Vsx2 inhibits *dmbx1a* to regulate retinal progenitor cell proliferation in zebrafish

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Cell and Systems Biology
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**Abstract**

Vsx2 is a gene expressed in RPCs during development and in a subset of bipolar cells and Müller glia of the differentiated neural retina. Dmbx1a is involved in the development and maintenance of numerous cell types and is important for the development of the midbrain, hindbrain, and retina. Since the mRNA expression patterns of *vsx2* and *dmbx1a* are non-overlapping, the present study investigates the molecular networks regulating the transition between RPCs to post-mitotic neurons with these genes. The primary objective is to test the hypothesis of mutual antagonism between *vsx2* and *dmbx1a*. Results suggest that *vsx2* is required on and off in a carefully timed balance for the proper expansion of progenitor cells to occur during retina development. It also implicates that this gene has an effect on proper retina cell differentiation and laminar organization. Finally there is evidence that *dmbx1a* is needed but not sufficient in regulating *vsx2* expression.
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Chapter 1
Introduction

Functional visual systems in vertebrates are vital for avoiding predators and securing food. The development of zebrafish (*Danio rerio*) as a model organism began in 1981 by George Streisinger. The molecular control and morphology of development in zebrafish is similar to that in higher vertebrates like humans (Glass and Dahm, 2004). As well, the genetic basis of retinal development is conserved in most vertebrates (Bassett and Wallace, 2012). Zebrafish embryonic development occurs quite rapidly and after 24 hours post fertilization (hpf) the major organs are visible. During early growth, the eyes form at about 13 hpf and the optic lobes also start to separate from the brain (Avanesov and Malicki, 2010). One physiological importance of the zebrafish is their retina cellular layout, which closely resembles the human retina, making it a good genetic model system to study retinal development. Zebrafish are ideal to use because they have functional genes that are similar to humans and are easy to manipulate. Microphthalmia was one of the first mouse mutants to showcase retina development defects and since human congenital eye malformations, such as microphthalmia, have been linked to the human VSX2 gene, we have an opportunity to advance our understanding of how this gene functions in normal and pathological circumstances. Currently there is no cure for microphthalmia, which invariably leads to blindness and this can be modeled and investigated using zebrafish so that we can gain a better understanding of how gene variants associated with this class of disorders, such as vsx2, are functionally altered. This can aid in development better prevention and treatment strategies.
1.1 Retinal Growth:

Vertebrate eye formation begins early during development with the eye field being organized during gastrulation, and soon after, two lateral optic pits form from the ventral forebrain (Graw 2010). The eye is formed from different embryonic tissues; the lens and cornea arise from the surface ectoderm and the retina and pigmented epithelium derive from the neural tube (Graw, 2010). The optic vesicles, the first visible manifestation of eye development, form through bilateral evagination. During this time there is a period of very close contact between the vesicles and the surrounding surface ectoderm, which allows the surface ectoderm to thicken and give rise to the lens placode. Thereafter the lens placode and the optic vesicle undergo invagination, forming the lens vesicle and the optic cup (Canto-Soler, 2007) (Figure 1.1). Cells in the outer layer of the invaginated optic cup differentiate into a ciliated cuboidal monolayer epithelium called the retinal pigmented epithelium (RPE) that produces melanin pigment (Graw, 2010). The inner layer of the optic cup (at this stage known as the neuroretina) transforms itself from a pseudostratified neuroepithelium (specified retinal progenitor cells, RPCs) into an expanded multilayered structure with several distinct classes of neurons and glia. Three major cellular layers can be identified in the embryonic eye: the retinal ganglion cell layer (RGC) consisting of RGC’s and a subset of amacrine cells; the inner nuclear layer (INL) consisting of amacrine, Müller glia, bipolar cells and horizontal cells; and the out nuclear layer (ONL) formed by the rod and cone photoreceptor cells (also called the photoreceptor layer). RPCs differentiate into these neurons and glia in a temporal order that is conserved among vertebrates (Figure 1.2). Ganglion cells are the first to differentiate, followed closely by amacrine cells, cone photoreceptors, and horizontal cells. Bipolar cells, rod photoreceptors, and Müller glia are formed at later stages (Trimarchi et al, 2008). This double layered optic cup is connected to the diencephalon by the optic stalk. The dorsal side of the optic vesicle invaginates to form the bilayered optic cup,
Figure 1.1: Overview of vertebrate optic cup development. The optic vesicle invaginates to form the optic cup and the lens vesicle. Then the optic cup invaginates to form a bilayered retina, with the outer layer forming the RPE and in the inner layer forming the neural retina. The optic cup contains undifferentiated RPCs (yellow) and as development progresses, these cells begin to exit the cell cycle (purple) centrally. Adapted from Bilitou and Ohnuma, 2010.
Figure 1.2 Temporal schedule of neuronal differentiation in the developing retina. These cells follow a conserved histogenetic order. During vertebrate retinogenesis, RPCs become restricted in fate competence and proliferation potential before their final cell divisions. These progenitors then differentiate into the six classes of neurons and one type of glia. Adapted from Vitorino et al, 2009.
while the more ventral aspect converges into the choroid fissure. This fissure will close and contribute to the formation of the optic nerve, where retinal ganglion cell axons will grow towards the brain (Canto-Soler, 2007). These axons will cross the midline at the optic chiasm and project to diencephalic structures, such as the lateral geniculate nucleus (LGN) in mammals, and the mammalian superior colliculus (SC) or its homolog the optic tectum in other vertebrates, in the contralateral brain (McLaughlin and O’Leary, 2005). The dorsal-ventral patterning of the optic cup is regulated by opposing signals that originate in the neural tube (Canto-Soler, 2007). Many of the transcription factors needed to form the eye field are conserved and are thus named eye-field transcription factors (EFTFs). These include, but are not limited to $Rx1$, $Six3$, $Pax6$, $Lhx2$, $Ath5$. Failure to form the eye field leads to eyeless phenotypes (anophthalmia) and if it forms but does not divide into two hemispheres cyclopia occurs (Winkler et al, 2000; Stigloher et al, 2006; Geng et al, 2008). This process has been studied in numerous models, such as mice, amphibians, and fish (Mathers et al, 1997; Winkler et al, 2000, Zuber et al, 2003).

Like most developing tissues, formation of the retina begins with multipotent cells that will eventually become terminally differentiated cells. RPCs have stem cell like features and they are defined by their ability to self-renew in their relatively undifferentiated state. As well, RPCs and retinal stem cells share similar transcription factor expression patterns such as $pax6a$, $six3$, and $rx1$ (Raymond et al, 2006). Similar to adult neural stem cells, RPCs are in close contact with both the apical surface and the basal lamina and are nearby large blood vessels. The maintenance of these stem cell properties in RPCs per se was not the focus of my Master’s research; instead, I was interested in understanding how RPCs transition into differentiated cells through the control of the cessation of cell proliferation.
1.2 Cell Cycle and Cell Fate:

The control of cell proliferation and self renewal in the retina depends on an array of intrinsic and extrinsic factors. During optic cup formation, retinal precursor cells undergo a proliferative stage in order to generate enough RPCs (Martins et al, 2008). Cyclin-dependent kinases (CDKs) and proliferating cell nuclear antigen (PCNA) are cell cycle activators that are turned on to propel this proliferative stage. During retinogenesis, regulation of RPC proliferation and cell cycle exit follows a tightly controlled schedule to produce the required neurons (Bilitou and Ohnuma, 2009) (Figure 1.3). The zebrafish retina maintains two populations of stem cells, the ones located in the ciliary marginal zone (CMZ) and the radial glia (Müller cells) (Meyers et al 2012). The stem cell properties of Müller glia are most evident during regeneration in older retinas (Raymond et al, 2006; Fischer and Bongini, 2010), but since this is not the focus of my research I will not be discussing this any further. We believe that RPCs during embryogenesis behave like the progenitor cells in the CMZ. One study has presented evidence that the CMZ contains three main regions or zones (Raymond et al, 2006), where starting closest to the periphery and moving centrally there is the (1) CMZ periphery, (2) CMZ middle, and (3) CMZ central. They then assessed through in situ hybridization what genes may be detected in each region during early development. The most peripheral region had the highest signal levels of $rx1$, $vsx2$, and $pax6a$ expression, supporting the idea that this is a region of RPCs. It has also been suggested that the CMZ may be further divided into 4 (Perron and Harris, 2000) or 5 main regions (Bilitou and Ohnuma, 2009). At the very periphery where the RPCs reside, there is lower mitotic activity with transcriptional down-regulation of CDKs and eye field genes. Moving centrally in the CMZ, the next two regions have an up-regulation of CDKs to activate the cell cycle and increase the pool of RPCs. Thus, CDKs activity rises and falls as cells move through
Figure 1.3: Model of retina organization and cell fate determination. The timing of cell fate determination is correlated with the down regulation of the cell cycle. Following this, post-mitotic cells differentiate, starting centrally, and mature into one of the six retinal neuron types or one type of glia. Adapted from Bilitou and Ohnuma, 2010.
the cell cycle. Zone 4 and 5 are more centrally located, where proliferation is down-regulated and cell fate determinants are activated. These cells will differentiate into mature retinal neurons. Regardless of the number of zones that can be reliably identified, the general progression in cell fate changes in RPCs from periphery to central in the CMZ mimics what is observed throughout the central retina during early embryogenesis, such that a similar molecular mechanism for neurogenesis persists even in the older stages.

Cyclical changes in CDK activity are controlled by regulator proteins called cyclins and CDKs need to be bound to cyclins in order to have protein kinase activity. CDK levels remain steady while cyclins undergo a cycle of synthesis and degradation with each cycle. During the early stages of the G1-phase, proliferative cells need mitogenic signals to maintain progressing through the cell cycle. Once in late G1, mitogens are no longer required, and cells are committed to move through to M phase. During this stage FGF, EGF, TGF, IGF, SHH, and Wnt families have been shown to act as mitogens on RPCs (Cameron et al 1998). G1 phase proteins, such as RB, D-cyclins, and Kip1, are all necessary for vertebrate retinal development (Levine and Green, 2004) as their interactions are required to ensure a successful transition to the S-phase. Checkpoints are vital and are arranged to regulate each part of the cycle. When DNA replication does not occur properly at the G2/M boundary, the checkpoint protein CDK1 will block progression into mitosis and either allow for repairs or enter an abortive state (Vitale et al, 2011). The differences of proliferation in RPCs may involve differences in the cell cycle machinery and region specific regulators of proliferation. Homeobox genes such as Pax6 and Six6 are some of the regulators of proliferation in the developing retina, which drive G1 progression (Levine and Green, 2004). RPCs need to exit the cell cycle in a timely manner in order to produce post-mitotic neurons and the final cell division in neurons has been found to be very important for
cell-fate determination (Bilitou and Ohnuma, 2009; Baye and Link, 2008). It is likely that certain neuronal transcription factors may be activated during this final cycle.

There are multiple pathways involved in the regulation of RPC proliferation and/or self-renewal. Many ligand-induced pathways (such as Wnt, FGF, Shh, and Notch) are believed to be the regulators of RPC proliferation (Bilitou and Ohnuma, 2009) and studies have suggested that Notch signaling is involved in RPC self-renewal (Perron and Harris, 2000), but others show that Notch signaling may instead promote a glial cell identity in the retina (Scheer et al, 2001). On the other hand, receptor tyrosine kinase signaling (e.g. FGF and EGF), has a critical role in RPC proliferation, cell survival, and cell growth and appears to function through a PI3K-Akt transduction pathway (Lillien and Cepko, 1992; Farrell et al 2011). Extracellular signal proteins that act through receptor tyrosine kinases consist of a large variety of growth factors and hormones. The binding of the signal protein to the ligand-binding domain on the outside of the cell activates the intracellular tyrosine kinase domain. Once activated, the kinase domain transfers a phosphate group from ATP to selected tyrosine side chains. The noncatalytic regions on the cytoplasmic domain also undergo phosphorylation which allows for the availability of docking sites for downstream cytoplasmic targets (Pawson, 2002). These docking sites can be diverse but most share an SH2 and/or PTB domain.

