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Research Report

Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina

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ABSTRACT

Chx10/Vsx2 and *Vsx1* are the only Paired-like CVC (Prd-L:CVC) homeobox genes in the mouse genome. Both are expressed in the retina and have important but distinct roles in retinal development. Mutations in *Chx10/Vsx2* cause reduced retinal progenitor cell (RPC) proliferation and an absence of bipolar cells, while mutations in *Vsx1* impair differentiation of cone bipolar cells. Given their structural similarities and importance in retinal development, we sought to determine if a regulatory interaction exists between these genes and whether inactivation of both genes blocks initiation of retinal development. We found that Chx10/Vsx2 binds to a specific sequence in the Vsx1 5′-intergenic region and represses the activity of a luciferase reporter under the control of the Vsx1 promoter. This is consistent with our observation that there is an inverse relationship between the levels of Chx10/Vsx2 and Vsx1 immunostaining within the bipolar cell class. Furthermore, *Vsx1* mRNA is upregulated in the RPCs of *Chx10/Vsx2* deficient mice and zebrafish embryos injected with a *chx10/vsx2* morpholino. In mice deficient for both *Chx10/Vsx2* and *Vsx1* and zebrafish embryos co-injected with *chx10/Vsx2* and *vsx1* morpholinos, the changes in embryonic retinal development and marker expression are similar in magnitude to embryos with *Chx10/Vsx2* loss of function only. From these studies, we propose that *Vsx1* is a direct target of Chx10/Vsx2-mediated transcriptional repression. Although *Vsx1* mRNA is upregulated in Chx10/Vsx2 deficient RPCs, Vsx1 does not genetically compensate for loss of Chx10/Vsx2, demonstrating that Prd-L:CVC genes, although important, are not absolutely required to initiate retinal development.

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Abbreviations: Prd-L:CVC, Paired-like:CVC; HD, homeodomain; RPCs, retinal progenitor cells; NBL, neuroblast layer; MO, morpholino; *orJ*, *ocular retardation J*; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; DCL, differentiated cell layer; RPE, retinal pigmented epithelium; EOM, extraocular mesenchyme; OAR motif, orthopedia/aristaless/rax motif; RV motif, rinx/vsx1 motif; BS, Black Swiss

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1. Introduction

A multitude of homeobox genes is required for retinal development, from the initial patterning events of the optic vesicle to the completion of terminal differentiation (reviewed in Chow and Lang, 2001; Dyer, 2003; Fuhrmann et al., 2000a; Levine and Green, 2004; Lupo et al., 2000; see also De Melo et al., 2003). In many cases, paralogous homeobox genes retain close relationships as indicated by similarities in expression patterns and mutant phenotypes. It is not uncommon, however, for these genes to develop cross-regulatory networks and diverge in function (for examples, see Czerny et al., 1999; Plouhinec et al., 2005). Identifying these relationships is essential for sorting out the complex transcription factor networks driving retinal development.

Prd-L:CVC proteins comprise a subgroup of homeodomain (HD) proteins based on two distinguishing structural characteristics: the presence of a Paired-like HD with a glutamine at position 50 (Prd-L, Q_{50}; Galliot et al., 1999), and a region of approximately 60 amino acids of unknown function positioned immediately C-terminal to the HD. This region of extended conservation is referred to as the CVC domain after the four genes from which it was originally identified, ceh-10 (*C. elegans*; Svendsen and McGhee, 1995), and *vsx1* and *vsx2* (goldfish; Levine et al., 1994), and *Chx10* (mouse; Liu et al., 1994). Whereas *ceh-10* is the only identified Prd-L:CVC gene in *C. elegans*, essentially all vertebrate genomes examined to date have two Prd-L:CVC genes that parse into two ortholog clusters, Chx10/*Vsx2*-like and Vsx1-like, named after their founding members. It is likely that duplication of a single Prd-L:CVC gene occurred prior to the vertebrate radiation to give rise to the Chx10/Vsx2-like and Vsx1-like paralogs (Chow et al., 2001; Passini et al., 1998).

Two conserved features of Prd-L:CVC genes during development are their expression in interneurons and importance in differentiation. *ceh-10* is expressed in a restricted set of sensory interneurons and has a role in their fate specification (Altun-Gultekin et al., 2001; Svendsen and McGhee, 1995). In vertebrates, Chx10/Vsx2 and Vsx1 are expressed in interneuron populations in the spinal cord, hindbrain, and in retinal bipolar cells. Both genes are required for bipolar cell differentiation, although in different ways (see below). Biochemical studies also suggest a high degree of functional overlap between Prd-L:CVC proteins. Chx10 and Vsx1 proteins bind with high affinity to the same DNA sequence (Ferda Percin et al., 2000; Hayashi et al., 2000; Heon et al., 2002) and overexpression of human CHX10 or VSX1 protein represses transcription from the same heterologous reporter construct (Dorval et al., 2005). All Prd-L:CVC proteins contain an octapeptide motif that functions as a nuclear export signal (Knauer et al., 2005) and a nuclear localization signal (Kurtzman and Schechter, 2001). These motifs may work together to regulate the sub-cellular localization of Prd-L:CVC proteins.

Despite their close structural and biochemical relationships, Chx10 and Vsx1 differ in several ways. Sequences N-terminal of the HD and C-terminal of the CVC domain vary considerably and contain motifs not shared across the paralog groups such as the OAR motif in Chx10 orthologs (named for *othopedia/aristaless/Rax*; Furukawa et al., 1997) and the RV motif in Vsx1 orthologs (named for *Rinx/Vsx1*; Hayashi et al., 2000). Chx10 and Vsx1 also differ in their expression patterns. Chx10 expression (mRNA and protein) initiates during optic cup formation in presumptive retinal progenitor cells (RPCs) and remains expressed until RPCs exit the cell cycle and in the adult, Chx10 is expressed in bipolar cells and a subset of Muller glia (reviewed in Levine and Green, 2004; see also Rowan and Cepko, 2004). Vsx1 mRNA is detected during embryonic retinal development in several species (Chen and Cepko, 2000; D'Autilia et al., 2006; Passini et al., 1997, 1998). In contrast, Vsx1 protein is first detected late in retinal development and is expressed in differentiating bipolar cells (Chow et al., 2001; Decembrini et al., 2006; Ohtoshi et al., 2001; this study). In the adult, Vsx1 is expressed in a subset of cone bipolar cells.

Evidence for distinct contributions of *Chx10* and *Vsx1* to retinal biology is illustrated by their mutant phenotypes. Mutations in Chx10 cause microphthalmia in humans (Bar-Yosef et al., 2004; Ferda Percin et al., 2000) and mice (Burmeister et al., 1996), and antisense-*chx10* RNA injected into zebrafish embryos causes a small eye phenotype (Barabino et al., 1997). Studies in *ocular retardation J* (*orJ*) mice, which carry a spontaneously-derived nonsense mutation in the HD (Y176stop) of Chx10 ($Chx10^{orJ}$; Burmeister et al., 1996; Theiler et al., 1976), show that in addition to a lack of bipolar cells, the $Chx10^{orJ}$ homozygote ($Chx10^{orJ/orJ}$) retina exhibits a profound decrease in RPC proliferation, a propensity to transdifferentiate along a pigmentation pathway, delays in embryonic neurogenesis, persistent neurogenesis in the adult retina, and an enrichment of adult ciliary epithelium derived retinal stem cells (Bone-Larson et al., 2000; Burmeister et al., 1996; Coles et al., 2006; Dhomen et al., 2006; Green et al., 2003; Horsford et al., 2005; Livne-Bar et al., 2006; Rowan et al., 2004; Rutherford et al., 2004).

On the other hand, retinal abnormalities associated with Vsx1 mutations are considerably less severe and more restricted. Microphthalmia is not observed and retinal histology appears normal in Vsx1 knockout mice (Chow et al., 2004; Ohtoshi et al., 2004). However, humans and mice with Vsx1 mutations have abnormal photopic electroretinogram (ERG) profiles associated with cone bipolar cell dysfunction (Heon et al., 2002; Mintz-Hittner et al., 2004; Valleix et al., 2006). Consistent with this, a restricted set of cone bipolar cells fails to complete their differentiation even though the full cohort of bipolar cells appear to be specified in Vsx1 knockout mice (Chow et al., 2004; Ohtoshi et al., 2004). Additionally, Vsx1 mutations in humans are also associated with corneal dystrophies such as keratoconus, possibly because of a role in corneal wound repair that is independent from its retinal function (Barbaro et al., 2006).

Since Chx10 and Vsx1 have several features in common but also mediate distinct aspects of retinal development, we set out to determine if a regulatory interaction exists between Chx10 and Vsx1 to control their expression. We also wanted to determine if Vsx1 promotes what remains of histogenesis in the Chx10 deficient retina. Data presented here provide evidence that Chx10 negatively regulates Vsx1 expression by direct transcriptional control. However, once relieved from this regulation, Vsx1 does not fill in for Chx10 during embryonic retinal development.

