 g for 15 s to wash the spin column membrane.
   iv). Add 500 µl of buffer RPE to the column, spin down at > 8000 g for 15 s to wash.
   v). Add another 500 µl of buffer RPE to the column, spin down at > 8000 g for 2 min to wash.
   vi). Spin the column at full-speed for 1 min (the collection tube can be changed in this step).
   vii). Transfer the column to a new 1.5 mL tube. Elute with 30 µl of RNase-free H2O, incubate at room temperature for 5 min. Spin down at > 8000 g for 1 min to elute the RNA.

8). Stored the total RNA at -80 °C.

A small portion was kept to quantitate the RNA with the Nanodrop ND-1000 and evaluated with the Bio-Rad Experion Automated Electrophoresis System. Pure RNA has an A260/A280 ratio of 1.9~2.1 in 10 mM Tris-Cl, pH7.5. Since water is not buffered, the pH and the resulting A260/A280 ratio can vary greatly. Lower pH results in a lower A260/A280 ratio and reduced sensitivity to protein contamination. Isolated RNA is run on the Bio-Rad Experion Automated Electrophoresis System. Electrophoresis on the gel matrix and the fluorescence should show discrete bands of high molecular weight RNA between 3 kb and 4 kb in size (composed of mRNA’s and hnRNA’s), two predominant ribosomal RNA bands at ~5 kb (28S) and at ~2 kb (18S), and low molecular weight RNA between 0.1 and 0.3 kb (tRNA, 5S).
Primary results

Initial attempts were made to isolate $\alpha$-gustducin:GFP expressing putative Type 7 bipolar cells. The flow cytometry data for these attempts were used to generate the GFP expression data to quantitate the number of GFP expressing cells and levels of GFP in wild type and $Vsx1$-null mice (see Fig. 15). The O.D. readings of the RNA purified from the sorted cells in this experiment, however, indicated that the RNA quality was poor, so a microarray experiment was not conducted.

Frozen retina RNA samples from P6.5 wild type (n = 3) and $Vsx1$-null (n = 3) mice were sent to the Microarray Facility, the Centre for Applied Genomics, the Hospital for Sick Children in Trontano. Samples were processed by using Affymetrix 3’IVT Expression Kit and applied to Mouse MOE 430 GeneChip® Expression Analysis. The data was sent back to lab and is awaiting further analysis.

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Sheet of TRIzol Reagent (Invitrogen, Cat. # 15596-026)