While FGF signaling is involved in a wide array of developmental processes, it has been shown to organize the optic vesicle into the neural retina and RPE (Hyer et al, 1998). By removing the surface ectoderm (the source of FGF signaling) and injecting an FGF virus into the mesoderm of the optic vesicle, they found that those cells exhibited characteristics typical of the neural retina. Downstream targets in this pathway include PKC, PI3k-Akt, and MAPK/Erk protein transduction pathways. Mutations in FGF-related genes, such as in the FGF8 mutant acerebellar,
can cause defects in neural formation (Reifers et al, 1998). There are four known FGF-receptor genes in vertebrates (FGFr1, FGFr2, FGFr3, FGFr4), which have been shown to have overlapping expression patterns during development, as well as at least 22 FGF ligands in many vertebrates (Green et al, 1996), but their localization in zebrafish is not well known. An estimated 20 FGF ligands have been seen in zebrafish (Ota et al, 2009). FGF signaling has been shown in numerous studies to be important for lens formation (Garcia et al, 2005, Zhao et al, 2008) and the loss of FGFr expression in the mouse retina had resulted in not only lens defects but also microphthalmia. FGFr2 has been shown to be expressed in early zebrafish development in the ventral telencephalon, anterior diencephalon and midbrain (Tonou-Fujimori et al, 2002), but further work is needed to provide detailed retinal localization of these receptors. A recent study by Picker and Brand (2005) presented data implicating a role for FGF signaling in nasal-temporal axis patterning during optic vesicle formation. By inhibiting FGF signaling they found a shift in nasal patterning by assessing *epha4b*, a receptor that is expressed temporally in the presumptive retina and *efna5a*, which is expressed nasally. In these inhibited embryos the expression pattern of *epha4b* expanded nasally and the expression of *efna5a* was lost. The similar outcome seen in the *ace* mutants and this inhibiting study lead us to believe that other FGFs may act in a similar mechanism.

A unique feature of RPC proliferation is the process known as inter-kinetic nuclear migration (IKNM) and its coordination with the RPC cell cycle. These movements involve the stochastic migration of nuclei from apical to basal and back during the G1 and G2 phases of the cell cycle in progenitor cells. Mitotic nuclei are located to the apical pole, while nuclei undergoing DNA synthesis are displaced basally (Norden et al, 2009). One hypothesis is that IKNM regulates the duration and level of exposure of progenitor nuclei to neurogenic signals. For example, Del Bene et al. (2008) showed that between cycles of mitosis, an RPC nucleus will move twice through a
Notch spatial gradient. If the nucleus remains close the apical side, it will receive high levels of Notch and both of the daughter cells will likely remain proliferative. In contrast, if the nucleus remains close to the basal side, there will be low Notch levels and the progeny will become differentiated (Del Bene et al, 2008). This is an appealing model that integrates the phenomenon of IKNM with RPC cell cycle kinetics, but in this context differentiation is viewed as a passive outcome relying on the absence of Notch signaling. However, it is not yet clear if Notch signaling maintains vsx2 expression in these RPCs, which facilitates self-renewal, or whether other regulators of RPC proliferation, such as the RTKs, fulfill this role. Furthermore, how other factors that actively promote RPC cell cycle exit interact with these pathways to regulate the balance between RPC proliferation and differentiation is poorly understood.

The number of cells in a tissue, such as the retina, relies on growth from proliferating RPCs and cell death. Contrary to cell necrosis, which signals neighbouring cells to die, apoptosis is a process of programmed cell death without necessarily damaging neighbouring cells. Apoptosis leads to the fragmentation of DNA, shrinkage of the cytoplasm, and membrane changes. The organic components left by the cell are quickly taken up and recycled. In zebrafish, waves of cell death have been shown to take place prior to waves of cell birth (Biehlmaier et al, 2001). In the retina an initial wave of cell death occurs in the RGC layer, followed by cell death in the INL and ONL. Though here, the timing of these events do not necessarily mirror the timing of neurogenesis (Stenkamp, 2007). The intracellular mechanics responsible for apoptosis is similar across vertebrates. Bax is believed to initiate apoptosis by forming a pore in the mitochondrial membrane, causing a release of cytochrome c into the cytoplasm and the subsequent activation of caspases (Jin and El-Deiry, 2005). Caspases are a family of intracellular proteases that are involved in initiating the cellular events of apoptosis (Jin and El-Deiry, 2005). These enzymes are inactive as procaspases that can be activated by cleavage at aspartic acids, which go on to
activate others thereby amplifying a proteolytic cascade. During this cascade numerous factors are activated or repressed and this intracellular pathway is similar is most animal cells. The intrinsic and extrinsic cues in regulating the timing of RPC proliferation and differentiation are integrated during retinogenesis. These processes may be similar in the central embryonic retina and later in the CMZ.

1.3 RPC Transcription Regulation:
As mentioned earlier the mature retina contains six types of neurons and one type of glial cell that arise from multipotent RPCs during development. RPC differentiation is controlled by a variety of transcriptional regulators. Retinal identity is specified by a network of signals from the eye field transcription factors that include, but are not limited to, \textit{Six6}, \textit{Six3}, \textit{Rx1}, and \textit{Lhx2} (Zuber et al, 2003). Slight variations of these factors may exist between vertebrate species. Other factors that are required for neuronal specification included are \textit{Math5} for ganglion cells, \textit{NeuroD} for photoreceptors, \textit{Math3} for amacrine cells, \textit{vsx2} for bipolar cells, and \textit{Hes1} for Müller glia (Bassett and Wallace, 2012). The combinations of these transcription factors are necessary for proper specification of RPCs in each of the layers. \textit{Ath5} is expressed during the G2 phase of RPCs and enables those cells to differentiate into RGCs (Poggi et al, 2005). The protein atonal homolog 7 (\textit{Atoh7/Math5}) is not only required for ganglion cell production as it is found to be expressed in non-ganglion cells (Feng et al, 2010). RPCs that express \textit{Prox1} differentiate into horizontal and amacrine cells (Dyer et al, 2003). This suggests that other interacting factors may be needed for proper cell fate for each retinal neuron. Many homeobox genes are required for retinal development and identifying their relationships is necessary for understanding the networks that drive this process. In mice, loss of \textit{Rax} (\textit{rax3} in zebrafish) has been found to result in an eyeless phenotype (Mathers et al, 1997). The RPE and neural retina have some genes that are specific to only those regions. \textit{Mitf} and \textit{Otx1/Otx2}, and are a few of the genes encoding
transcription factors necessary for RPE specification. On the other hand, \textit{Chx10/Vsx2} is one of the earliest known genes to be expressed specifically in the presumptive neural retina that controls neuroretinal specification (I will refer to this gene as \textit{Vsx2} throughout).

### 1.4 \textit{Vsx2}

\textit{Vsx2} is a conserved paired-like homeobox transcription factor expressed in RPCs during development and in a subset of bipolar cells and Müller glia of the differentiated neural retina (Burmeister et al, 1996; Horsford et al, 2005; Clark et al, 2008; Vitorino et al, 2009). \textit{Vsx2} encodes a paired-class homeodomain and a CVC domain. The CVC domain was named after the three proteins which were recognized: CHX10 and VSX1 from goldfish and CEH10 from nematodes (Svendsen and McGhee, 1995). Vertebrates also have a Vsx2 paralog called Vsx1, and both are expressed early during eye development (Clark et al, 2008). During mouse development, at around E9.5, \textit{Vsx2} is highly expressed in RPCs and later during differentiation expression is restricted to the INL (Burmeister et al, 1996). The \textit{Ocular retardation (or)} mouse mutant has microphthalmia, a cataractous lens, a thin retina that is morphologically poorly differentiated, and a lack of an optic nerve and is therefore blind (Burmeister et al, 1996). They also found that proliferation was reduced in these mutants. This phenotype is caused by a loss of function mutation in \textit{Vsx2}, and similar losses of function mutations in VSX2 have been associated with human microphthalmia (Percin et al, 2000). Later studies in mice further identified \textit{Vsx2} as a transcriptional repressor during retinal development that is likely acting downstream of FGF signaling (Horsford et al, 2005). The \textit{or} mutant mice displayed not only an increased RPE, but also an increase in \textit{Mitf} (a transcription factor required for RPE specification). Whether or not \textit{Vsx2} represses this factor directly or indirectly is unknown.

Another recent study has further implicated \textit{Vsx2} as a repressor, and in this case it is repressing its paralog \textit{Vsx1} (Clark et al, 2008). Their knock out studies suggest that Vsx1 and Vsx2 do not
compensate for each other and mRNA expression analysis found that \textit{vsx1} is not expressed in \textit{vsx2} positive RPCs. \textit{Vsx1} is instead turned on postnatal in cone bipolar cells. Surprisingly in chick the \textit{Vsx1} ortholog \textit{Chx10-1} is highly expressed in RPCs while \textit{Chx10} is at relatively lower levels (Chen and Cepko, 2000). In zebrafish, the expression of \textit{vsx2} occurs in all RPCs at 16-28 hpf prior to the onset of differentiation (Vitorino et al, 2009). \textit{Vsx2} is highly expressed throughout the neuroretinal epithelium at a time when RPCs are expanded in number, and then the expression is down-regulated as cells differentiate into the various classes of mature retinal cell types. Even though \textit{vsx2} has been shown to be required for RPC proliferation during early retinal development, it also has a later postnatal role in promoting bipolar cell differentiation and inhibiting rod development (Livne-bar et al, 2006). Therefore, \textit{vsx2} is actively expressed in RPCs during proliferation, it can function as a transcriptional repressor (repressing \textit{vsx1} and \textit{Mitf}), and also acts as a key determinant of bipolar cell and rod development, indicating that this gene could have multiple roles governed by the stages of development.

1.5 \textit{Dmbx1a}

Our lab has been investigating the role of the paired-type diencephalon/mesensephalon homeobox genes \textit{dmbx1a} and \textit{dmbx1b} in zebrafish (Chang et al, 2006), which are conserved orthologs of the chordate Dmbx1 gene (Holland and Takahashi, 2005). \textit{Dmbx1a} was originally shown to be involved in the genetic control of the development of the midbrain, hindbrain, and retina in zebrafish (Kimura et al, 2005). Through loss of function and biochemical assays, Kimura et al (2005) found that mouse Dmbx1 directly regulates and represses Otx2, indicating that these two genes may interact with each other in order to regulate target genes during brain development. \textit{In situ} hybridization in mice provided spatial and temporal expression information about \textit{Dmbx1} (Ohtoshi et al, 2002). They found that \textit{Dmbx1} is expressed in the anterior head folds between 7.5 and 8.5 days postcoitum (dpc). At 9.5 dpf, expression is found in the caudal
diencephalon, mesencephalon, and is restricted to the neuroepithelium. In zebrafish, both paralogs are needed during neural development but they have differences in onset, spatial distribution, and relative abundance during the first 48 hpf of embryogenesis (Chang et al., 2006). They found that during shield and tailbud stages, \textit{dmbx1a} is expressed in the presumptive midbrain region, the RPCs, and the progenitors in the diencephalic lineages. Conversely, the expression of \textit{dmbx1b} is almost imperceptible during these stages. Between 24 hpf and 48 hpf both paralogs have partially overlapping domains of expression in the midbrain and hindbrain. At 72 hpf the expression of \textit{dmbx1a} was expressed in the optic tectum and in the retina. Both are expressed throughout the retinal INL, but \textit{dmbx1a} appears enhanced and localized to the central region of the INL, whereas \textit{dmbx1b} expression is relatively diffuse and significantly less abundant throughout most layers (Wong et al, 2010). Although both genes have conserved regions of expression (i.e. midbrain), there are differences in the onset of expression and spatial distribution, and these are correlated with the changes of the genomic sequence conservation between the paralogs (Chang et al, 2006). The knockdown of \textit{dmbx1a} significantly reduces ganglion cell and amacrine cell differentiation, and virtually eliminates the differentiation of bipolar cells, Müller glia and all photoreceptors (Wong et al, 2010). Thus, since \textit{dmbx1a} appears to have a more important role on the retina, I chose to focus my research on \textit{dmbx1a} only. Wong had found through \textit{in situ} hybridization that RPCs continued to proliferate when Dmbx1a was knocked down. A defect in RPC cell cycle exit is proposed to underlie this phenotype (Wong et al, 2010). The targets of Dmbx1a are unknown, but one leading candidate is \textit{vsx2}, since this gene is up-regulated in \textit{dmbx1} knockdown retinas and is likely to promote the maintenance of an RPC identity in this context. As part of its role in maintaining an RPC identity, Vsx2 functions to repress genes that would otherwise specify different retinal cell fates. Vitorino et al. (2009) found that \textit{vsx2} is a transcriptional repressor that targets \textit{vsx1}, \textit{ath5}, and \textit{foxn4}. When \textit{vsx2} is
down-regulated, vsx2 negative progenitors escape vsx2 repression and thus are able to express factors that restrict lineage potential and become specific retinal cell types. Therefore, it is also possible that dmbx1a is a target of vsx2 repression that enables the maintenance of an RPC identity.