2. Results

2.1. *Vsx1 is a candidate direct target of transcriptional repression by Chx10*

We generated a polyclonal antiserum against a peptide corresponding to a unique sequence in the C-terminal variable region of mouse Vsx1. The Vsx1 antis serum detects nuclei positioned in the INL in a pattern consistent with previous reports of Vsx1 expression in bipolar cells of the adult wild type retina, whereas retinal sections from adult Vsx1 null mice (*Vsx1*$^{\tau lacz/\tau lacz}$) are devoid of any staining Figs. 1A, B). Western blots from retinal lysates P14 and older show that the antiserum detects a band of approximately 39 kDa in the wild type retina, which is consistent with the predicted size of the endogenous Vsx1 protein, and this band is absent in lysates from the *Vsx1*$^{\tau lacz/\tau lacz}$ retina (Fig. 1C). A second band at 64 kDa is detected in both lysates and is therefore assumed to not originate from the *Vsx1* locus. In addition, embryonic retinal extracts contain a band of the same size as Vsx1 that persists in the *Vsx1*$^{\tau lacz/\tau lacz}$ lysates (data not shown). Importantly, immunohistochemical detection of these 'non-Vsx1' proteins in cryosections is not apparent. These observations demonstrate the utilities and limitations of our antiserum for analyzing Vsx1 expression in the mouse retina.

Using double-label indirect-immunofluorescence, we directly compared the expression patterns of Chx10 and Vsx1 in the postnatal mouse retina (Figs. 1D–I). As expected, both proteins were localized to cells in the outer half of the inner nuclear layer (INL; Figs. 1D–F). However, cells showing the brightest staining with each antibody segregated into distinct populations as indicated by the lack of yellow nuclei in the merged images (Figs. 1F, I). Closer examination (Figs. 1G–I) revealed that most cells with Chx10 staining had little or no Vsx1 staining (closed arrowheads); some cells with low Chx10 staining had high Vsx1 staining (arrows); and occasional cells with low Chx10 staining also had low Vsx1 staining (open arrowheads). These data show that Chx10 and Vsx1 can be co-expressed in the same cells but not in a manner in which both proteins are expressed at their highest levels.

Prior to bipolar cell differentiation, Chx10 expression predominates. Chx10 expression is activated to relatively high levels in RPCs at the earliest stages of optic cup formation

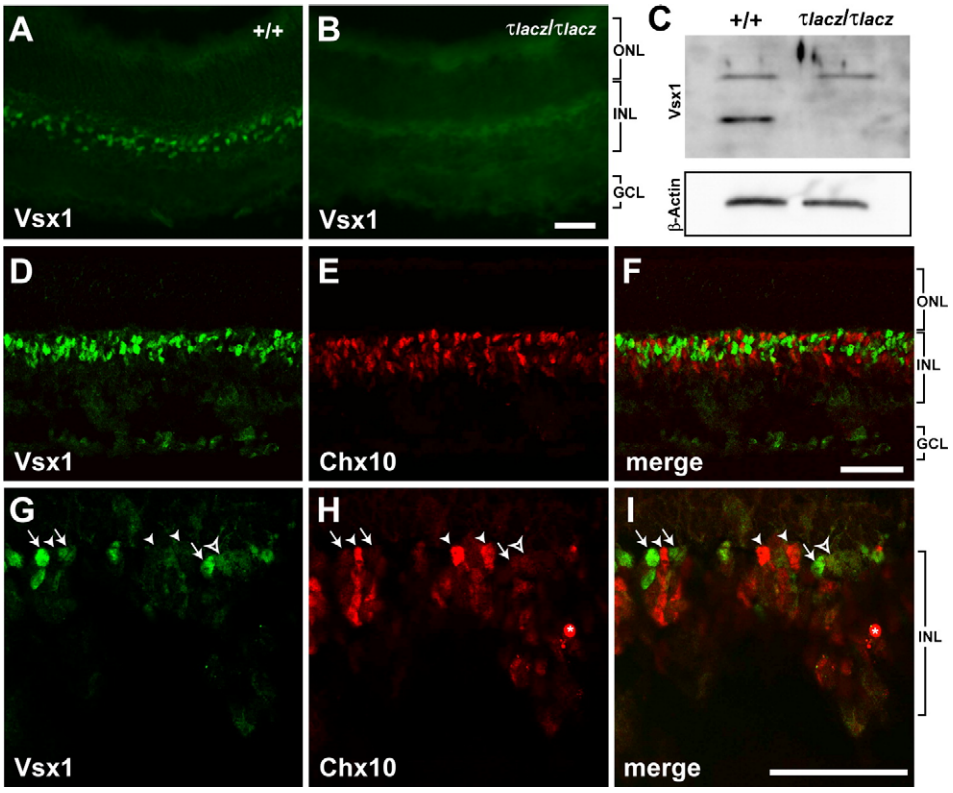

Fig. 1 – Endogenous expression pattern of Vsx1 and Chx10 in postnatal retina. (A–C) The specificity of Vsx1 antibody was determined by immunohistochemistry (A, B) and by western blot analysis (C) on retinal samples from wild type (+/+) and *Vsx1*$^{\tau lacz/\tau lacz}$ (*τlacz/τlacz*) mice at P14 or older. The nuclear pattern of Vsx1 staining in the INL is observed in wild type (A) and absent in *Vsx1*$^{\tau lacz/\tau lacz}$ retina (B). (C) A band of the expected size (approximately 39 kDa) is present in wild type and absent in *Vsx1*$^{\tau lacz/\tau lacz}$ P14 retinal lysates. Another band of approximately 64 kDa is observed in both lysates. β-Actin was used as a loading control. (D–I) Confocal images showing Vsx1 (D, G) and Chx10 (E, H) expression patterns in P8 wild type retina. Merged images are shown in F and I. Arrows point to examples of cells with high Vsx1 and low Chx10 expression. Arrowheads point to examples of cells with low Vsx1 and high Chx10 expression. The open arrowhead points to a cell that co-expresses both proteins at low levels. Asterisks in H and I indicate a staining artifact. Scale bars: 40 μm. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

whereas Vsx1 is expressed at low levels, if at all. Two exceptions are the Vsx1 orthologues *Chx10-1* in chick and *vsx1* in *Xenopus*, whose transcripts are robustly detected in RPCs (Chen and Cepko, 2000; D'Autilia et al., 2006). Even so, the sum of the expression data suggests a regulatory mechanism exists to keep Vsx1 expression at low levels in Chx10 expressing cells. Consistent with this, *Vsx1* mRNA is upregulated in the newborn *Chx10*$^{orJ/orJ}$ retina (Fig. 2A).

Since Chx10 can act as a transcription repressor, we asked whether Vsx1 is a target of Chx10. Based on a collection of known Chx10 binding sequences, Dorval and colleagues defined the following sequence as a consensus for Chx10 binding: PyTAATTPuPu (Py, pyrimidine; Pu, purine; Dorval et al., 2006). A scan of genomic DNA associated with the Vsx1 locus revealed two potential sites: one at −576 nt (TTAATTAG) and another at −1275 nt (CTAATTGG) relative to the Vsx1 transcriptional start site as predicted by ensembl (www.ensembl.org). Chx10 preferentially binds at or near the site positioned at −576 as determined by chromatin immunoprecipitation (ChIP) using native neonatal retinal lysates (Fig. 2B). Consistent with this, electrophoretic mobility shift assays (EMSA) show that in vitro translated Chx10 binds to the ^{32}P-labeled Vsx1 probe containing the site at −576 (Vsx1$_{wt}$; Fig. 2C). The binding of Chx10 to this probe is diminished with unlabeled Vsx1$_{wt}$ oligonucleotide in excess, but not by a variant containing a mutated Chx10 binding site (Vsx1$_{mut}$). These data show that the Chx10 binding is dependent on the sequence conforming to the consensus. Addition of Chx10 antibody to the binding reaction resulted in a supershifted band, which indicates that the band observed in these assays is due to the association of Chx10 protein with the Vsx1$_{wt}$ probe.

To further explore a potential transcriptional regulation of Vsx1 by Chx10, a Chx10 expression construct was co-transfected with the pGL3E luciferase reporter construct into HEK293 cells (Fig. 3). Approximately 2.6 kb of Vsx1 genomic DNA containing the two putative Chx10 binding sites (−1275 and −576), the Vsx1 promoter, and 0.4 kb of exon 1 was cloned directly upstream of the luciferase cDNA. Consistent with its role as a transcriptional repressor, we found that Chx10 significantly inhibits luciferase activity (p-value<0.0001). We also tested a variant of Chx10 containing an arginine to glutamine replacement at residue 200 (Chx10^{R200Q}). This mutation resides in the DNA binding helix of the HD (residue 53) and eliminates high affinity binding to Chx10 consensus sites and causes microphthalmia when inherited in a homozygous manner in humans (Ferda Percin et al., 2000). Chx10^{R200Q} does not repress expression from the construct containing both Chx10 binding sites (pGL3E-Vsx1^{wt}; p-value 0.076). Additionally, the level of reporter activity is significantly higher in the presence of Chx10^{R200Q} compared to Chx10 (p-value<0.0001).