1.6 Objectives and Hypothesis:
Given the central role of Vsx2 as a positive regulator of RPC identity and a negative regulator of differentiation, and the function of Dmbx1a as a positive regulator of RPC cell cycle exit and differentiation, my thesis focused on testing the hypothesis that Vsx2 and Dmbx1a function in regulating zebrafish RPCs through mutual antagonism. Since the mRNA expression patterns of both genes are predominantly non-overlapping, the present study investigates how these factors interact to regulate the transition between RPCs to post mitotic cells. I will test this hypothesis by addressing two specific aims.

1.6.1 Aim 1: To investigate the role of FGF signaling as an upstream regulator of vsx2 expression
Evidence from the literature indicates that vsx2 is regulated by a RTK signaling mechanism, more specifically FGF signaling (Horsford et al, 2005; Clark et al, 2008; Zhao et al, 1997). Previous work indicated that the addition of FGF2 coated beads lead to the development of an ectopic neural retina that did not express Mitf protein. Adding FGF2 coated beads in Chx10^op-op^-J mice did not produce an ectopic retina, indicating that Chx10 (vsx2) lies downstream of FGF (Horsford et al, 2005). Whether this pathway is conserved in zebrafish is unknown, thus I explored the elements upstream of vsx2 by assessing where in the retina FGF signaling may be occurring during these early time points.
1.6.2 Aim 2: To determine if vsx2 and dmbx1a mutually antagonize each other

Loss of dmbx1a function resulted in the failure of RPCs to exit the cell cycle and differentiate properly, which is supported by the fact that cells in the retina maintain a RPC profile at a time when most of the cells are normally post-mitotic, differentiated and functionally networked (Wong et al, 2010). One hypothesis to account for this phenotype is that Dmbx1a normally represses RPC gene expression to facilitate the transition from a RPC to mature retinal neuron or Müller glia cell. A corollary of this hypothesis is that this transition is not initiated too early in normal development because RPC genes, such as vsx2, repress dmbx1a expression. Indeed, the mRNA expression patterns of both these genes are mostly, but not completely, non-overlapping. There are at least two possible mechanisms by which Vsx2 can antagonize Dmbx1a function. First, Vsx2 can act as a transcriptional repressor of dmbx1a gene expression. Second, Vsx2 can regulate Dmbx1a protein function either directly or indirectly through its regulation of other genes. I will test the hypothesis that over-expression of vsx2 will inhibit the expression of dmbx1a, resulting in RPCs remaining proliferative and failing to mature into functional neurons.

To complement the over-expression study, I will ask if knocking down Vsx2 at an early stage causes the precocious expression of dmbx1a.

In more recent studies in zebrafish, loss of vsx2 function was found to affect bipolar cells fate at the expense of photoreceptors (Vitorino et al, 2009). But these knock down studies were assessed at a later time, 80 hpf, when the morpholino may not be effective and the retina may have begun to recover. Therefore I wanted to assess changes due to loss of Vsx2 early on and pinpoint when vsx2 expression is needed for cells to maintain their proper fate. The ability to visualize when phenotypic changes take place and assessing them in more detail will provide information into the functions of Vsx2.
2.1 Zebrafish husbandry

Adult zebrafish (*Danio rerio*) used in this study were maintained at 28°C on a 14-hour light/10-hour dark cycle and housed in an automated re-circulating system (Aquaneering). Animals were treated in accordance with the regulations on animal experimentation established by the Canadian Council on Animal Care. The maintenance breeding and experimental procedures were approved by the University of Toronto Animal Care Committee. Embryos were staged and reared according to standard procedures (Westerfield, 2007). The wildtype strain used was AB originally received from the Zebrafish International Resource Center (ZIRC). The transgenic strains used were: Tg(hsp70l:dnfgfr1-EGFP) obtained from ZIRC, and Tg(dusp6:GFP) was a kind gift from Dr. Brian Ciruna (Hospital for Sick Children Research Institute, Toronto, Canada).

2.2 Cryosectioning

Embryos at 1-4 day post fertilization (dpf) were fixed with 4% paraformaldehyde overnight at 4°C and washed in a sucrose series (from 5% to 30% sucrose in PBS) for cryoprotection. Samples were left in 30% sucrose:OCT (2:1 ratio) at -20°C before processing into 18-20 µm sections with a cryostat (Leica).

2.3 BrdU labelling

To label cells that were in the S-phase of the cell cycle, embryos at 24hpf, 48hpf, or 72hpf were placed into 10 mM of 5’-bromo-2’-deoxyuridine (BrdU) and fixed the embryos with 4%
paraformaldehyde two or three hours later. Cryosectioning procedures were performed as described. Sections were post-fixed in 4% paraformaldehyde for 10 minutes and washed with PBS + 0.1% DMSO + 0.1% Tween-20 (PBDT). They were incubated in 10mM sodium citrate for 30 minutes at 70°C, followed by washes in PBDT at room temperature. Slides were incubated in 2N HCl for 1 hour at 37°C followed by washes in PBDT at room temperature. Sections were blocked for two hours and incubated in mouse anti-BrdU (1:500 Roche) primary antibody overnight at 4°C, which was detected with Cy3 secondary antibody (1:500 Jackson ImmunoResearch Laboratories, Inc). Images were obtained from mounted slides using a Leica TCS SP5 II Confocal Microscope and analyzed with Leica LAS AF software.

2.4 Antisense morpholino oligonucleotide injection

Antisense morpholinos oligonucleotides (MOs) were obtained from Gene Tools, Inc. Vsx2-MO1 was complementary to the sequences that flanked the ATG start codon and Vsx2-MO2 was complementary to the first exon/intron boundary, as previously described and validated (Vitorino et al, 2009). I also verified the translational blocking morpholino by Western blot with an anti-Vsx2 antibody. Where indicated, a p53-MO was used to determine the extent of p53 dependent apoptosis in specific treatments (Robu et al, 2007). This is also a translational blocking morpholino that binds just upstream of the ATG start codon that has been previously validated by Robu et al (2007). The dmbx1-MO is complementary to the start codon and partially overlaps the 5'UTR, which has also been validated (Wong et al, 2010).

The sequences of the MOs are as follows:

Vsx2 MO1: 5’-ACATCATCTGAATCTGAGCTGGCAG-3’
Vsx2 MO2: 5’-AGGCATGTACCCCGTACCTGAGCTG-3’
dmbx1a MO1: 5’-ACTCCGTAGTGCTGCATGATTCACA-3’
p53 MO: 5’-AGAATTGATTTTGCCGACCTCCTCTC-3’
Unless otherwise noted, embryos were injected with 5ng of either MO into the yolk at 1- to 4-cell stage.

2.5 vsx2 over expression
To over-express vsx2 in vivo, the pSGH:vsx2 plasmid (gift from Thomas Czerny; Bajoghli et al, 2004) was microinjected (100pg) into the cytoplasm at the 1-4 cell stage. This construct is a bi-directional meganuclease transgene vector containing a fragment with eight heat shock elements upstream of a CMV minimal promoter, driving the expression of GFP gene. A similar cassette with the same promoter was in the opposite orientation with untranslated regions and pA containing a polylinker where the full length cDNA of vsx2 was inserted. Injected embryos were incubated at 28.5°C until needed. Heat shocking was done in 50 ml falcon tubes containing 30-40 embryos that were placed into a 37°C water bath. Embryos were heat shocked at 24 hpf, 48 hpf, or 72 hpf, with 3 pulses (60 minutes in water followed by 60 minutes normal incubation temperature) and then allowed to develop normally overnight. The following day (24 hours post heat shock) embryos were screened for GFP positive ones and imaged using Zeiss fluorescent stereoscope (SteREO Lumar.V12) and software (Zen Imaging software).

2.6 FGF Signalling knock down
The Tg(hsp70l:dnfgfr1-EGFP) embryos were heat shocked for 60 minutes at desired time points (24 hpf, 48 hpf, and 72 hpf) for only one pulse at 37°C as described above. They were allowed to develop normally overnight and the following day (24 hours post heat shock) embryos were screened for GFP expression. To chemically knock down FGF signalling, a 10 mM stock solution of SU5402 was prepared in DMSO and a 40 uM solution of SU5402 was made by dilution in facility water. Embryos were incubated in this solution for ~12 hours followed by a
series of washes in fresh facility water. Control embryos were placed in 0.02% DMSO in place of SU5402. Images were obtained using a Zeiss fluorescent stereoscope (SteREO Lumar.V12) as above.

2.7 Western blotting

Whole embryos (deyolked in 1x Ringers solution) were placed in a ice cold lysis buffer (20 mM MOPS, pH 7, 2 mM EGTA, 5 mM EDTA, protease inhibitor cocktail (Sigma), Halt phosphatase inhibitor cocktail (78420, Pierce), 1% Triton X-100, and 1mM dithiothreitol) and sonicated on ice for 10 seconds. These samples were centrifuged at 4°C for 30 minutes at 13.2 rpm and the lysate was stored at -20°C. Protein quantification was performed using Bradford reagent and BSA as a standard control. The supernatants were prepared for electrophoresis with sample buffer NuPAGE LDS (Invitrogen) with 5% ß-mercapthanol and they were incubated for 10 min at 70°C. Cooled samples were loaded onto a 4-12% Bis-Tris SDS gel (Invitrogen) and submitted to electrophoresis followed by transfer to a PVDF membrane (Hybond ECL, GE Healthcare Pharmacia Biotech). Membrane blots were blocked in 5% BSA (in TBS-T) for 1 hour at room temperature and placed overnight in primary antibody (in blocking solution) at 4°C. Membranes were then washed 3x10 minutes in TBS-T and placed in secondary antibody (in TBS-T) at room temperature for 45 minutes. A series of 3x10 minute washes was performed and the membrane was incubated for 5 minutes in ECL prime (Invitrogen) for film development. Primary antibodies used were 1/500 p-ERK1/2 (Cell Signaling), 1/500 ERK1/2 (Cell Signaling), 1/000 actin (Cell Signaling), and 1/1000 vsx2 (kind gift from Dr. Higashijima). Secondary antibodies used were 1/5000 goat-anti-rabbit-hrp, goat-anti-mouse, and goat-anti-guinea pig (Jackson ImmunoResearch).
2.8 Immunohistochemistry

1-4 dpf embryos were fixed in 4% paraformaldehyde overnight at 4°C and washed in sucrose series (from 5% to 30% sucrose in PBS) for cryoprotection and cryosectioning. Sections were rehydrated with 1xPBS and blocked with 0.2% Triton X-100 + 2% goat serum in PBS at room temperature for 1 hour. Primary antibody in blocking solution was incubated with sections overnight at 4°C. Sections were washed extensively with 1xPBS + 0.1% Tween-20 (PBST) and incubated with secondary antibody (1:500 Cy3, Jackson ImmunoResearch Laboratories, Inc) for 3 hours at 4°C. Counterstaining of nuclei was done with 1µg/ml Hoechst (Sigma, 861405) before mounting the slides. GFP antibody was conjugated to Alexa Fluor 488, therefore no secondary antibody was needed. The following primary antibodies were used: rabbit anti-PKC (1:200 Santa Cruz Biotechnology, Inc.), mouse anti-GS (1:400, Chemicon), mouse anti-Zpr1 (1:200, ZIRC), mouse anti-Zpr3 (1:200, ZIRC), rabbit anti-Caspase 3 (1:200, Cell Signaling), mouse anti-Zn5 (1:400, ZIRC), rabbit anti-GABA (1:400, Sigma-Aldrich), guinea pig anti-vsx2 (1:400, kind gift from Dr. Higashijima). Images were taken from Leica TCS SP5 II Confocal Microscope and analyzed with Leica LAS AF software.