To determine the importance of the Chx10 consensus sites for transcription from the Vsx1 promoter, we tested reporter constructs with mutations in the sites at −576 and −1275, either alone or in combination. Chx10 still inhibits luciferase activity

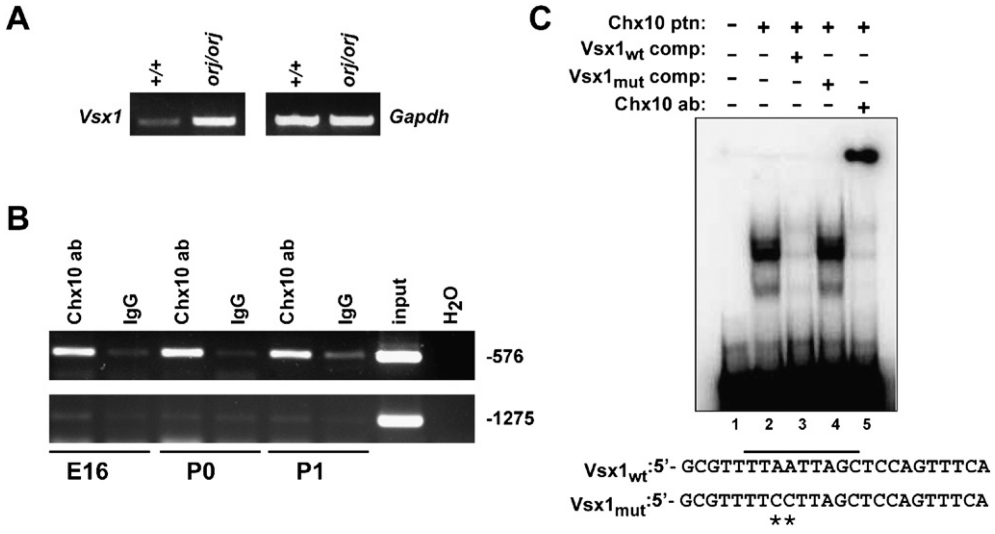

Fig. 2 – *Vsx1* mRNA levels are increased in the *Chx10*^{orJ} retina and Chx10 binds to a site upstream of the Vsx1 gene in vivo and in vitro. (A) RT-PCR amplification products using wild type and *Chx10*$^{orJ/orJ}$ P0 retinal RNA and primers for *Vsx1* and *Gapdh*. (B) Chromatin immunoprecipitation (ChIP) assay. Native chromatin-protein lysates were isolated from E16, P0, and P1 wild type retinas and immunoprecipitated with a sheep polyclonal Chx10 antibody (Chx10 ab). The precipitated chromatin was amplified by PCR using two non-overlapping primer sets that flank sequences conforming to the Chx10 consensus at positions −576 and −1275 relative to the Vsx1 transcription start site. The input lane shows amplification from chromatin not subjected to immunoprecipitation. Sheep IgG or H_2O were used as negative controls. (C) Electrophoretic mobility shift assays (EMSA). Lane 1 shows the pattern of $^{[32]}$P-labeled oligonucleotide probe containing the Chx10 consensus site at −576 (Vsx1$_{wt}$) without addition of Chx10 protein. Lane 2 shows the resulting band pattern when in vitro translated Chx10 protein is mixed with the $^{[32]}$P-labeled Vsx1$_{wt}$ probe in a binding reaction. Lane 3 and 4 show results of competition assays when unlabeled Vsx1 oligonucleotides are added in excess to the binding reaction (Vsx1$_{wt}$ comp (Lane 3) and Vsx1$_{mut}$ comp (Lane 4)). Addition of Chx10 ab to the binding reaction (lane 5) results in a supershifted band. The line over the sequences shown indicates the position of the Chx10 binding site sequence and asterisks indicate the nucleotide changes in the Vsx1$_{mut}$ oligonucleotide.

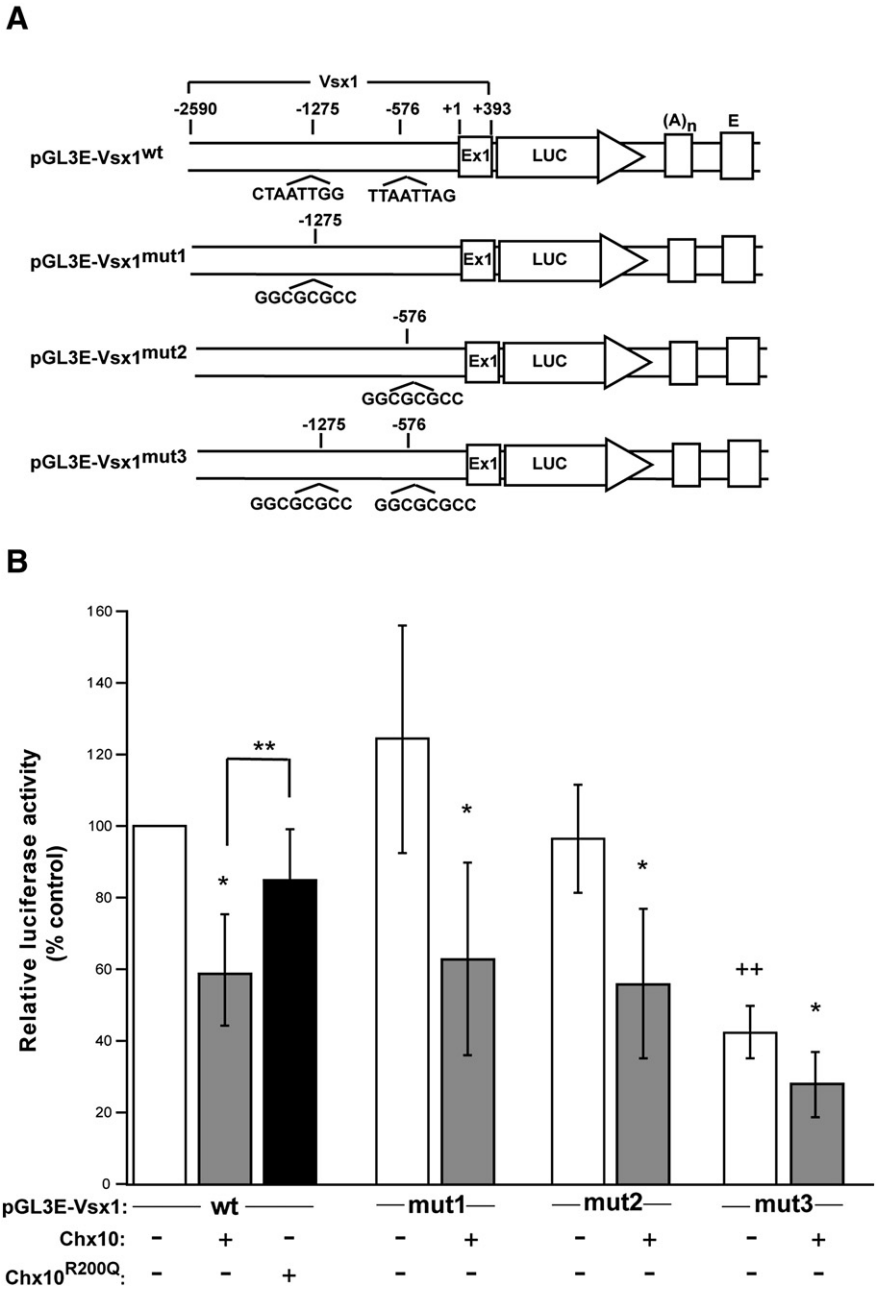

Fig. 3 – Chx10 expression inhibits luciferase activity in a DNA-binding dependent manner and Chx10 binding sites near the Vsx1 promoter are required for high luciferase activity in HEK293 cells. (A) Schematic diagrams of reporter constructs with Vsx1 promoters that contain both Chx10 binding sites (pGL3E-Vsx1^{wt}) or with site specific mutations in these sites (pGL3E-Vsx1^{mut1}, mutated at −1275; pGL3E-Vsx1^{mut2}, mutated at −576; pGL3E-Vsx1^{mut3}, mutated at both sites). Region bracketed corresponds to Vsx1 genomic DNA and numbering is relative to predicted Vsx1 transcription start site. (B) HEK293 cells were transfected with each reporter construct, pRL-TK to monitor transfection efficiency, and either an empty expression vector (pCMV; white bars), pCMV-Chx10 (gray bars), or pCMV-Chx10^{R200Q} (black bar). Relative luciferase activity is normalized to the pGL3E-Vsx1^{wt} co-transfected with empty expression vector (the first white bar) and is arbitrarily assigned as 100% activity. The standard deviation for each test condition is shown. Statistical significances (p-values) were tested by two-way repeated measures ANOVA and Tukey tests for multiple comparisons with values transformed by log2. $^{*}p<0.001$ (p-value of each gray bar compared to each respective white bar); $^{**}p=0.0001$; $^{++}p<0.001$ (p-value of pGL3E-Vsx1^{mut3} (last white bar) compared to other reporter constructs (white bars)). Abbreviations: Ex1, exon 1 of Vsx1; LUC, luciferase cDNA; (A)n, SV40 late poly(A) signal; E, SV40 enhancer.

in the context of the single site mutations (mut1 and mut2; p-value<0.001) and inhibition is not completely eliminated when both sites are mutated (mut3). Interestingly, the basal level of luciferase activity is significantly lower with the mut3 reporter than observed for the other promoters (wt, mut1, and mut2; p-value<0.001, the same value for all three comparisons).