2.9 In situ hybridization

Embryos were fixed in 4% paraformaldehyde and processed as previously described for cryosectioning. The following anti-sense RNA probes were used: *dmbx1a* (cloned by Dr. Loksum Wong), *vsx2* (Open Biosystems), *fgfr1, fgfr2, fgfr3* (gift from Dr. Laure Bally-Cuif; Topp et al, 2008). Slides were rehydrated in 1xPBS and post-fixed in 4% paraformaldehyde for 10 minutes and washed in 1xPBS. Slides were incubated in pre-hybridization solution for 1 hour at 65°C in a humid chamber with 5XSSC and left overnight in hybridization + probe solution at 65°C. The next day slides were washed 4x15 minutes in 0.2x SSC at 65°C and incubated in TN.
at room temperature for 5 minutes. Slides were blocked in TNB for 30 minutes followed by a series of washes in TNT. Anti-Dig-POD antibody (Fluorescence) or anti-Dig-AP (colour) (Roche) in TNB was placed on slides for 60 minutes at room temperature. Next sections underwent a series of washes in TNT. For fluorescence staining, signal amplification was done with a Cy3 TSA kit (PerkinElmer). Slides were washed with PBS-T and counterstained with Hoechst before mounting. Images were taken from Leica TCS SP5 II Confocal Microscope and analyzed with Leica LAS AF software. For colorimetric staining, slides were washed 3x5 minutes in AP buffer. NBT and BCIP were added to the AP buffer to make the staining solution. Slides were stained in fresh solution from 30 minutes to 4 hours. Slides were then washed with PBS-T and mounted. Images were taken from Leica Compound Microscope (SP5) and LAS-AF software.

2.10 Statistics
Statistical significance was determined by unpaired t-tests using Microsoft Excel, 2007.

2.11 qRT-PCR
RNA was isolated from head tissue only in all untreated and treated groups (n=50) at 24 hpf, 48 hpf, or 72 hpf. Tissue was placed in Trizol and extracted according to the manufacturer’s protocol and RNA was quantified using a Nanodrop analyzer. RNA was reverse transcribed using Superscript III First Strand Synthesis system (Invitrogen). Primer sequence information can be found in Table 2.1. Quantitative RT-PCR was performed on cDNA samples obtained as described above. cDNA was amplified in the Rotor Gene 3000 using LightCycler 480 SYBR Green I Master Mix (Roche). Values obtained for genes of interest were normalized to ef1α expression, and the average is presented in arbitrary units ± standard deviation. All cases had a
no template control (NTC) with water in place of the cDNA template in order to rule out non-specific contamination of PCR reaction. cDNA amplification was done without reverse transcriptase (-RT) for all RNA samples to account for genomic DNA contamination of RNA samples.

Table 2.1: Primers used for gene expression analysis with qRT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ef1α</td>
<td>CATACATCAAGAAGATCGGCTACAACC</td>
<td>GTAGTACCGCTAGCATTACCCTCCTTG</td>
<td>154</td>
</tr>
<tr>
<td>Vsx2</td>
<td>CCTGGAGGTTATTCCGTCTTTCTACAGTT</td>
<td>CTGGCTCAGTGAAGACTTTGACATTTT</td>
<td>186</td>
</tr>
<tr>
<td>Dmbx1</td>
<td>CGAGCTAGAAGCAAGAAATATCA</td>
<td>GAGTTCATGGCGTGGAGAGAGTA</td>
<td>115</td>
</tr>
</tbody>
</table>
Chapter 3
Results

3.1 Upstream regulation of vsx2 expression by FGF signaling

Results from mouse studies indicate that vsx2 expression is regulated by FGF signalling (Horsford et al, 2005; Clark et al, 2008; Zhao et al, 1997). For example, it was shown that activating FGF signalling lead to the development of an ectopic neural retina that did not express Mitf protein (Nguyen and Arnheiter, 2000). In contrast, FGF signalling in Chx10<sup>or-1or-1</sup> mice did not produce an ectopic retina, indicating that Chx10 (vsx2) lies downstream of FGF and is required for ectopic retinal formation (Horsford et al, 2005). However, the extent to which the upstream regulation of vsx2 expression by FGF is conserved among vertebrates is not clear. I hypothesized that FGF signalling is required for the regulation of vsx2 expression in RPCs during zebrafish embryonic retinal development.

3.1.1 Expression of FGF receptors in the embryonic retina

To verify that embryonic RPCs are competent to receive FGF signals, I examined the expression of three of the four conserved vertebrate FGF receptors by in situ hybridization using fgf1<sub>1</sub>, fgf2<sub>1</sub>, and fgfr3 RNA probes. All three receptors were expressed in the retina (Figure 3.1), with fgf2<sub>1</sub> being the most pronounced. Fgf3 expression was mostly concentrated in the lens and brain with relatively less expression in the central retina (Figure 3.1C). Fgf1 was dispersed throughout the retina and lens (Figure 3.1A), while fgf2 was mostly localized in the retina (Figure 3.1B). These expression patterns are consistent with previous findings (Tonou-Fujimori et al., 2002).
Figure 3.1: FGF receptor *in situ* hybridization on embryos. Embryos at 48 hpf were cryosectioned and probed with *fgfr1*, *fgfr2*, or *fgfr3* RNA probes. The top panel presents merged fluorescent and bright field images, while the bottom is fluorescent only. The *fgfr2* expression pattern was the most intense in comparison to *fgfr1* and *fgfr3* in the retina (n=5 for each probe). Scale bar = 30µm.
3.1.2 FGF signaling is active in the embryonic retina

The expression of FGF receptors in the embryonic retina suggested that FGF signaling is active in retinal cells, and others have shown that this signaling pathway is involved in early retinal morphogenesis and patterning (Reifers et al, 1998; Picker and Brand, 2005). In order to confirm that FGF signaling is active in the retina during the later stages of retinal neurogenesis, I analyzed the expression of two known markers of this pathway: Dusp6 and phosphorylated-Erk 1/2 (pErk1/2). Dusp6 is a highly conserved downstream transcriptional target of FGF signaling that is believed to be a negative feedback regulator in this pathway (Li et al, 2007). MAPK activation is a major target of FGF receptor signal transduction (Turner and Grose, 2010) and is activated in an FGF-dependent manner in early stages of zebrafish development (Li et al, 2007).

I examined the *Tg(dusp6:GFP)* transgenic line that contains a destabilized GFP variant with a relatively short half-life, providing a temporal readout of activated FGF signaling in the embryo. From 24 hpf to 72 hpf, GFP expression was evident in the retina (Figure 3.2A-C), with varying degrees of intensity along the dorso-ventral axis, especially at 24 hpf, consistent with a wave-like patterning of FGF signaling and proliferation reported previously (Picker and Brand, 2005).

To confirm that GFP expression was dependent upon FGF signaling, transgenic 24 hpf embryos were treated with 40µM of a pharmacological inhibitor of FGF receptors (SU5402) for 12 hours. Untreated and DMSO control treated embryos retained their GFP expression (Figure 3.2D, E), but embryos treated with SU5402 appeared to lose GFP expression (Figure 3.2F). Furthermore, those embryos that were incubated in 40µM SU5402 were subjected to Western blotting and showed a ~ 60% decrease in the levels of p-ERK1/2 protein (Figure 3.2G; n = 3 separate experiments), which is a target of FGF signaling in early development (Turner and Grose, 2010). Together these data suggest that FGF signaling is active during the later stages of retinal development.
Figure 3.2: Tg(dusp6:GFP) embryos imaged at (A) 24 hpf, (B) 48 hpf, and (C) 72 hpf. Dusp6 expression changes as development progresses in a wave-like manner. This indicates that FGF signaling may be occurring in a similar pattern during early eye growth (n=15). Tg(dusp6:GFP) embryos in SU5402 showed a decrease in GFP expression, indicating that dusp6 is likely downstream of FGF in zebrafish. (D) Control in water, (E) embryos in 0.02% DMSO, and (F) embryos in 40uM SU5402 at 48 hpf (n=10). Western blotting showed a decrease in pERK 1/2, compared to ERK 1/2, using actin as a control. A 60% reduction in expression was found when comparing intensity levels using Image J (n=3). Scale bar = 50 µm.
3.1.3 Blocking FGF signaling leads to reduced vsx2 expression in the embryonic retina

The aforementioned observations indicate that FGF signalling is active during retinal neurogenesis, when RPCs express high levels of vsx2 and are actively proliferating and generating new retinal cells. To directly test if FGF signalling is required for vsx2 expression in RPCs, I utilized the transgenic line, Tg(hsp70l:dnfgfr1-EGFP), to inhibit FGF signalling in a heat inducible manner driven by the zebrafish hsp 70-like promoter (Lee et al., 2005). The zebrafish dnfgfr1 cassette is based on the Xenopus dominant-negative FGFr1 construct from Amaya and Krischner (1991), where the tyrosine kinase domain was replaced with the EGFP-coding sequence. This construct, with a 3’ truncated fragment of the zebrafish fgfr1 gene, is predicted to heterodimerize with all FGF receptor subtypes, thereby competitively and broadly blocking FGF signalling (Lee et al, 2005). Thus, using this transgenic I tested whether FGF signalling is required for vsx2 expression during neurogenesis.

To begin to address this hypothesis, I had to first optimize the heat shock parameters in order to determine the best strategy to examine the effects of blocking FGF signaling in the retina, while avoiding the widespread detrimental effects on development. The results of my optimization trials, which examined GFP expression, morphology and developmental milestones, are shown in Table 3.1. The controls in these experiments included non-heat shocked and heat shocked wildtype (AB strain) embryos, which did not result in any abnormal retinal development, as well as non-heat shocked transgenic embryos, which did not express GFP and were morphologically normal. Optimization of heat shock experimentation resulted in a single pulse of heat shock at 37°C for 60 minutes at 24 hpf, 48 hpf, or 72 hpf and placing embryos back in normal rearing temperature of 28°C for 24 hours. Embryos heat shocked at 24 hpf (Figure 3.3A, B) and 48 hpf (Figure 3.3C, D) were GFP positive with reduced eye diameter (Figure 3.3E), which is an
Table 3.1: Results of optimization trials using the transgenic line Tg (hsp70l:dnfgfr1-EGFP). Embryos were heat shocked for 60 minutes at 37°C at 24, 48, or 72 hpf and assessed 24 hours post heat shock. Embryos that were heat shocked at 24 hpf and viewed at 48 hpf had the most intense GFP expression, along with the most morphological changes including small eye phenotype.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GFP Expression</th>
<th>Morphology</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shocked @ 24 hpf Viewed @ 48 hpf</td>
<td>Intense expression throughout embryo</td>
<td>Severe eye and head growth retardation</td>
<td>22/30 (73%)</td>
</tr>
<tr>
<td>Heat shocked @ 48 hpf Viewed @ 72 hpf</td>
<td>Moderate expression in head region</td>
<td>Mild growth defect</td>
<td>24/30 (80%)</td>
</tr>
<tr>
<td>Heat shocked @ 72 hpf Viewed @ 96 hpf</td>
<td>Little to no expression</td>
<td>Normal</td>
<td>28/30 (93%)</td>
</tr>
</tbody>
</table>
Figure 3.3: Knock down of FGF signaling leads to a small eye phenotype. Control embryos at (A) 48 hpf, and (C) 72 hpf were compared to heat shocked (B, D) Tg[hsp70l(dnfgfr1:EGFP)] embryos that were heat shocked for 60 minutes (n=15 for each group). Scale bars = 50 µm. (E) Transgenic embryos that heat shocked at 24 hpf showed a significant decrease in eye growth by 48 hpf, measured using ImageJ. Bars represent the mean ± standard deviation (s.d.) of 7 replicate experiments.
indirect measure of retinal growth. This phenotype was significant only after heat shock in 24 hpf embryos. The effect was much less severe in embryos heat shocked between 48 hpf and 72 hpf when the vast majority of RPCs have already differentiated (data not shown), indicating that FGF signaling is likely necessary for proper RPC proliferation, differentiation or survival of progenitor cells during the peak of neurogenesis, but not for the survival or maintenance of differentiated cells under the heat-shock parameters that were used.

I next examined the expression level of \textit{vsx2} in 48 hpf embryos with and without heat shock at 24 hpf using RT-qPCR (Figure 3.4). A significant decrease in \textit{vsx2} expression was observed, implying that FGF signaling is important for maintaining \textit{vsx2} expression in RPCs. Interestingly, I observed the opposite effect on \textit{dmbx1a} expression, whereby the levels of \textit{dmbx1a} transcripts were significantly increased in heat-induced embryos (Figure 3.4). Thus, these data suggest that FGF signaling positively regulates \textit{vsx2} expression, and negatively regulates \textit{dmbx1a} expression, but the relationship between these components are not fully understood. I further examined this issue in the sections that follow.

### 3.1.4 Reduced FGF signaling compromises RPC survival, but not proliferation

One possible explanation for why blocking FGF signaling at 24 hpf or 48 hpf caused a small eye phenotype 24 hours post-induction is that in the presence of reduced levels of FGF signaling, fewer RPCs were proliferating and instead underwent premature differentiation. This is supported by extensive evidence showing that RTK signaling (especially EGF and FGF) has an important role in regulating stem and progenitor cell proliferation in the brain and retina (Hyer et al, 1998; Russell, 2003; Picker and Brand, 2005; Cohen et al, 2010; Dorey and Amaya, 2010).
Figure 3.4: Gene expression levels of \textit{vsx2} and \textit{dmbx1a} when FGF signaling is knocked down in \textit{Tg[hsp70(dnfgfr1:EGFP)]} embryos at 48 hpf. RT-qPCR was used to quantify RNA from three pools of 50 embryos collected in non-heat shocked and heat shocked transgenic embryos. Embryos were heat shocked at 37°C for 60 minutes and allowed to develop normally for 24 hours. Levels of \textit{vsx2} significantly (p=0.016) decreased and \textit{dmbx1a} significantly increased (p=0.0139) with loss of FGF signaling, + s.d. from 3 replicate experiments.
To examine this possibility, \textit{Tg(hsp70l:dnfgfr1-EGFP)} embryos that were heat shocked at 24 hpf or 48 hpf and maintained at normal temperatures for 24 hours post-induction were placed in a 10 mM BrdU bath for 3 hours and then immediately processed for BrdU immunohistochemistry. I found that regardless of the age at which embryos were heat-shocked (Figure 3.5A-D), no significant change in the number of proliferating (BrdU+) cells was observed (Figure 3.5E), suggesting that the small eye phenotype was not primarily due to a decrease in proliferation, although more subtle changes in the cell cycle cannot be ruled out by this experiment.

An alternative explanation for the small eye phenotype in the heat-induced \textit{Tg(hsp70l:dnfgfr1-EGFP)} embryos was that cell survival was compromised when FGF signaling was reduced. I next investigated whether there was an increase in cell death by examining the expression of activated (cleaved) Caspase-3, which is a marker of apoptosis (Jin and El-Deiry, 2005). Interestingly, there was a significant increase (Figure 3.6E) in the number of apoptotic (Caspase-3+) cells when embryos were heat shocked at 24 hpf (Figure 3.6A, B) and examined 24 hours post-induction. The effect was not consistently observed at 48 hpf, which is in line with our eye diameter data. This increase in apoptosis is likely the reason behind this phenotype, indicating that the lack of FGF signaling may lead to cell death during development.

Reducing the levels of FGF signaling during retinal development can give rise to microphthalmic eyes, which are likely due to an increase in apoptosis. From these results I sought to identify if there were specific cell types dying or changes in retinal composition during these time points. I specifically examined the expression of a bipolar cell marker (pkcß1), retinal ganglion cell (RGC) marker (zn5), cone photoreceptor marker (zpr1), Müller glial cell marker (glutamine synthetase, GS), and a GABAergic amacrine cell marker (GABA) using immunohistochemistry in heat-induced and control embryos. Bipolar cells (Figure 3.7 I-L) and cone photoreceptors
Figure 3.5: Levels of proliferating cells remained normal when FGF signaling was knocked down. Control and heat shocked embryos were placed in 10mmol of BrdU and fixed after 3 hours. Cryosections of 20µm were labeled with anti-BrdU and counterstained with Hoechst (n=10, + s.d. of 5 replicate experiments). Scale bars = 50 µm.
Figure 3.6: Significant increase in cell death with loss of FGF signaling. Cryosections of 20 µm from control and heat shocked transgenic embryos were labeled with cleaved-caspase 3 and counterstained with Hoechst. Cell death was maintained at a higher level at 72 hpf (n=15, ± s.d. of 5 replicates). These counts are based on a 45 cell area using the optic nerve as a marker. Arrows point to caspase-3 positive cells. Scale bars = 50 µm.
Figure 3.7: Knock down of FGF signaling led to a delay in neuronal differentiation. Embryos were heat shocked with one pulse at 37°C and observed 24 hours later. Control and heat shocked Tg[hsp70l(dnfgfr1:EGFP)] embryos were cryosectioned (20µm) (n=8). Most cell types formed normally at the appropriate time and location. Cells in the (A-D) RGC layer appear to be developing slower and (Q-T) Müller glia production was delayed by 24 hours (+ s.d. of 4 replicates). Scale bar = 50 µm.
(Figure 3.7 M-P) arose in a normal spatial and temporal pattern. RGCs that were Zn5+ (Figure 3.7 A-D) were formed in the proper location, but were disorganized and likely delayed in their maturation. Müller glia (Figure 3.7 Q-T) and GABA+ amacrine cells (Figure 3.7 E-H) appeared to be most affected by the reduced levels of FGF signaling with apparently fewer cells expressing these markers. For example, at 72 hpf, a time when Müller glia should be well defined, there were few GS+ cells in the heat shocked embryos. One limitation in my analysis is that since I did not quantify each cell subtype in the heat shocked embryos and compared them to controls, I cannot conclude with certainty that only some cell types were affected compared to others. Nonetheless, given that proliferation in heat shocked embryos was unaffected at both 48 hpf and 72 hpf, that there was an overall small eye phenotype, and that there were elevated levels of apoptotic cells, a plausible explanation is that reduced levels of FGF signaling generally compromised post-mitotic RPC survival leading to microphthalmia.

3.2 Knockdown of Vsx2 leads to microphthalmia with an increase in dmbx1a expression and compromised RPC survival

The microphthalmic phenotype observed in the zebrafish embryos with reduced levels of FGF signaling was tightly correlated with reduced expression of vsx2. Previous studies (Barbino et al, 1997; Clark et al., 2008; Vitorino et al, 2009) showed that knockdown of zebrafish vsx2 leads to microphthalmia, which is reminiscent of the Chx10mutation mouse mutant (Burmeister et al, 1996). Thus, although FGF inhibition likely affects many different developmental processes and gene expression patterns, it is possible that the downstream effects on vsx2 expression specifically are required for the observed retinal growth defects. To test this possibility, I injected previously
described \textit{vsx2} morpholinos (MO) targeting either the start codon or a splice junction (Barbino et al., 1997), as well as control MO, and examined the retina at 24 hpf, 48 hpf and 72 hpf.

### 3.2.1 Validating the Vsx2 knockdown phenotype

I confirmed that \textit{vsx2} MO injections gave rise to embryos with a microphthalmic phenotype with a significant decrease in eye diameter by 24-48 hpf (Figure 3.8 A-C). Vitorino et al (2008) validated the splice inhibiting morpholinos via RT-PCR. Despite the use of these MOs in the literature, it is unclear whether \textit{vsx2} protein levels are reduced in the morphants. In order to further validate the knockdown phenotype, I examined expression of Vsx2 protein by Western blot and showed that protein levels were reduced in the knockdown embryos compared to controls (Figure 3.8 D), which also correlated with inhibition of \textit{vsx2} mRNA splicing (Vitorino et al., 2008). Thus, my data demonstrate that the \textit{vsx2} MO leads to a small eye loss of function phenotype, consistent with the mouse mutant data.

### 3.2.2 Loss of Vsx2 expression leads to an increase in \textit{dmbx1a} expression

Since my previous findings indicated that reduced FGF signaling resulted in a decrease in \textit{vsx2} expression and a concomitant increase in \textit{dmbx1a} expression, I wanted to test whether a specific loss of \textit{vsx2} was sufficient to cause the same inverse change in \textit{dmbx1a} expression. First, \textit{in situ} hybridization on 20\textmu m sections with \textit{vsx2} and \textit{dmbx1a} RNA probes was used to evaluate broad spatial changes in mRNA levels. At 48 hpf, there was a more widespread distribution of \textit{dmbx1a} expression in the morphant retina compared to controls, which was less obvious by 72 hpf (Figure 3.9 A-D). To quantify changes in expression levels I used RT-qPCR.
Figure 3.8: Loss of Vsx2 lead to a decrease in eye size. Translation blocking or splice inhibiting morpholinos were injected at the 1-4 cell stage. Control (A, B) and morpholino injected embryos (A’, B’) were imaged at 24 hpf and 48 hpf, respectively. (C) To validate the translational blocking morpholino, a western blot of control and \textit{vsx2} morpholino (MO) injected embryos was performed. A decrease in \textit{vsx2} was observed in the morphant embryos (with actin as a control). (D) Eye diameter was measured with ImageJ and a significant decrease in eye size was observed at 48 hpf (n=10, + s.d. of 5 replicate experiments). Scale bar = 50 µm.
Figure 3.9: Increase in $dmbx1a$ expression when Vsx2 is knocked down. (A-D) *in situ* hybridization with $dmbx1a$ RNA probes on control and vsx2 morphant embryos ($n=8$). Both cases an increase in $dmbx1a$ expression was observed. (E) RNA samples from 3 pools of 50 embryos at 48 hpf and 72 hpf for RT-qPCR with $dmbx1a$ primers. A significant increase in $dmbx1a$ expression occurred when vsx2 was knocked down at 48 hpf ($p=0.0128$) (+ s.d. from 3 replicate experiments). Scale bar = 50 µm.
Consistent with the in situ data, there was a significant increase in \( dmbx1a \) expression in the morphant compared to controls only at 48 hpf (Figure 3.9 E). These findings suggest that \( vsx2 \) is an upstream negative regulator of \( dmbx1a \) expression.