Although Chx10 continues to inhibit luciferase activity in the absence of both Chx10 binding sites (see Section 3.1), our results indicate that both of these sites are necessary for high levels of transcription from the Vsx1 promoter and adjacent regions, and the sum of our observations support the model that Chx10 is a direct negative regulator of Vsx1 transcription.

2.2. *Vsx1 fails to compensate for the loss of Chx10 function during embryonic retinal development*

Although histogenesis is severely affected by the absence of Chx10, several aspects of retinal development are still evident. This suggests Chx10 is not absolutely required and Vsx1 may partially compensate for the Chx10 deficiency. The upregulation of *Vsx1* mRNAs in the *Chx10*^{orJ} retina (Fig. 2A) supports this idea. Therefore, we crossed *Chx10*^{orJ} mice with *Vsx1*$^{\tau lacz}$ mice to produce compound mutants and compared the resultant phenotypes.

During normal retinal development in mice, histogenesis is well underway by E14.5. RPC proliferation and differentiation into postmitotic neurons are robust as indicated by the expression patterns of PCNA and CycD1 in proliferating RPCs found in the neuroblast layer (NBL; Figs. 4A, C) and Tuj1 in differentiating neurons that are scattered through the NBL

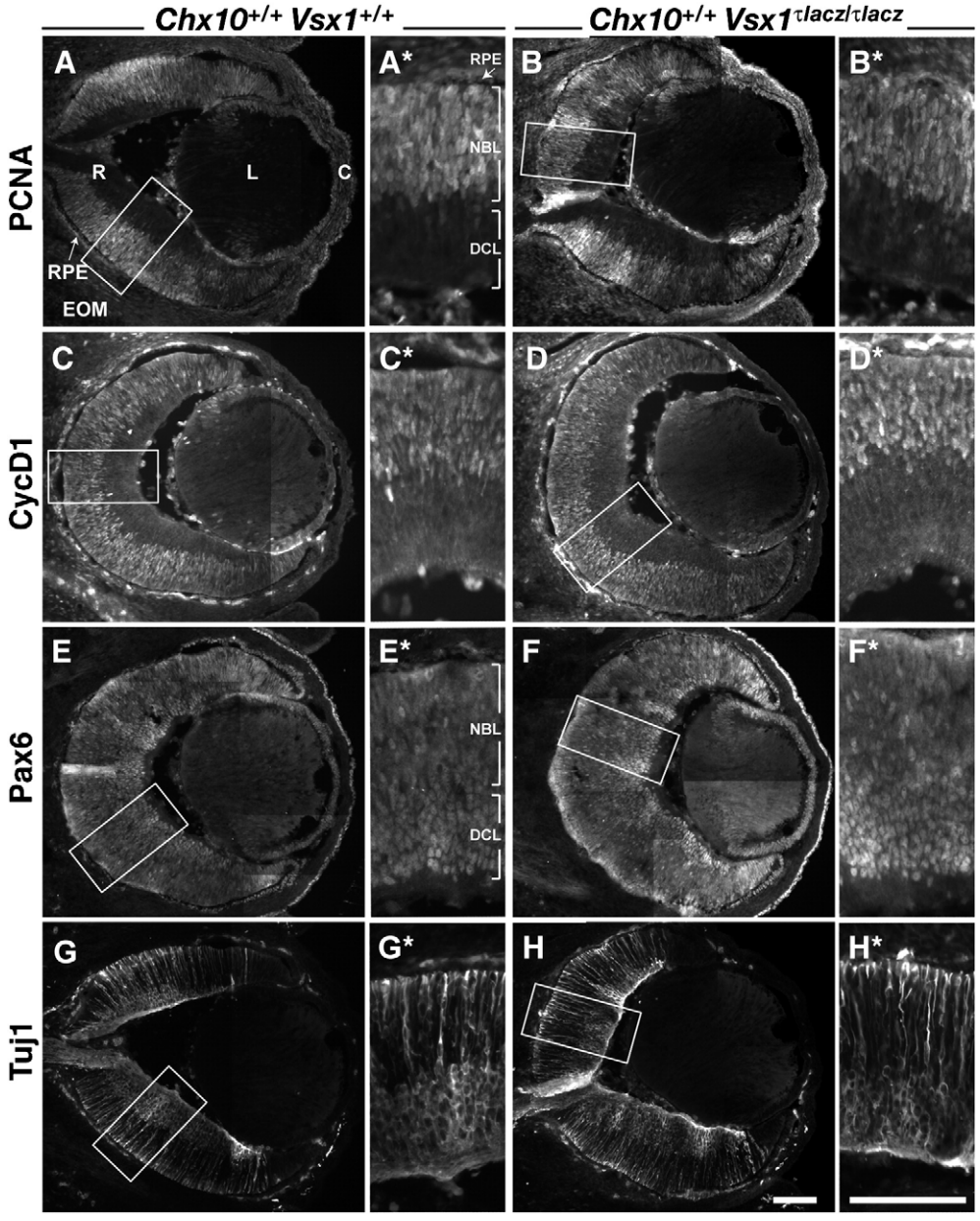

Fig. 4 – Immunohistochemical analysis of E14.5 eyes from wild type and *Vsx1*$^{\tau lacz/\tau lacz}$ mice. Expression patterns of PCNA (A, B), CycD1 (C, D), Pax6 (E, F), and Tuj1 (G, H) proteins in wild type (A, C, E, G) and *Vsx1*$^{\tau lacz/\tau lacz}$ (B, D, F, H) eyes. All panels with an * show the images in the boxes from each corresponding panel. These images were rotated such that the apical surface of the retina is pointed down. Scale bars: 100 μm. Abbreviations: RPE, retinal pigmented epithelium; R, retina; EOM, extraocular mesenchyme; L, lens; C, cornea; NBL, neuroblast layer; DCL: differentiated cell layer.

and accumulating in the nascent ganglion cell layer and presumptive INL (collectively defined here as differentiated cell layer, DCL; Fig. 4G). Pax6, which is important for optic vesicle development, RPC proliferation, and maintenance of the multipotential-state in RPCs (reviewed in Ashery-Padan and Gruss, 2001), is expressed initially in RPCs and subsequently shifts to differentiating neurons. At E14.5, Pax6 is detected in most if not all cells, and appears to be detected at higher levels in differentiated cells compared to RPCs (Fig. 4E). Compared to the wild type retina, overall ocular morphology and expression of PCNA, CycD1, Pax6, and Tuj1 are unaffected in the *Vsx1*$^{\tau lacz/\tau lacz}$ retina (Figs. 4B, D, F, H). Consistent with these observations, Chx10 expression appears normal in the *Vsx1*$^{\tau lacz/\tau lacz}$ retina (data not shown). In the *Chx10*$^{orJ/orJ}$ mouse, overall eye size is reduced, but retinal expression of PCNA is still abundant (Fig. 5A). CycD1 is also expressed but in a more dispersed pattern compared to wild type and *Vsx1*$^{\tau lacz/\tau lacz}$ retinas (Fig. 5C). Tuj1 expression is more centrally restricted (Fig. 5G) consistent with a delay in neurogenesis (Bone-Larson et al., 2000; Rutherford et al., 2004; Green et al., submitted). Pax6 is widely expressed in a pattern consistent with RPCs, and this could be due to developmental delay (Fig. 5E). Compared to the *Chx10*$^{orJ/orJ}$ mouse, ocular morphology and the expression patterns of the markers analyzed are similar in the *Chx10*$^{orJ/orJ}$, *Vsx1*$^{\tau lacz/\tau lacz}$ compound mutant (Figs. 5B, D, F, H). Although retinal development appears enhanced in the compound mutant, the phenotype is within the range of variation observed for Chx10orJ/orJ single mutants. From these obser-