### 3.2.3 RPC proliferation and cell death increased in the Vsx2 knocked down embryos

Previous research in our lab suggests a role for Dmbx1a in promoting zebrafish RPC cell cycle exit (Wong et al., 2010). More recent observations indicate that \( dmbx1a \) over expression could cause premature RPC cell cycle exit leading to either enhanced differentiation of at least one early cell type (Zn5+ RGCs), but more commonly to an increase in RPC apoptosis (Wong, 2013). Thus, the increase in \( dmbx1a \) expression in the Vsx2 knock down embryos may cause the RPCs to prematurely exit the cell cycle and differentiate, or to prematurely die. If true, the microphthalmic phenotype in the Vsx2 knockdown embryos may be due to reduced numbers of proliferating RPCs (especially at 48 hpf), as was observed in embryos over expressing \( dmbx1a \) (Wong et al., 2013). To test this possibility, I exposed \( vsx2 \) MO and control injected embryos to 10 mM BrdU for 3 hours at the specified developmental time points and processed the embryos immediately for immunohistochemistry. As expected, sections of control embryos showed cells proliferating throughout the retina at 24 hpf, and as embryos with proliferation of RPCs becoming increasingly confined to the CMZ by 72 hpf (Figure 3.10 A-F). In contrast, the pattern of RPC proliferation in the Vsx2 knock down embryos showed that while the number of BrdU+ cells was similar at 48 hpf, this number was significantly increased in the morphant retinas at 72 hpf, with an expanded BrdU+ domain at the periphery and proliferating RPCs located centrally (Figure 3.10 G). The overall decrease in retinal size and the fact that RPC proliferation was at normal levels at 48 hpf suggests that these RPCs are not actually
Figure 3.10: Vsx2 morphants display a delayed increase in proliferating cells and an increase in cell death. Embryos were injected with translational or splice inhibiting morpholinos at the 1-4 cell stage. To assess proliferation, embryos were placed in a 10mmol BrdU bath for 3 hours at 24, 48, or 72 hpf, and were then fixed. (A-F) Cryosections of 20 µm were labeled with anti-BrdU and counterstained with Hoechst. (G) A significant increase in proliferating cells was maintained higher up at 72 hpf (n=8, + s.d. of 4 replicate experiments). (H-O) Control and injected embryos were also fixed at 24, 48, or 72 hpf and cryosections were labeled with anti-cleaved-caspase 3. Arrows indicate caspase-3 positive cells. (P) A significant increase in cell death was observed in knock down embryos at 48 hpf (n=10, + s.d. of 4 replicate experiments). These counts are based on a 45 cell area that was regulated by using the optic nerve as a marker. Scale bar = 50 µm.
proliferating more in the morphant retinas compared to the controls, but rather that they may be prematurely undergoing cell death, before exiting the cell cycle. Thus, control and vsx2 MO injected embryos were processed for cleaved Caspase-3 immunohistochemistry to detect apoptotic cells. The control embryos only had a few Caspase-3 positive cells per retinal section while in the Vsx2 morphant embryos there was a significant increase in apoptotic cells (Figure 3.10 H-P). Morpholinos are known to sometimes have off target toxic effects, including p53 mediated cell death in early embryos regardless of the expression pattern of the target gene (Robu et al, 2007). I co-injected the vsx2 MO with a p53 MO previously shown to effectively reduce p53 protein levels (Robu et al, 2007). The results were the same as those without the p53 MO. For simplicity, I presented only the data with just single morpholino injections. Therefore, the lack of Vsx2 may cause RPC cell death via a different pathway, such as the p53 up-regulated modulator of apoptosis (PUMA) pathway (Tuffy et al, 2010), which can be both p53-dependent and p53-independent. Cell death could be occurring through Fas-associated protein with Death Domain (FADD) and capase-8 (Bush et al, 2001; Kaufmann et al, 2012) but additional experiments are required to further determine the mechanism of cell death.

3.2.4 Widespread differentiation delay in Vsx2 morphants

Vsx2 MO injected embryos and controls were fixed at 24 hpf, 48 hpf, and 72 hpf, cryosectioned and immunolabeled with retinal cell type markers: bipolar cells (pκβ1), RGC layer (zn5), cone photoreceptors (zpr1), Müller glia (GS), and GABAergic amacrine neurons (GABA) (Figure 3.11). Fewer differentiated cells were present between 48 hpf and 72 hpf, which is consistent with the fact that RPC survival was compromised, but not completely abolished in the morphant retinas before they had a chance to differentiate. Differentiated cells in the RGC layer and
Figure 3.11: Loss of Vsx2 lead to a delay in neuroretinal differentiation. Vsx2 morpholino injected embryos were fixed at 24, 48, or 72 hpf, followed by cryosectioning. 20µm sections were labeled with the mentioned retinal markers and a delay in differentiation by 24 hours was detected for most retinal cell types (n=8). The later types, such as (M-P) cone photoreceptors and (Q-T) Müller glia, were even more prolonged in their developmental time. Scale bar = 50 µm.
GABAergic amacrine cells that are normally present by 48 hpf were not present until 72 hpf in the morphant embryos (Figure 3.11 E-H). By 72 hpf, when all retinal cell types should be present, cone photoreceptors and Müller glia were barely detectable, and bipolar cells were present but in a disorganized manner (Figure 3.11 I-T). Overall, these findings suggest that although vsx2 must ultimately be down regulated in RPCs in order for them to differentiate into most cell types, expression of this gene is still required in RPCs during the transition to a post-mitotic state.

3.3 Over expression of vsx2 results in microphthalmia with decreased dmbx1a expression

Vsx2 is expressed initially in RPCs throughout the retina, and as cells exit the cell cycle its expression is restricted to the RPCs within the CMZ. My findings indicated that the ability of RPCs to exit the cell cycle and undergo differentiation may depend on vsx2 levels being maintained during this transition, but ultimately down regulated. I asked whether over expressing vsx2 would slow or prevent the transition from proliferating RPCs to differentiated cells.

3.3.1 Validating the pSGH:vsx2 over expression construct

To accomplish this I began by optimizing the use of the inducible full length vsx2 over expression construct pSGH:vsx2 and determining critical time points for over expression. To over-express vsx2 in vivo 100 pg of the pSGH:vsx2 plasmid (Bajoghli et al, 2004) was microinjected into embryos at the 1-4 cell stage. This construct is a bi-directional meganuclease transgene vector containing a fragment with eight heat shock elements upstream of a CMV
minimal promoter, driving the expression of GFP gene. A similar cassette with the same promoter was in the opposite orientation with untranslated regions and poly-A containing a polylinker where the full-length cDNA of vsx2 was inserted. The expression patterns of vsx2 and dmbx1a in 48 hpf and 72 hpf control embryo sections were consistent with previously published data (Wong et al, 2010; Vitorino et al, 2009). Vsx2 is expressed at high levels throughout the retina at 24 hpf, while dmbx1a is just beginning to turn on at 48 hpf. Using this as a starting point, I created a protocol for an over expression procedure (see methods), where embryos were assessed 24 hours following the final heat shock, when GFP expression was most robust (Figure 3.12 A-D). Mosaic GFP expression was accompanied by a noticeable small eye phenotype in embryos that were heat shocked at 24 hpf. This phenotype was less severe when embryos were heat shocked at 48 hpf. This could be due to dilution of the plasmid by 48 hpf, or it could be that over expression of vsx2 at this time point has a different role on RPCs. Instead of affecting RPC survival, the vsx2 could be playing a role in the development of later cell types, particularly Müller glia. Eye diameter was measured from both heat shocked and non-heat shocked groups and a significant decrease in size was found in those that were heat shocked at 24 hpf (Figure 3.12 E).

The mosaic expression of the transgene 24 hours post heat shock is expected since the construct is presumably not integrated into genome of cells after injection. One possibility to improve this and reduce the mosaic expression would be to co-inject a meganuclease enzyme to improve integration and expression, but this was not done during these experiments. However, the consistent small eye phenotype would indicate that there might have been more widespread expression early post heat shock that then diminished with time. Alternatively, over expressing vsx2 in cells causes non-autonomous effects on retinal growth. To distinguish between these two possibilities, I first examined the expression of Vsx2 protein levels in 48 hpf embryos 24 hours
post heat shock. Under normal conditions, Vsx2 protein is already mostly confined to the CMZ with a few Vsx2+ cells in the central INL, which are presumptive Muller glia or bipolar neurons (Figure 3.13). In the heat-shocked embryos, Vsx2 expression was widespread and overlapped only partially with GFP+ cells at 48 hpf. I next examined GFP expression 4 hours post heat shock in 24 hpf embryos injected with the pSGH:vsx2 construct and found that there is evidence that there are more widespread GFP+ and Vsx2+ double labeled cells (Figure 3.13 F, I). This suggested that I am maintaining the over expression of Vsx2 that is initially widespread in the retina and presumably affecting most RPCs. The lower amount of co-labeled positive cells at 48 hpf may be due the somewhat faster degradation of GFP or more likely the unequal expression of GFP and Vsx2 from the plasmid at later stages.
Figure 3.12: vsx2 over expression in zebrafish embryos causes microphthalmia. Control (A, C) and pSGH:vsx2 injected (B, D) embryos were heat shocked at 24 hpf (top) or at 48 hpf (bottom). Mosaic GFP expression indicates where vsx2 over expression is occurring. (E) Quantification of change in eye size was done by measuring eye diameter in both groups 24 hours post heat shock using Image J and a significant decrease in eye size was observed in those that were heat shocked at 24 hpf (n=8, + s.d. of 5 replicate experiments). Scale bar = 50 µm.
Figure 3.13: Over expression of vsx2 increased Vsx2 protein expression. Plasmid injected embryos were heat shocked at 24 hpf with 3 pulses at 37°C and fixed the following day. Cryosections of 20µm were labeled with anti-vsx2 and anti-GFP. (D-F) Co-labeling of Vsx2 and GFP was observed in those embryos that were heat shocked between 24-28 hpf (n=5). (G-I) Embryos heat shocked between 24-48 hpf also showed an increase in vsx2 expression (n=5, ± s.d. of 2 replicates). Scale bar = 50 µm.
3.3.2 Vsx2 over expression causes a decrease in *dmbx1a* expression

One prediction from our previous findings (Wong et al., 2010) is that the onset of *dmbx1a* expression in the retina during normal development occurs as a downstream consequence of the reduced *vsx2* expression in RPCs as they undergo cell cycle exit and differentiation. However, it is not known whether Vsx2 is a negative regulator of *dmbx1a* expression. Therefore, I determined whether the over expression of *vsx2* could lead to a decrease in *dmbx1a* expression by first evaluating gene expression changes in control and *vsx2* over expressed embryo using *in situ* hybridization with *vsx2* and *dmbx1a* RNA probes. As expected, the heat shocked embryos had higher *vsx2* expression, but I also observed a decrease in *dmbx1a* expression (Figure 3.14 A-F). Next, to complement these results I quantified the changes in expression with RT-qPCR, which confirmed my *in situ* data (Figure 3.14 G-H). Since the expression of these genes only transiently overlaps in the retina as RPCs undergo cell cycle exit, our data indicate that *vsx2* might be an upstream repressor of *dmbx1a* expression, but whether this is a direct or indirect repression remains to be further investigated. This could be accomplished by utilizing ChIP-qPCR to assess if *vsx2* protein directly binds to the *dmbx1a* locus.
Figure 3.14: Over expression of vsx2 results in decreased in dmbx1a expression. (A-F) Control and pSGH:vsx2 injected heat shocked embryos were cryosectioned at 20µm for in situ hybridization with vsx2 and dmbx1a RNA probes. This qualitative data was corroborated with quantitative RT-PCR (G-H). Three different experimental pools of RNA, of 50 embryos each, were extracted at two different time points, 48 hpf and 72 hpf. Student t-test was performed with the heat shock values and the control. Levels of vsx2 expression significantly increased at 48 hpf and 72 hpf (p=0.0023 and p=0.0168 respectively). Levels of dmbx1a significantly decreased at 48 hpf and 72 hpf (p=0.0135 and p=0.0011 respectively) (+ s.d. from 3 replicate experiments). Scale bar = 50 µm.
3.3.3 Over expression of Vsx2 does not alter RPC proliferation, but increases cell death

Wong et al (2010) had shown that the knock down of dmbx1a leads to an increase in vsx2 expression, which I was able to confirm (Figure 3.15 F), and a defect in cell cycle exit. Interestingly, the increased levels of vsx2 did not correlate with increased RPC proliferation, but instead correlated with a slowing of the cell cycle length at 72 hpf when most RPCs should have differentiated (Wong et al., 2010). This suggested that the function of vsx2 in progenitor cell fate is uncoupled from progenitor cell proliferation. To test this hypothesis, I asked if increasing the levels of vsx2 was sufficient to promote the maintenance of a “proliferating” RPC identity in the embryonic retina. I incubated embryos that were induced to over express vsx2 at 24 hpf or 48 hpf in 10 mM BrdU for 3 hours (24 hours post heat shock) and subsequently processed the tissue for immunohistochemistry. Sections of control embryos showed proliferating BrdU+ cells throughout the retina at 24 hpf, and increasingly localized proliferation toward the retina peripheries by 72 hpf (Figure 3.15 A-D). The vsx2 over expression did not cause a significant increase or decrease in proliferation at all time points examined (Figure 3.15 E), which supports the hypothesis that vsx2 does not regulate RPC proliferation during the time that neurogenesis is peaking in the retina. This finding is surprising because of the microphthalmic phenotype in observed embryos over expressing vsx2.
Figure 3.15: vsx2 over expressing embryos did not exhibit changes in proliferation rates. To assess this, pSGH:vsx2 injected embryos were heat shocked with 3 pulses at 37°C and placed in a 10mM BrdU bath for 3-4 hours and fixed the following day. Cryosections of 20µm were labeled with anti-BrdU and no significant change was observed in those embryos that were heat shocked at 24-48 hpf (n=8, + s.d. of 4 replicate experiments). (F) RNA samples from 3 pools of 50 embryos were extracted from control and dmbx1 morpholino (3 ng) injected embryos at 48 hpf and at 72 hpf for RT-qPCR. A significant increase in vsx2 expression was seen in the morphant embryos at 48 hpf (p=0.0067) (+ s.d. from 3 replicate experiments). Scale bar = 50 µm.
I next investigated the possibility that over expression of \textit{vsx2} resulted in cell death. Control and over expressing embryos were sectioned and stained with (cleaved) caspase-3 (Figure 3.16). As expected in the control group only a few positive cells appeared (Figure 3.16 A-B, E-F). In contrast, in the \textit{vsx2} over expressing group there was a significant increase in the number of Caspase-3+ cells (Figure 3.16 C-D, G-H). I performed injections of pSGH:\textit{vsx2} with a \textit{p53} morpholino and heat shocked them according to the same established protocol. The number of Caspase-3+ cells in the retina of heat-shocked embryos remained elevated (Figure 3.16 I), suggesting that this effect is primarily due to specific increase in the levels of \textit{vsx2} resulting in non-\textit{p53} mediated cell death.

\subsection{3.3.4 Bipolar cell and Müller glia differentiation is delayed in embryos over expressing \textit{vsx2}}

The preceding results have provided preliminary detail on how Vsx2 may be regulating retinal development, but it is still unclear whether the increases in cell death were affecting specific cell types or if it was an overall effect. Therefore, I examined what retinal cell types may be affected in \textit{vsx2} over expressed embryos. Plasmid injected, heat shocked, GFP positive embryos from each time point were cryosectioned and immunolabeled with retinal cell type markers: bipolar cells (pkcß1), retinal ganglion cell (RGC) layer (zn5), cone photoreceptors (zpr1), Müller glia (GS), and GABAergic amacrine neurons (GABA). Embryos heat shocked at 24 hpf and assessed at 48 hpf displayed the typical differentiation patterns of early cell types (Figure 3.17 A-H). Ganglion and amacrine cells were among those to differentiate normally, but at reduced numbers compared to control embryos at 48 hpf. It may be that by the time enough \textit{vsx2} is expressed after the heat shocks, those early cell types are already forming and are less affected. Those that were heat shocked at 48 hpf and viewed at 72 hpf had more obvious irregularities.
Figure 3.16: vsx2 over expression lead to an increase in cell death. pSGH:vsx2 injected embryos were heat shocked with 3 pulses at 37°C and fixed the following day. (A-H) Cryosections of 20μm were labeled with anti-caspase 3 and a significant increase was observed in those (I) embryos that were heat shocked between 24-48 hpf (plasmid injected alone p-value =0.007; plasmid and p53 morpholino injected p-value =0.002). Arrows indicate caspase-3 positive cells. These counts are based on a 45 cell area that was regulated by using the optic nerve as a marker (n=10, + s.d. of 5 replicates). Scale bar = 50 μm.
Figure 3.17: Embryos over expressing vsx2 led to a delay and disorganization of retina neuronal differentiation. Cryosections of 20µm of control and vsx2 injected heat shocked embryos were immunolabeled with the mentioned retinal cell type markers. (A-D) Early differentiated cells (RGC’s) did form but in a lower amount, while later derived cells (I-T) (bipolar and Müller glia), with the exception of (M-P) cone photoreceptors, were delayed and appeared disorganized (n=7). Scale bar = 50 µm.
The cone photoreceptors overall appeared to be normal, but conversely there were defects in bipolar cell and Müller glia development. On average the bipolar cells appeared disorganized and were less apparent, with some embryos having none at that time. Müller glia appeared disorganized with a delay in differentiating. Together these data indicate that Vsx2 is an important factor, not only in maintaining RPCs, but also in the proper timing of retinal cells exiting the cell cycle. Future experiments focused on detailed quantification will help to support these qualitative observations.
Chapter 4
Discussion

4.1 Summary of observations

In this study I investigated the role of FGF signaling as an upstream regulator of Vsx2 expression during early vertebrate retinal development, and as well the possible function of Vsx2 and Dmbx1a in regulating zebrafish RPCs through mutual antagonism. Corroborating with evidence from Tonou-Fujimori et al, (2002) I have also shown the presence of three receptors, FGFr1, FGFr2, and FGFr3, during peak stages of retinal neurogenesis and that FGF signaling is active at this time. FGFr2 appeared to be more prominent in the retina while FGFr1 was more dispersed in the retina and FGFr3 was found to be expressed at low levels in the retina and more highly in the lens. From there I was able to infer the pattern of active FGF signaling during early growth using the transgenic line \textit{Tg(dusp6:GFP)}, which demonstrated that FGF signaling appeared to be migrating in a dorsal to ventral progression. This could be in concert with a wave of proliferation or survival of RPCs (Picker and Brand, 2005). The role of FGF signaling in patterning and organizing the developing eye has been defined by a few loss of function assays (Hyer et al., 1998; Nguyen and Arnheiter, 2000; Picker and Brand, 2005). I further found that the loss of FGF signaling resulted in a decrease in \textit{vsx2} expression and an increase in \textit{dmbx1a} expression. This suggests that FGF signaling may be upstream of \textit{vsx2} and \textit{dmbx1a}.

Results found from knocking down \textit{vsx2} during early critical developmental stages not only suggest that this leads to microphthalmia, but also effects the formation of mature retinal cells. This is consistent with the ocular retardation phenotype seen in mice (Burmeister et al, 1996). Previous studies (Vitorino et al, 2009) examined these changes at a later time point at 80 hpf, and found that all retinal cell types generally form normally. This may have occurred because they
looked past a time when the \textit{vsx2} morpholino would remain effective. I decided to look earlier, between 24 hpf and 72 hpf, and validated the effect of the knockdown by Western blot. When \textit{vsx2} was knocked down the levels of \textit{dmbx1a} expression increased, suggesting that \textit{vsx2} may be repressing \textit{dmbx1a} expression. Many post-mitotic retinal cells in the neural retina express \textit{dmbx1a} and this early increase in expression could be impacting the cells exiting the cell cycle. The increase in BrdU and caspase 3 expressions suggests that RPC proliferation and maintenance are being affected by Vsx2, but likely in an indirect manner. Another interesting finding was the apparent delay and disorganization of differentiating retinal neurons in the Vsx2 knock down embryos because previous studies had shown that this was not the case (Vitorino et al, 2008). This indicates that cells were not prematurely exiting the cell cycle and differentiating.

The over expression of \textit{vsx2} was done at critical developmental time points and assessed soon afterwards. This resulted in a small eye phenotype with a decrease in \textit{dmbx1a} expression. I found that the levels of proliferating cells remained normal and the retina size decrease is more likely due from the resulting increase in cell death. Heat shocked embryos labeled with BrdU maintained relatively normal proliferative levels, indicating that the pool of actively dividing RPCs remained normal. Similar to the Vsx2 loss of function analysis, retinal cell maturation was delayed in all cell types by about 24 hours. The earlier differentiating cell types (amacrine, ganglion) arose normally, while the later differentiating ones (bipolar, Müller glia) were delayed by about 24 hours.

4.2 Cell cycle regulation: where does \textit{vsx2} and \textit{dmbx1a} fit in the broader scheme

Similar to the findings of Horsford et al (2005), where in mice FGF signaling was found to be upstream of \textit{Chx10}, I found that FGF signaling is likely upstream of \textit{vsx2} in zebrafish. This leads
me to conclude that this pathway may be conserved in vertebrates. It may be that FGF signaling organizes the retina by activating \textit{vsx2} and other neural retina genes. These may also act on other FGF genes, such as \textit{Fgf8}, to continue retina development and maintenance.

The formation of some retinal neurons in embryos with inhibited FGF signaling hints that other signaling pathways, such as Notch, may likely be functioning in parallel to FGF signaling. It has been suggested that Notch2 is expressed in the embryonic and post-natal retina of mice, and furthermore downstream genes of Notch were detected in \textit{Vsx2} expressing cells (Zhu et al, 2013). To identify if both pathways are needed simultaneously for proper retinal differentiation, a double knockdown of FGF signaling and Notch could be accomplished using the \textit{Tg(hsp70l:dnfgfr1-EGFP)} transgenic line and an inhibitor for the Notch pathway. For example \textit{N-[N-(3,5-difluorophenacetyl)-1-alanyll-S-phenylglycine \textit{t}-butyl ester} (DAPT) efficiently blocks the presenilin-\(\gamma\)-secretase complex, which prevents the activation of the Notch response (Crawford and Roelink, 2007). If both pathways are operating in parallel, then I would expect to find a greater delay in retinal neurogenesis when they are both knocked down.

When FGF signaling was knocked down I had found there was no significant change in proliferative levels, and this leads me to believe that during these later retinal developmental times FGF signaling may not be responsible for proliferation in RPCs. Although, when I performed the BrdU assay, I placed the embryos into the BrdU bath immediately after heat shocking, but when I over expressed \textit{vsx2} I waited 24 hours post heat shock before placing embryos into BrdU. This is because that was when I noticed changes in eye size, cell death, and differentiation. Thus, if I waited 24 hours post heat shock with the \textit{Tg(hsp70l:dnfgfr1-EGFP)} embryos before placing into BrdU, then there may be a relative change in proliferative levels.
The increase in cell death along with delayed neurogenesis leads me to speculate that during retinal growth FGF signaling may be not only signaling to these RPCs to promote survival, but also to exit the cell cycle and differentiate at the appropriate time in the retina. These findings help to further lay more groundwork in understanding how FGF signaling regulates neurodevelopment.

It has been reported that Vsx2 may act like a gate-keeper on retinal lineages, to ensure they arise at the correct time (Vitorino et al, 2009). Misexpression of vsx2 in previous studies (Clark et al, 2005; Vitorino et al, 2009) did not show detailed acute responses resulting from knocking down or over expression of vsx2. Thus here the knockdown and over expression of vsx2 were done at critical developmental time points and assessed soon after. In both cases there was a decrease in eye size, an increase in cell death, and delayed and disorganized retinal formation. A recent study on Pax6, a transcription factor necessary for RPCs and retina development, had a similar resulting pattern when Pax6 was over expressed. Manuel et al (2008) provided evidence that over expressing Pax6 in transgenic mice also led to a microphthalmic eye and retinal dysplasia. BrdU analysis indicated no change in proliferation in these transgenics. Another paper (Hsieh and Yang, 2009) established that the over expression and loss of Pax6 in chick embryo retinas had lead to microphthalmia in both cases that was a result of increased cell death. With this in mind, these results lead me to speculate that the balanced levels of vsx2 are very important in allowing for proper retina development but more specifically the survival of RPCs. Tipping the balance in either direction causes cells to enter an abortive mitosis state that ultimately leads to apoptosis. In chx10 deficient mice, the expression of mitf (an important transcription factor for RPE cell specification) increased and a possible antagonistic relationship was identified between chx10 and mitf (Horsford et al, 2005). Similar to mitf, dmbx1a has been found necessary for cell cycle exit and cell specification in zebrafish (Wong et al, 2010) and if vsx2 was a repressor of
then expression levels would decrease when vsx2 was over expressed. Since Vsx2 is also known for promoting/maintaining cells in a RPCs identity (Horsford et al, 2005), one would expect that over expression of vsx2 would likely promote cells to remain proliferative and that the retina would continue to grow. My results indicated that dmbx1a levels are reduced in the retina and that retinal cells were still exiting the cell cycle and differentiating. This leads me to believe that Vsx2 may require a co-factor in maintaining a RPC identity and that Vsx2 may also have dual roles in RPCs: (1) maintaining the RPC pool and (2) regulating the transition from a mitotic cell to a post-mitotic one. Examining changes in cell fate after vsx2 over expression provided some clues as to the role of Vsx2 in promoting the transition of progenitor cells to exit the cell cycle.