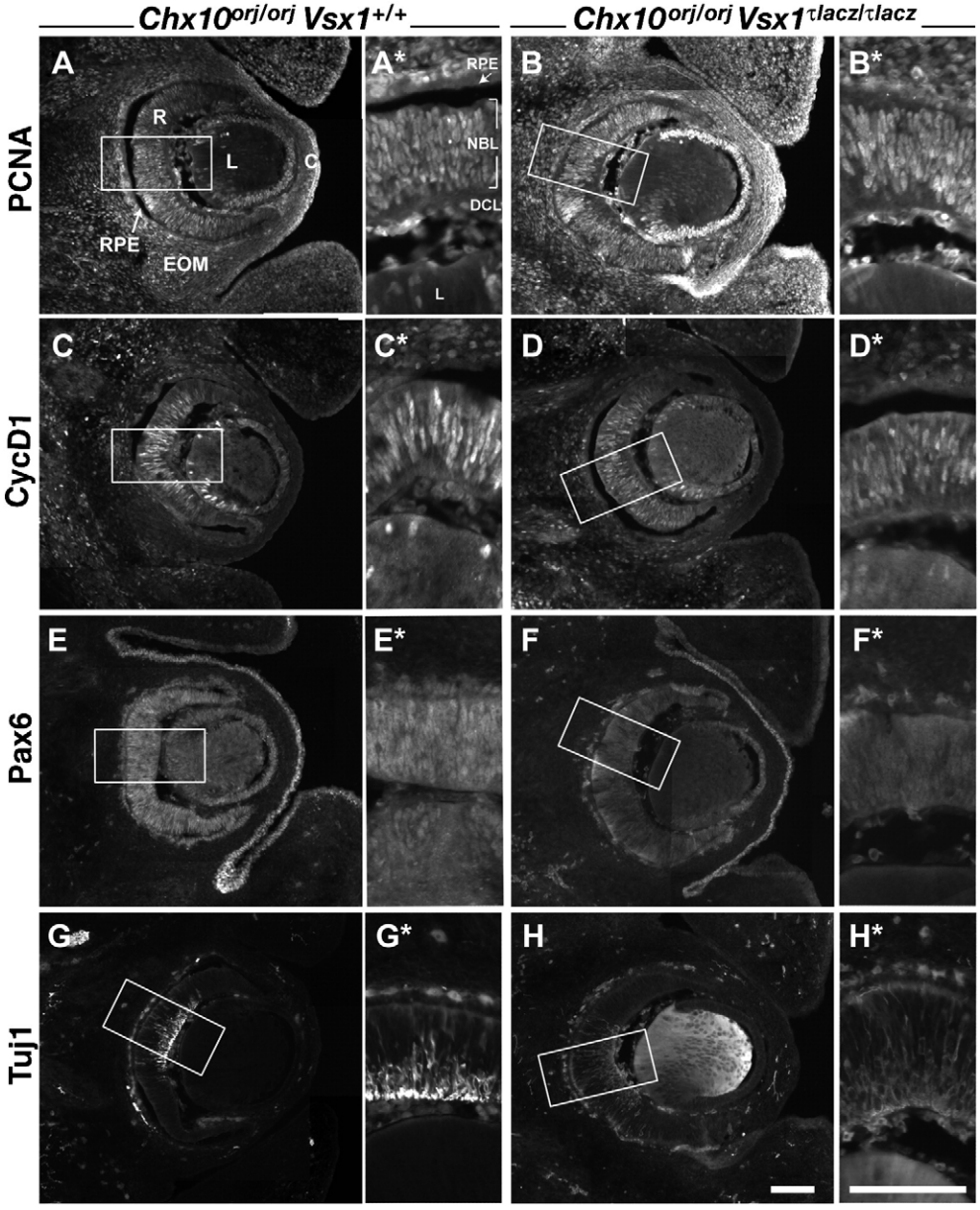

Fig. 5 – Immunohistochemical analysis of E14.5 eyes from *Chx10*$^{orJ/orJ}$ and *Chx10*$^{orJ/orJ}$, *Vsx1*$^{\tau lacz/\tau lacz}$ mice. Expression patterns of PCNA (A, B), CycD1 (C, D), Pax6 (E, F), and Tuj1 (G, H) proteins in *Chx10*$^{orJ/orJ}$ (A, C, E, G) and *Chx10*$^{orJ/orJ}$, *Vsx1*$^{\tau lacz/\tau lacz}$ (B, D, F, H) eyes. All panels with an * show the images in the boxes from each corresponding panel. These images were rotated such that the apical surface of the retina is pointed down. Scale bars: 100 μm.

vations, we conclude that Vsx1 is dispensable in early retinal development and does not compensate for the loss of Chx10.

Marker expression was examined at P0 to determine whether Vsx1 compensates for the loss of Chx10 in neonatal RPCs (Fig. 6). The expression patterns of PCNA, CycD1, and Tuj1 in the $Vsx1^{\tau lacz/\tau lacz}$ retina are similar to wild type (Figs. 6A, B, E, F, I, J), and the expression of these markers in the $Chx10^{orJ/orJ}$, $Vsx1^{\tau lacz/\tau lacz}$ compound mutant retina is similar to the $Chx10^{orJ/orJ}$ single mutant (Figs. 6C, D, G, H, K, L). We also examined the expression of Brn3b, which marks a major fraction of the retinal ganglion cell population (RGCs; Xiang et al., 1993). Whereas Brn3b-positive cells are observed in all genotypes (Figs. 6M–P), the $Chx10^{orJ/orJ}$ and $Chx10^{orJ/orJ}$, $Vsx1^{\tau lacz/\tau lacz}$ mutants showed obvious dispersion of these cells into the NBL. The cause of this dispersion is not known, but possibilities include the delay in neurogenesis, migration defects, or lamination defects. Importantly, these observations extend our finding that Vsx1 is largely dispensable for retinal development through birth, either in the presence or absence of Chx10.

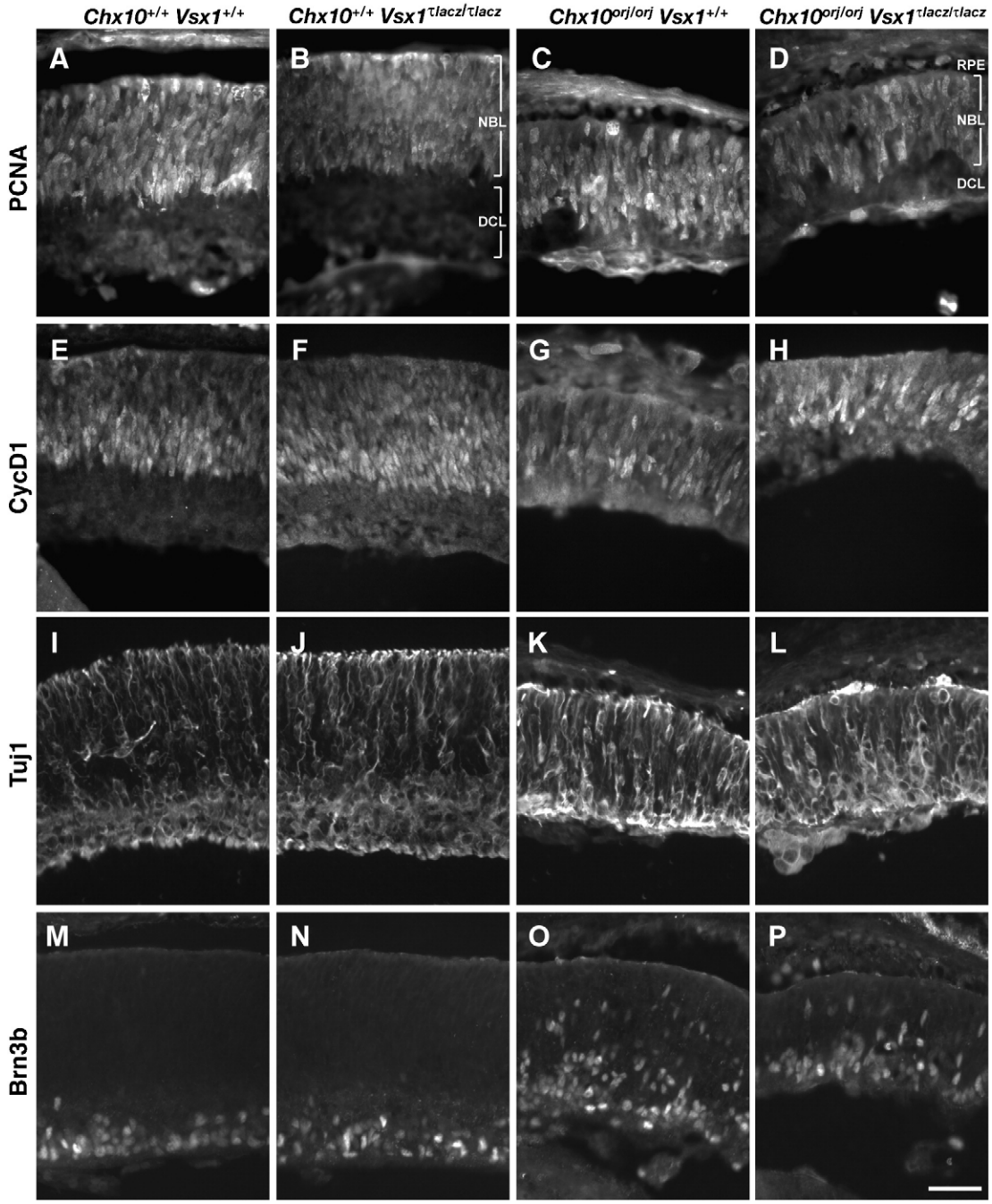

Fig. 6 – Immunohistochemical analysis of P0 eyes. Expression patterns of PCNA (A–D), CycD1 (E–H), Tuj1 (I–L), and Brn3b (M–P) in wild type (A, E, I, M), $Vsx1^{\tau lacz/\tau lacz}$ (B, F, J, N), $Chx10^{orJ/orJ}$ (C, G, K, O), and $Chx10^{orJ/orJ}$, $Vsx1^{\tau lacz/\tau lacz}$ (D, H, L, P) eyes. Scale bar: 40 μm.

To determine whether this is a common feature of vertebrate retinal development, we performed similar experiments in zebrafish (Fig. 7). Translation and splice blocking morpholino oligonucleotides (MO) targeted against *chx10* and *vsx1*, respectively, were injected into one-cell stage zebrafish and embryos were examined at 24 hpf, a stage of development when RPC proliferation is robust, but neurogenesis has not yet begun. Fig. 7A shows that the *vsx1* MO blocks splicing of *vsx1* mRNA in a dose dependent manner. To assess the effects of the MOs on retinal development, the expression patterns of *vsx1*, *cycD1*, and *pax6a* were examined by in situ hybridization (Figs. 7B–M) and the sum of our observations on *vsx1* and *cycD1* expression are shown in Figs. 7N and O. Chx10 knockdown led to an increase in *vsx1* expression specifically in the eye, as well as to a decrease in eye size (Figs. 7B, C). An eye-specific decrease in *cycD1* expression was also observed in *chx10* morphant embryos (Figs. 7F, G). Vsx1 knockdown had little or no effect on the expression of *vsx1* (Fig. 7D), *cycD1* (Fig.