By 24 hpf, when I began the heat shocks, production of ganglion cells had begun and it could be that an earlier induction of the plasmid may be needed to show if these earlier neurons would have the same type of delay as seen in the later born cell types. Vsx2 has been found to be expressed in only a small subset of bipolar cells, while vsx1 expression appears to be on in the remaining bipolar cells (Vitorino et al, 2009). When I over expressed vsx2 I observed fewer bipolar cells that were also GFP positive. Since Vsx2 negatively regulates vsx1 (Clark et al, 2008), it may be that the over expression not only reduced vsx1 expression, but also reduced the production of most bipolar cells. Repression of vsx1, and subsequently the repression of most bipolar cell types, may help explain this phenomenon. It has been shown that RPCs located adjacent to differentiating neurons in the central retina are exposed to different cues from the RPCs located closer to the retina peripheries (Oron-Karni et al, 2008). Similar to that idea, it may also be that if some RPCs are unable to exit the cell cycle at the right time, then when they do exit the appropriate cues needed for each neuronal type may have passed or they too are delayed. Thus, the delay and disorganization of differentiating neurons may have resulted from a
misexpression of the required cues. My findings on the effects of vsx2 during retina development indicates a better understanding of how pertinent it is to have the appropriate levels of critical developmental factors strongly regulated.

Wong et al (2010), found when Dmbx1a was knocked down the cell cycle length increased dramatically. Therefore, an increase of dmbx1a expression may cause the cell cycle length to be shortened and more cells may be BrdU positive at the same time. This may be the case when there was a significant increase in dmbx1a in the Vsx2 morphants. These morphants had an increase in BrdU positive cells throughout the retina at 72 hpf when they should localized towards the peripheries. This increase in proliferative cells was also accompanied by an increase in cell death. Since Vsx2 is required for RPC maintenance, the loss of it may be leading to the instability of RPCs and ultimately abortive mitoses. In one of the first few studies to show a genetic interaction between a homeobox gene and a cell cycle regulatory protein, Vsx2 was implicated in cell cycle check point regulations (Green et al, 2003). It has been suggested that Chx10 in mice may be influencing p27Kip1 at a post-transcriptional level through interactions with cyclin D1 (Green et al, 2003) and it is entirely possible that Vsx2 may follow this pattern in zebrafish. Wong et al (2010) proposed that Dmbx1a may be involved in regulating cell cycle components, such as cyclin D1. It may be the increase in dmbx1a expression in the Vsx2 morphants could have resulted in a decrease in cyclin D1 and an increase in p27Kip1, leading cells to prematurely exit the cell cycle. Vsx2 may also potentially be a factor that is involved in the mitotic regulation of RPCs in zebrafish. In the Vsx2 knockdown embryos the increase in BrdU and caspase 3 positive cells may indicate that RPCs are entering-phase, but they are unable to complete mitosis and thus abort. Using phospho-histone H3 (pHH3), a marker of cells in M phase, may help indicate whether these same cells are initially able to enter M-phase. Checking the expression level of cyclin B may help indicate whether Vsx2 is involved in regulating the
G2/M transition. One other way cells may be entering an abortive state may be that RPCs are being signaled by other factors to proliferate normally, but without Vsx2 RPCs are unable to survive to get to the point of exiting the cell cycle, and they die. These Vsx2 results lead me to speculate that if RPCs exit too early then the right cues would not yet be present for each neuronal type. This is in contrast to previous findings by Vitorino et al (2009), where they did not notice any significant changes in neuronal maturation. I believe in their case this is likely due to the morpholino dilution over time because as mentioned earlier, by 72 hpf the effects of morpholinos begin to dissipate. They had analyzed those retinas at such a later time, 80 hpf, that the remaining neurons either differentiated normally and or/ the retina has already begun to recover.

### 4.3 Proposed model

When FGF signaling and Vsx2 function were knocked down, the levels of \textit{dmbx1a} increased and when \textit{vsx2} was over expressed the levels of \textit{dmbx1a} decreased. These findings imply that FGF signaling is upstream of both \textit{vsx2} and \textit{dmbx1a} and furthermore, it indicates that Vsx2 and Dmbx1a may be functioning antagonistically (Figure 4.1). Dmbx1a knock down embryos showed increased \textit{vsx2} expression, but when it was over expressed (Wong, 2013) the levels of \textit{vsx2} remained normal. This suggests that Vsx2 represses \textit{dmbx1a} and it also suggests that Dmbx1a represses \textit{vsx2} but it is not sufficient on its own. A threshold level of \textit{vsx2} may be needed to get RPCs to the point of exiting the cell cycle before other factors, like \textit{dmbx1a}, take over. During the transition from proliferative state to a post-mitotic state Vsx2 functions to maintain RPC identity and these results lead me to speculate that balanced levels of \textit{vsx2} are
Figure 4.1: Proposed model of RPC regulation during development. FGF signaling is upstream of both vsx2 and dmbx1. Vsx2 is a repressor of dmbx1a and Dmbx1a represses vsx2 but it is not sufficient on its own. During the transition from proliferative cell to a post-mitotic one, Vsx2 functions to maintain RPC identity and survival, and these results lead me to speculate that balanced levels of vsx2 are critical for proper RPC behaviour.

\[ \text{Fgf} \rightarrow \text{vsx2} \rightarrow \text{dmbx1} \rightarrow \text{Cell cycle exit} \]
critical for proper RPC behaviour. Thus, my model proposes that FGF signaling and Vsx2 are important for RPC survival during the transition to a post-mitotic state.

4.4 Testing the model in future studies

Future work should be aimed at further characterizing this relationship and understanding the molecular pathways involved. In order to gain better insight between these molecular mechanisms, we need to understand whether these genes are really mutually antagonistic. To address this question I would first perform a double knockdown of Vsx2 and Dmbx1a in order to determine if any phenotypic recovery of eye size, cell death, and/or differentiation can be obtained. I would next clone dmbx1a into the pSGH heat shock inducible plasmid to create a dmbx1a over expression construct and I would then inject it alone and also co-inject embryos with the dmbx1a and the vsx2 over expression construct. This would allow me to over express both genes at later developmental time points. If I heat shock just dmbx1a over expressing embryos early at 24 hpf, when the retina consists of RPCs, I would expect those cells to prematurely exit the cell cycle. These cells may have a disorganized retina with early retinal differentiation, which can be identified with immunohistochemical labeling.

One key aspect that I would like to understand is whether these two genes are directly interacting. One method that can be employed for this would be to perform a chromatin-immunoprecipitation combined with qPCR or sequencing (ChIP-qPCR; ChIP-seq) to possibly identify if either of these two proteins directly binds the others promoter. Here, specific DNA sites in direct physical contact with transcription factors and other proteins can be isolated (Ho et al, 2011). This process will enrich specific cross-linked DNA-protein complexes using the Vsx2 or Dmbx1a antibody. Once these molecules are pulled down the DNA can be purified and amplified. If a complex is found, I would still not be able to infer whether it is a direct effect.
Since both these genes are homeobox genes I would expect them to directly interact, but Vsx2 could bind to another promoter to activate it which would go on to repress dmbx1.

Misexpression of Vsx2 led to two different outcomes but in both cases there was an increase in cell death and a delay in neuronal differentiation. In order to clarify what may be occurring here there are two experiments I propose. First of all, to further facilitate identifying these effects I would like to perform blastomere transplantations from either Vsx2 knock down embryos or vsx2 over expressing embryos to wildtype embryos. Here I would co-inject and label donor cells that have either vsx2 RNA, or vsx2 morpholino, and a fluorescent compound, such as rhodamine-dextran, or perform these injections in transgenic donor embryos [e.g. Tg(beta-actin:GFP)]. Assessing these cells at a later time point, such as 30 hpf would allow me to determine if these transplanted cells remain normal or continue with their irregular behaviour and organization autonomously. This would provide insightful information on (1) whether only these donor cells show changes in gene expression and if neighbouring cells are also affected; and (2) if the increase in cell death is cell specific to the donor cells or does it become an overall effect of vsx2 misexpression in the retina. Second of all, it would be ideal to generate a conditional Vsx2 knock out transgenic line in order to ubiquitously remove Vsx2 from the retina and verify my results. But at this moment there is no reliable method to produce a conditional knock out transgenic line in zebrafish, but I could create a vsx2 over expressing transgenic line. Co-injecting embryos with the over expression construct and the meganuclease enzyme could allow me to develop a heat shock inducible transgenic. To confirm the results I obtained from my morpholino experiments I could instead produce a Vsx2 mutant line using the transcription activator-like effector nuclease (TALENs) method and asses the changes in gene levels. Here double-stranded DNA breaks are induced at the Vsx2 locus by these synthetic nucleases called TALENs, which have sequence-specific heterodimer endonucleases (Dahlem et al, 2012). Host repair results to the generation of
insertion and deletion mutations at the targeted locus. A Vsx2 mutant line would allow me to monitor \textit{dmbx1a} expression pattern changes during early development. As well, I would be able to do an assessment of retinal size and composition with a Vsx2 mutant line and possibly observe if a functioning visual system does eventually develop.

One of the hallmarks of this project was the similar results that arose from \textit{vsx2} over expression and Vsx2 knock out experiments. In both cases a significantly higher amount of caspase 3 activated cell death occurred. It would be useful to construct a fluorescent reporter line of activated cleaved caspase 3 and thus, we would be able to utilize live imaging to reconstruct the spatial and temporal patterns induced by apoptosis in cells when \textit{Vsx2} is being manipulated. This would let us know whether the onset of apoptosis occurs as \textit{vsx2} is being over expressed, or if it is a later effect. A second surprising outcome was the increase in proliferative retinal cells when Vsx2 was knocked down, which we believe is through abortive mitosis. If this is the case then there could be a decrease in pH3 positive cells if they die before entering M-phase.

Alternatively, there could be an accompanying increase in pH3 positive cells, indicating that these cells die after completing M-phase. In line with this idea it would be informative to check if there are changes in cyclin D, which governs the G1-S process, or cyclin B, that is essential for the G2-M transition. Firstly I would perform \textit{in situ} hybridization with RNA probes for \textit{cyclinD1} and \textit{cyclinB1} on \textit{vsx2} over expressed and Vsx2 knock down embryos. I would expect in the Vsx2 knock down embryos to have an increase in \textit{cyclinD1} expression and a decrease in \textit{cyclinB1} expression. This may help explain the increase of cells in S-phase that will eventually enter an abortive mitosis cycle. To complement this I could use chemical inhibitors of CDKs. Experiments such as these, and the ones mentioned earlier, will have the potential to unravel the complex role of Vsx2 and identify \textit{dmbx1a} as a target for repression.
4.5 Conclusion

In conclusion, the ultimate goal of this study was to understand the role of Vsx2 during retinal development and identify the relationship between Vsx2 and Dmbx1a. The data obtained has helped further validate that FGF signaling as an upstream regulator of Vsx2 in the zebrafish retina and that this pathway does play a critical role during these early neuro-developmental stages. This appears to be a conserved feature among vertebrates (Horsford et al, 2005). The Vsx2 over and under expression data has provided insight into a unique situation where tipping the balance in either direction produces a similar outcome, indicating that Vsx2 levels are stringently controlled in RPCs. A threshold or spatial level of vsx2 may be needed to get RPCs to the point of exiting the cell cycle before other factors, such as dmbx1a, take over. Vsx2 may be needed for progenitor cells to transition from a mitotic state to a post-mitotic one. When vsx2 was over expressed the levels of dmbx1a decreased and when Vsx2 was knocked down dmbx1a expression increased. The loss of Dmbx1a lead to an increase in vsx2 expression but preliminary dmbx1a over expression analysis showed that vsx2 expression levels were not affected. This suggests that Vsx2 and Dmbx1a may be antagonistic and that Vsx2 is likely necessary and sufficient in repressing dmbx1a. This also suggests that Dmbx1a is a necessary repressor but is not sufficient on its own. This leads me to speculate that is more likely an indirect antagonistic interaction.

These results demonstrate the importance of understanding the mechanisms that regulate RPCs. By deciphering the roles and pathways of important retinal genes, such as Vsx2, we can identify possible targets to turn it on or off for diseases such as retinoblastoma. If the loss of Vsx2 does in fact lead to abortive mitosis, then down the road this may be one way to reduce tumour size. My research may provide new ways of manipulating RPCs using gene-based therapies to simulate
proper growth of the retina in those with retinal disorders. This information can be also used in understanding how to slow retinal cancer progression or direct the differentiation of RPCs in the context of transplant therapies.
References