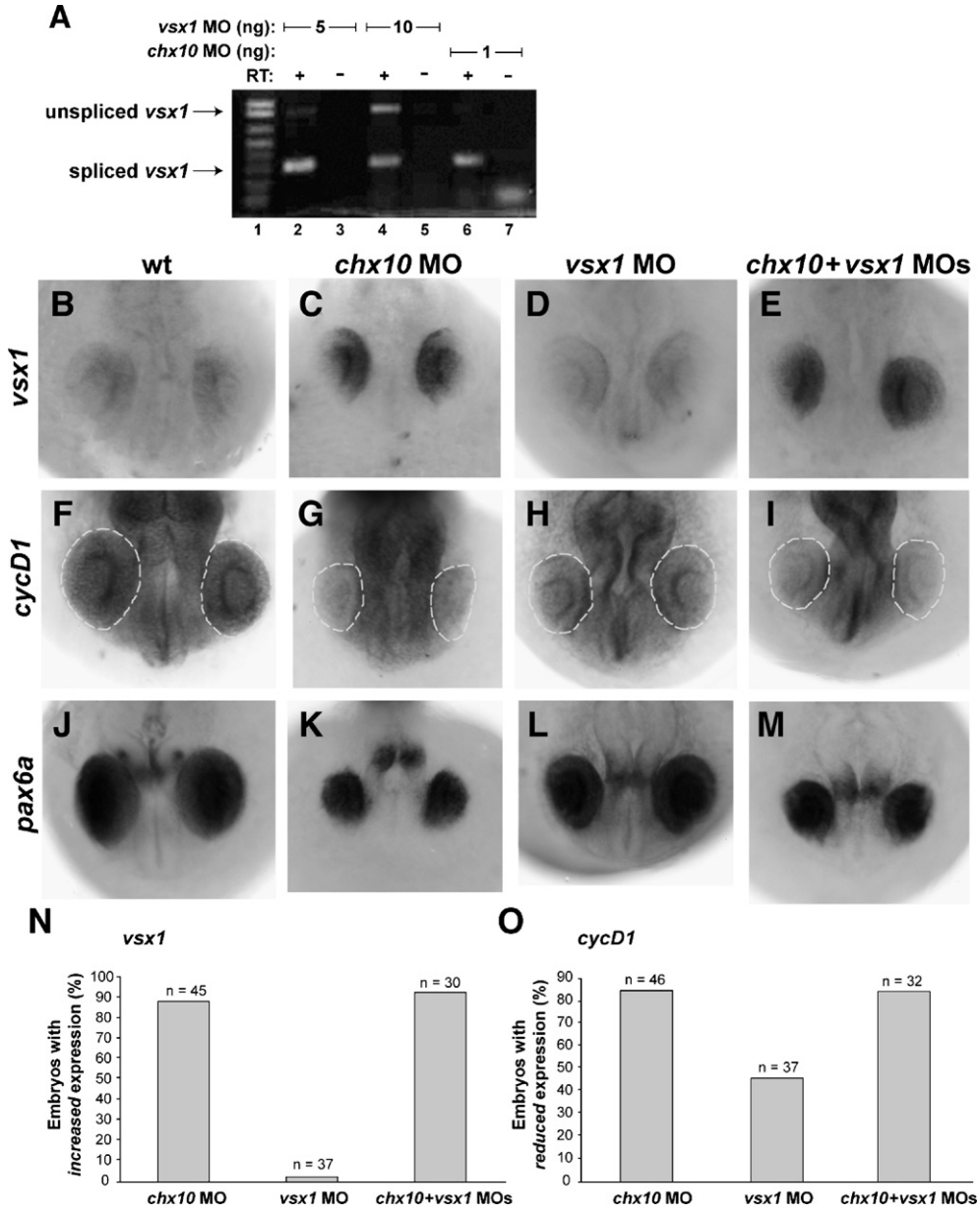

Fig. 7 – Vsx1 does not compensate for loss of Chx10 in zebrafish. (A) RT-PCR showing accumulation of unspliced *vsx1* mRNA in a dose-dependent manner following injection of a splice-blocking morpholino (MO) against vsx1. Lane 1 shows molecular weight marker. Lanes 2, 4, and 6 show spliced and unspliced Vsx1 RNA derived from zebrafish embryos injected with the indicated morpholinos. Lanes 3, 5, and 7 represent no template controls (reverse transcriptase omitted). (B–M) In situ hybridizations for *vsx1* (B–E), *cycD1* (F–I), and *pax6a* (J–M) in 24 h embryos that were uninjected (B, F, J), injected with 1.5 ng *chx10* MO (C, G, K), 15 ng *vsx1* MO (D, H, L), or co-injected with 1.5 ng *chx10* and 15 ng *vsx1* Mos (E, I, M). Eyes in F–I are within the dotted lines. (M, O) Quantification of relative changes in expression levels for vsx1 and cycD1 mRNAs among morphant embryos (*n*, number of embryos analyzed).

7H) or *chx10* (not shown). pax6a expression was unaffected relative to eye size in all morphant embryos (Figs. 7J–M), indicating that eye identity was not perturbed. If Vsx1 compensates for loss of *Chx10*, then it would be expected that *cycD1* expression and eye size would be more severely affected in *chx10*, *vsx1* double morphant embryos compared to *chx10* single morphants. We did not observe such phenotypes (Figs. 7G, I), suggesting that, as in the mouse, Vsx1 does not compensate for loss of Chx10 in zebrafish eye development.

3. Discussion

3.1. *Regulation of Vsx1 by Chx10*

In this study, we present evidence supporting a model in which Chx10 directly regulates expression of Vsx1 mRNA through a mechanism of transcription repression. Chx10 preferentially binds at or in close proximity to the Chx10 consensus sequence that is positioned close to the transcription start site (−576) in vivo and requires this site for binding in vitro. Chx10 inhibits luciferase activity when transcription is under the control of the Vsx1 promoter, which is consistent with the proposed role of Chx10 as a transcription repressor. We found that $Chx10^{R200Q}$ does not significantly repress reporter activity, suggesting that the ability of Chx10 to repress transcription is largely dependent on high affinity binding to sequences conforming to the consensus. These findings are in agreement with a previous report showing that mutation of another residue in the DNA binding helix of Chx10 (N51A) also reduces the efficiency of transcriptional repression by Chx10 (Dorval et al., 2005, 2006).

Interestingly, mutation of both consensus sites in the Vsx1 genomic sequence (mut3) causes a significant drop in basal reporter activity. Since Chx10 is a more effective repressor when it has high affinity for its consensus site, it is possible that Chx10 inhibits transcription by competing with activators for the same sites.

We found that Chx10 still inhibits luciferase activity in the absence of the two consensus sites (mut3). Although it is tempting to propose a mechanism of Chx10 repression that is independent of its binding to sequences fitting the consensus, two additional Chx10 consensus sites are present in the SV40 enhancer region of the reporter construct and it is likely that the repressive effect of Chx10 on the mut3 reporter is mediated through these sites. Currently, the simplest model is that Chx10 inhibits Vsx1 transcription by a mechanism that depends on binding to its consensus sequence, and the site at −567 is sufficient for Chx10 binding and negative regulation of Vsx1 transcription in vivo.

The expression patterns of Chx10 and Vsx1 during retinal development provide further evidence of a regulatory interaction. When Chx10 expression is high in RPCs, Vsx1 is low or nonexistent. Additionally, Vsx1 mRNA levels are increased in the Chx10 deficient retina of mice and zebrafish. Interestingly, the chick Vsx1 ortholog, *Chx10-1*, is expressed at high levels, and *Chx10* appears to be expressed at relatively lower levels in RPCs (Chen and Cepko, 2000). A simple scenario to explain this apparent reversal in expression profile is that *Chx10* regulation changed in the developing chick retina such that *Chx10* levels decreased, thereby allowing *Vsx1* levels to increase. *Xenopus vsx1* is also expressed at high levels in RPCs, but the status of Chx10 expression is not known (D'Autilia et al., 2006).

In the postnatal and mature retina of all vertebrates examined, *Chx10* and *Vsx1* mRNAs are expressed in bipolar cells, but their patterns are not in perfect correspondence. Where examined, *Chx10* is expressed at the earliest stages of bipolar cell differentiation and remains expressed in rod bipolar cells and a large subset of cone bipolar cells. Vsx1 expression is subsequently activated and is restricted to a subset of cone bipolar cells (off-cone). We found that the Chx10 and Vsx1 proteins can be co-expressed in the same cells of the postnatal retina, but in a complementary fashion: cells expressing high levels of Chx10 express Vsx1 at low levels, and vice versa, and these relationships are not time dependent (R.L. Chow, ms in prep). While these observations support the model that Chx10 antagonizes Vsx1 expression, they also leave open the possibility that Vsx1 could antagonize Chx10 expression as well.

Further insight into the transcriptional control of Chx10 and Vsx1 is gained from expression studies in Chx10 and Vsx1 single mutant mice. *Chx10* mRNA expression in $Chx10^{orJ}$ mice is not altered, suggesting that Chx10 does not regulate its own expression (Rowan and Cepko, 2004; Rowan et al., 2004; Rutherford et al., 2004; Green et al., submitted). Furthermore, the upregulation of *Vsx1* mRNA in the developing $Chx10^{orJ}$ retina does not appear to have a significant effect on *Chx10* expression as well, either because Vsx1 does not regulate Chx10 levels or its expression is not at sufficient levels to exert an effect. In contrast, Vsx1 may negatively regulate its own expression in bipolar cells (Chow et al., 2004; Ohtoshi et al., 2004).

The sum of these findings suggests a hierarchical transcriptional network shown in Fig. 8. In this model, Chx10 expression is not regulated either by Chx10 or Vsx1 in RPCs. However, RPCs are competent to express Vsx1 but are inhibited from doing so by cross-regulation from Chx10. Activation and/or maintenance of Chx10 expression may be mediated by interaction with the surface ectoderm (Hyer et al., 1998; Nguyen and Arnheiter, 2000) and/or exclusion of the extraocular mesenchyme (Fuhrmann et al., 2000b). Transcriptional regulation of Chx10 appears to be complex and involve multiple enhancers (Rowan and Cepko, 2004, 2005) and candidate pathways and factors for promoting Chx10 expression include Fgf signaling, Bmp signaling, Hes activity, Mab21l2, and Rx3 (Gotoh et al., 2004; Hatakeyama et al., 2004; Horsford et al., 2005; Loosli et al., 2001; Murali et al., 2005; Nguyen and Arnheiter, 2000; Winkler et al., 2000; Yamada et al., 2004). However, the mechanisms that account for their regulation of Chx10 are not known nor is it known whether any of these candidates act in a direct manner. Pou3f2 (Brn2) was recently identified as a candidate direct regulator of Chx10 expression in late RPCs and early, differentiating bipolar cells (Rowan and Cepko, 2005). In this case, however, it is not known if Pou3f2 is sufficient or required for Chx10 expression. As bipolar cells mature, Chx10 is downregulated in a subset of cone bipolar cells and Vsx1 is upregulated, albeit in a controlled manner because of Vsx1-mediated autoregulation (Chow et al., 2001, 2004; Ohtoshi et al., 2004). The factors that maintain Chx10 expression in mature bipolar cells are

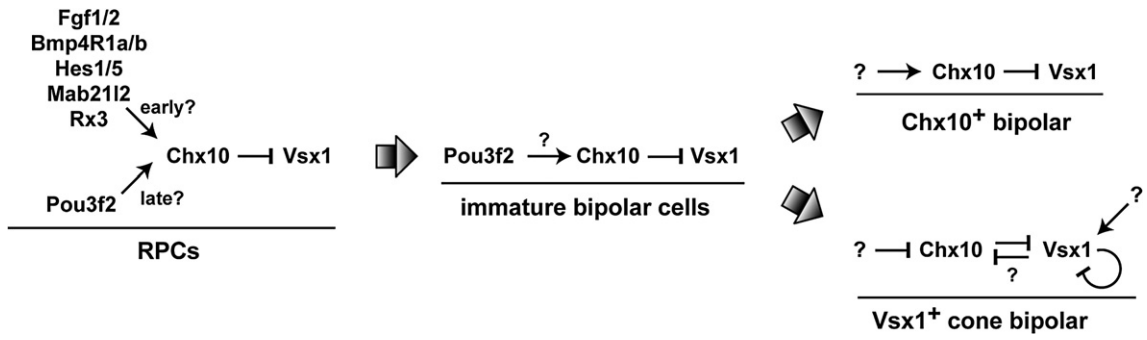

Fig. 8 – Proposed regulatory network for Chx10 and Vsx1 in RPCs and bipolar cells. Large arrows represent the indicated developmental transitions. See Section 3.2 for details.

not known, nor is it known whether negative regulators of Chx10 or positive regulators of Vsx1 feed into the proposed network.

3.2. *Prd-L:CVC genes in embryonic retinal development*

The increase in *Vsx1* mRNA in the developing *Chx10*$^{orJ/orJ}$ mouse retina and zebrafish *chx10*-morphant retina prompted us to test whether Vsx1 has an effect on retinal development prior to bipolar cell differentiation. Since the HD and CVC domain of Chx10 and Vsx1 are similar and both proteins act as transcription repressors with overlapping DNA binding specificities, we wanted to know if the residual histogenesis occurring in the *Chx10*$^{orJ/orJ}$ retina is dependent on Vsx1. In mice and zebrafish deficient for both Chx10 and Vsx1, we did not observe any significant changes in retinal development compared to Chx10 deficient animals. Additionally, Vsx1 deficiency did not have any obvious effect on retinal development compared to wild type animals. From these observations, we conclude that Vsx1 does not contribute to embryonic retinal development and that the residual histogenesis occurring in the Chx10 deficient retina is not dependent on Prd-L:CVC genes in general since Chx10 and Vsx1 are the only known Prd-L:CVC genes in mouse and zebrafish.

These findings begin to provide insight into how Chx10 fits into the hierarchy of transcription factors important for early retinal development. Homeobox genes such as Six3, Rx, Pax6, and Lhx2 are expressed in the developing eye field and are necessary for optic cup formation in mice or humans. In each case, genetic inactivation results in anophthalmia, which is a complete failure of eye development (reviewed in Fitzpatrick and van Heyningen, 2005; Graw, 2003). In addition, these genes induce ectopic eyes in *Xenopus* when expressed together and in combination with *six6/optx2, tll*, and *ET* (Zuber et al., 2003). As a result of these features, these genes have been termed the *eye field transcription factors* (EFTFs) and they are thought to form a network analogous to the *retinal determination gene network* (RDGN) in *Drosophila* (reviewed in Hanson, 2001). How Chx10 fits into this paradigm is not known. At present, it is the earliest expressed and most specific marker of the neural retina domain and its inactivation produces a severe and highly penetrant microphthalmia. Since genetic inactivation of both Chx10 and Vsx1 does not push the eye phenotype towards anophthalmia, it is now clear that Vsx1 is not compensating for loss of Chx10 in early eye development and Chx10 has a role distinct from, but possibly downstream of EFTFs such as Six3, Rx, Pax6, and Lhx2.

3.3. *Functional overlap between Chx10 and Vsx1*

We did not observe a change of phenotype in *Chx10*$^{orJ/orJ}$, *Vsx1*$^{\tau lacz/\tau lacz}$ compound mutants and *chx10, vsx1* double morphants compared to the *Chx10*$^{orJ/orJ}$ single mutants and chx10 single morphants, respectively. While this suggests that Chx10 and Vsx1 do not have overlapping functions such as shared transcriptional targets, it is premature to conclude this since we do not know if Vsx1 protein is upregulated to sufficient levels in RPCs lacking Chx10. Even in *Xenopus*, where *vsx1* mRNA is highly expressed in RPCs (D'Autilia et al., 2006), post-transcriptional regulation is thought to keep Vsx1 protein levels at a minimum until bipolar cell formation (Decembrini et al., 2006). Thus, it is still not known if the functional overlap between Chx10 and Vsx1 shown in biochemical studies has significance in retinal development.

Chx10 overexpression inhibits photoreceptor differentiation and several phototransduction genes are candidate direct transcriptional targets of negative regulation by Chx10 (Dorval et al., 2006; Livne-Bar et al., 2006; Toy et al., 2002). These findings combined with the shared DNA binding characteristics and repressive activities of Chx10 and Vsx1 led to the proposal that these genes could function in bipolar cells to prevent inappropriate expression of photoreceptor genes (Dorval et al., 2006). While possible, this scenario is not likely to fully explain how Chx10 and Vsx1 function in bipolar cells. In the Vsx1 deficient mouse retina, several markers of bipolar cells are downregulated in cells that normally express Vsx1 (Chow et al., 2004; Ohtoshi et al., 2004). However, Chx10 remains expressed in the same cells. This suggests that Chx10 is not able to compensate for Vsx1 in cone bipolar cells and that Chx10 and Vsx1 have distinct functions, possibly by targeting different genes for regulation.

Although more work is needed to understand more fully the functional relationships between Chx10 and Vsx1, our work shows that a regulatory relationship has evolved between these paralogs that could allow for overlap of some functions, but at the same time, also allow for functional divergence that contributes to the complex networks driving retinal development.

4. Experimental procedures

4.1. Mouse strains

Chx10 null mice (*Chx10*^{orJ}) were purchased from Jackson Labs. Vsx1 null mice (*Vsx1*$^{\tau acz}$; Chow et al., 2004) were mated to Chx10orJ mice to produce compound mutants. PCR based genotyping was done as previously described (Burmeister et al., 1996; Chow et al., 2004). The *Chx10*^{orJ} and *Vsx1*$^{\tau lacz}$ alleles originated from 129 genetic backgrounds and the mice analyzed for this study are mixed hybrids. For all animals used in this study, efforts were made to minimize pain and discomfort during procedures and in preparing for euthanasia.

4.2. Vsx1 polyclonal antibody production

15-Amino acid peptide, HLKKGANKDEDGPER (position 301 to 315), was synthesized and conjugated to KLH at the University of Utah peptide synthesis core facility. Polyclonal antibodies were prepared commercially (Harlan Bioproducts, Inc. Indianapolis IN). IgG fraction was purified using SulfoLink Kit according to manufacturer's instructions (Pierce, Rockford, IL).

4.3. Immunohistology

Mouse embryonic heads, postnatal whole eyes or isolated retinas were dissected in Hanks buffered saline solution (HBSS) and immediately fixed in 4% neutral-buffered formalin (PFA) for various lengths of time varying from 20 min at room temperature to overnight at 4oC. Following fixation, tissue was washed twice with PBS, then replaced with sucrose gradient and finally embedded in OCT (Sakura Finetek, Torrance, CA). 12 μm thick cryosections were used for immunostaining. The primary antibodies used were: rabbit anti-Vsx1 (1:200; this study); sheep anti-Chx10 (1:300; Exalpha Biologicals, Boston, MA); rabbit anti-CycD1 (1:400; Lab Vision, Fremont, CA); rabbit anti-Pax6 (1:300; Mastick et al., 1997); mouse monoclonal anti-PCNA (clone PC10; 1:500; Dako, Denmark); goat anti-Brn3b (1:50; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-neuronal class III tubulin (Tuj1; 1:4000; Covance, Richmond, CA). Primary antibodies were followed with species-specific secondary antibodies conjugated to either Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen/Molecular Probes, Eugene, OR).

4.4. Western blots

Mouse retinas were dissected in HBSS and stored at −80 °C. Frozen retinas were resuspended in a lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1% Triton X-100) complemented with protease inhibitors (Complete mini tablets, Roche, Indianapolis, IN) and sonicated on ice. Protein concentration was determined by a BCA protein assay (Pierce Biotechnology, Rockford, IL). 20 μg of protein lysates were subsequently run on 12% SDS PAGE, then transferred to nitrocellulose membranes. Blots were incubated with antibodies diluted in blocking buffer (5% dry milk, 0.04% Tween-20, TBS pH 7.6). Primary antibodies included rabbit anti-vsx1, mouse anti-β-Actin (Chemicon, Temecula, CA). Detection was performed using SuperSignal West Dura (Pierce Biotechnology, Rockford, IL) and documented by ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA).

4.5. RT-PCR of mouse RNA

Total RNA was extracted from P0 Black Swiss (BS) wild type and *Chx10*^{orJ} retinas by using Trizol (Invitrogen, Carlsbad, CA). The concentration of total RNA was assessed by spectrophotometry and adjusted to the same level among samples. First-strand cDNA was synthesized by using M-MuLV Reverse Transcriptase and Oligo(dT)$_{18}$ (Fermentas, Hanover, MD). RT-PCR was carried out in a 30-μl reaction mixture. RT-PCR for a housekeeping gene (Gapdh) was used as a control (22 cycles) to normalize the concentration of the cDNA samples (primer sequences available on request). RT-PCR for *Vsx1* was performed with different amounts of cDNA (4, 2 or 1 μl) to estimate an appropriate number of cycles to amplify in the linear range (data not shown). The primers for *Vsx1* were: 5′-GGATGAGGATGGACCTGAGA-3′ and 5′-AGGTGTTTGTCCAGCTTTGG-3′. PCR conditions were: 94 °C 30 s, 55 °C 30 s and 72 °C 30 s for 30–33 cycles. The size of the product is 208 bp.

4.6. Chromatin immunoprecipitation (ChIP)

Retinas from E16.0, P0 or P1 BS wild-type animals were dissected and cut into small pieces and then fixed in 1% formaldehyde for 15 min at room temperature. Following crosslinking, tissues were washed and sonicated to shear the DNA to lengths between 200 and 1000 bp. Sonicated supernatants were precleared with salmon sperm DNA (Invitrogen)//Protein G agarose beads (Roche, Indianapolis, IN), and incubated overnight at 4 °C with 5 μl sheep anti-Chx10 antibody (Exalpha Biologicals, Boston, MA) or sheep IgG (Sigma, St. Louis, MO). The chromatin–antibody complex was collected with salmon sperm DNA/Protein G agarose. Crosslinking was reversed with 5M NaCl at 65 °C for 4 h. DNA was purified by QIAquick PCR purification kit (QIAGEN, Valencia, CA). 4 μl IP DNA was employed for PCR amplification for potential Chx10 binding sites in Vsx1 promoter (−576 and −1275). The following primers were used: Vsx1 (−567): 5′-AGTTGTAAGCTGCCCTGTGG-3′ and 5′-CCTGACTGGCACGTAGGAAT-3′. Vsx1 (−1275): 5′-GCCGAAATTTGGATTTACGA-3′ and 5′-TGGATGAGTGGGGAGAAATC-3′.

4.7. Electrophoretic mobility shift assays (EMSA)

pET26b-chx10 plasmids were in vitro translated using rabbit reticulocyte lysates (Promega, Madison, WI). 2 pmol single-strand probes were end-labeled by T4 kinase (Invitrogen) with [γ-32P]ATP (MP Biologicals, Solon, OH) and were purified using BioSpin6 column (Bio-Rad, Hercules, CA). The probes were boiled for 5 min at 95 °C and cooled to room temperature for 3 h to form double-stranded probes. Probe sequences were: Vsx1 wild type (Vsx1$_{wt}$), top strand: 5′-GCGTTTTAATTAGCTCCAGTTTCA; Vsx1 mutant (Vsx1$_{mut}$), top strand: 5′-GCGTTTTCCTTAGCTCCAGTTTCA. EMSA assays were performed as described by (Dorval et al., 2005). Gels were dried and visualized with phosphoimager (Bio-Rad, Hercules, CA).

4.8. *Luciferase assays*

HEK293 cells were transfected with 0.04 μg reporter construct, 0.02 μg pRL-TK and either 0.2 μg pCMV-Chx10 or pCMV-Chx10^{R200Q} using the lipofectamine method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Reporter constructs were designed as follows: pGL3E-Vsx1^{wt} (wild type Vsx1 promoter), pGL3E-Vsx1^{mut1} (Vsx1 promoter mutated in −1275 Chx10 binding site), pGL3E-Vsx1^{mut2} (Vsx1 promoter mutated in −576 Chx10 binding site) and pGL3E-Vsx1^{mut3} (Vsx1 promoter mutated in both −1275 and −576 Chx10 binding sites).

Cell lysates were prepared 24 h after transfection. The activities of firefly and Renilla luciferase were assayed using a Dynex Technologies MRX Revelation microplate reader (Dynex Technologies, Denkendorf, Germany) using 100 μl D-luciferin reagent and 100 μl coelenterazine (Biotium, Hayward, CA). To standardize for transfection efficiency, the luciferase activities of all transfected cells were divided by the Renilla luciferase activities. Data are presented as mean ± S.D. from four separate experiments. Statistical significances were tested by two-way repeated measures ANOVA and Tukey tests for multiple comparisons.

4.9. *Zebrafish strains and staging*

Embryos were obtained from natural spawning of wild-type (AB-1) zebrafish lines. All developmental stages in this study are reported in hours post-fertilization (hpf) at 28.5 °C (Kimmel et al., 1995).

4.10. *MO injections*

chx10 translation blocking, and vsx1 splice-blocking MO antisense oligonucleotides were obtained from Gene Tools (*vsx1* MO: 5′-AGCAAAGTGATTCGTACCGGAGTAA-3′ and *chx10* MO: 5′-AAACAGCCCCATCCTTTCCTGTCAT-3′). Both MOs were injected into one-cell stage wild-type embryos at doses of 1.5 ng and 15 ng, respectively.

4.11. *RT-PCR of zebrafish RNA*

Fifty wild-type embryos and *vsx1* morphants were used for preparing RNA. Total RNA was isolated using Trizol reagent and standard protocols. Total RNA (1–5 μg/μl) was reverse transcribed by either random hexamers or a gene-specific primer using the Superscript first strand synthesis kit (Invitrogen) following the manufacturer's protocol. PCR was performed using an exon 1 forward primer (CGC AAT CAC AGA TCT CCT GG) and an exon 2 reverse primer (TCC ATC ATT GCG ATC ACC GG) for 30–35 cycles using an annealing temperature of 55 °C, and reactions were visualized on 1% agarose gels in TAE.

4.12. *In situ hybridization*

Probe synthesis and in situ hybridization were performed as described elsewhere (Oxtoby and Jowett, 1993), and visualized using BM Purple (Roche, Indianapolis, IN). The following three RNA probes were used: *vsx1* (amplified from published cDNA sequence (Passini et al., 1997); *cycD1* (869 bp PCR fragment); and *pax6a* (Krauss et al., 1991).

5. Note added in proof

As part of an effort to unify and update the nomenclature of human homeobox genes, the HUGO Gene Nomenclature Committee (www.genenames.org) has adopted VSX2 as the official name for the gene previously assigned as CHX10; the mouse ortholog will likewise be Vsx2. Chx10 and Alx (synonym of zebrafish Vsx2) will still be retained in databases to ensure retrieval of all relevant data.

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