Vsx2 Controls Eye Organogenesis and Retinal Progenitor Identity Via Homeodomain and Non-Homeodomain Residues Required for High Affinity DNA Binding

Changjiang Zou, Edward M. Levine*

Department of Ophthalmology and Visual Sciences, John A. Moran Eye Center, University of Utah, Salt Lake City, Utah, United States of America

Abstract

The homeodomain and adjacent CVC domain in the visual system homeobox (VSX) proteins are conserved from nematodes to humans. Humans with missense mutations in these regions of VSX2 have microphthalmia, suggesting both regions are critical for function. To assess this, we generated the corresponding mutations in mouse Vsx2. The homeodomain mutant protein lacked DNA binding activity and the knock-in mutant phenocopied the null mutant, ocular retardation J. The CVC mutant protein exhibited weakened DNA binding; and, although the corresponding knock-in allele was recessive, it unexpectedly caused the strongest phenotype, as indicated by severe microphthalmia and hyperpigmentation of the neural retina. This occurred through a cryptic transcriptional feedback loop involving the transcription factors Mitf and Otx1 and the Cdk inhibitor p27Kip1. Our data suggest that the phenotypic severity of the CVC mutant depends on the weakened DNA binding activity elicited by the CVC mutation and a previously unknown protein interaction between Vsx2 and its regulatory target Mitf. Our data also suggest that an essential function of the CVC domain is to assist the homeodomain in high-affinity DNA binding, which is required for eye organogenesis and unhindered execution of the retinal progenitor program in mammals. Finally, the genetic and phenotypic behaviors of the CVC mutation suggest it has the characteristics of a recessive neomorph, a rare type of genetic allele.

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* E-mail: ed.levine@utah.edu

Introduction

The homeodomain is a 60 amino acid DNA binding module composed of three alpha helices in a helix-turn-helix configuration. Homeodomain proteins are among the most numerous of transcription factors, second only to C2H2 zinc finger transcription factors in humans [1]. Structural studies of isolated homeodomains and site-directed mutants indicate that the properties needed for DNA binding are encoded within the homeodomain [2,3], and two recent DNA binding screens of 168 homeodomains and site-directed mutants indicate that the properties needed for DNA binding are encoded within the homeodomain [2,3], and two recent DNA binding screens of 168 homeodomains. Many homeodomains, however, exhibit inherently low sequence specificity or weak binding affinity, characteristics inconsistent with their high degree of functional specificity in vivo. Solutions to this problem include the incorporation of additional DNA binding domains (e.g. Pou, Paired) or protein interaction domains that recruit additional DNA binding proteins (e.g. LIM) [7–10]. Other solutions do not incorporate modular domains, but rather utilize non-homeodomain residues or motifs to assist the homeodomain. The DNA binding capacity of several Hox homeodomains is enhanced by a cooperative interaction with PBC homeodomain proteins and is mediated by the hexapeptide/YPWM motif, a stretch of conserved hydrophobic residues near the N-terminus of the Hox homeodomain. This interaction not only increases the complexity of the target sequence since both proteins bind DNA, but it also enhances the DNA binding affinity of the Hox homeodomain [11]. The C-terminal tail in PBC proteins is a helical region adjacent to the C-terminus of the homeodomain that increases the homeodomain’s DNA binding affinity, not by acting as a protein interaction motif or by directly contacting DNA, but through an intramolecular interaction that assists in properly positioning the third alpha helix (DNA recognition helix) into the major groove of its DNA binding site [12,13].

It is unclear whether non-homeodomain motifs are commonly used to enhance homeodomain function. Predicting which non-homeodomain residues or motifs are required for homeodomain function is difficult because these relationships depend on subtle and highly specific differences among homeodomains and to specific structural conformations of DNA sequences that comprise binding sites [14]. However, the hexapeptide and C-terminal tail have two properties in common: they are positioned close to the homeodomain and are evolutionarily conserved in a non-modular fashion, meaning that they are only found in proteins with similar homeodomains.
Author Summary

Problems with the early development of the mammalian retina can cause congenital eye defects such as microphthalmia, in which the eye is dramatically smaller and functionally compromised. Severe microphthalmia is associated with mutations in the retinal-expressed visual system homeobox 2 (Vsx2) gene, but how Vsx2 controls retinal development, and ultimately eye formation, has remained unclear. We assessed the impact of two missense mutations, discovered in humans, on Vsx2 function and eye development in mice. One mutation altered a highly conserved residue of the homeodomain, and the other altered a highly conserved residue in the CVC domain, a region of unresolved function. Both mutations impacted the DNA binding properties of the protein, although to differing extents. Likewise, both mutations caused microphthalmia and disruptions in retinal development, also to differing extents and by distinct mechanisms. Our data suggest that Vsx2 acts as a gatekeeper of the retinal gene expression program by preventing the activation of interfering or competing gene expression programs. We propose that the evolutionary stable association between the VSX-class homeodomain and CVC domain set the stage for Vsx2 or its archetype to assume a gatekeeper function for retinal development and ultimately eye organogenesis.

Another group of homeodomain proteins with a conserved, non-modular motif adjacent to the homeodomain are in the visual system homeobox family (VSX; also referred to as Prd-L-CVC or CVC paired like). These include Vx1 and Vx2 (formerly Chx10) in vertebrates and D. melanogaster and ceh-10 in Caenorhabditis elegans. VSX genes belong to the larger paired-like homeodomain class that include Rx, Arx, and Axl genes [10], but are unique in that they encode a region of approximately 60 amino acids extending from the C-terminus of the homeodomain and named the CVC domain for the genes in which it was initially discovered (Figure 1A) [15–17]. Genetic data suggest the CVC domain is essential for VSX function. In C. elegans, two missense mutations in the ceh-10 CVC domain cause embryonic lethality and neuronal differentiation defects similar in severity and timing to those in retinal development, and ultimately eye formation, has remained unclear. We assessed the impact of two missense mutations, discovered in humans, on Vsx2 function and eye development in mice. One mutation altered a highly conserved residue of the homeodomain, and the other altered a highly conserved residue in the CVC domain, a region of unresolved function. Both mutations impacted the DNA binding properties of the protein, although to differing extents. Likewise, both mutations caused microphthalmia and disruptions in retinal development, also to differing extents and by distinct mechanisms. Our data suggest that Vsx2 acts as a gatekeeper of the retinal gene expression program by preventing the activation of interfering or competing gene expression programs. We propose that the evolutionary stable association between the VSX-class homeodomain and CVC domain set the stage for Vsx2 or its archetype to assume a gatekeeper function for retinal development and ultimately eye organogenesis.

Addressing whether the CVC domain assists in homeodomain function is complicated by the likelihood that the CVC domain has multiple functions. Its deletion in VSX2 altered DNA binding and transcriptional properties although it is unclear whether these changes were interdependent, and whether they were specific to the CVC domain because other regions were also removed [34]. Its deletion in Vx1 reduced polyubiquitination suggesting a role in regulating protein stability [35]. Because deleting the entire CVC domain could lead to pleiotropic effects, another approach to identify functional requirements of the CVC domain and its relationship with the homeodomain is to study the effects of the missense mutations on protein function and eye development.

In this study, we generated the homeodomain mutation R200Q and CVC domain mutation R227W in the mouse Vsx2 ortholog and compared their functional properties. A predominant effect of these mutations is to reduce homedomain-dependent DNA binding but to different degrees. Since Vsx2 regulates eye size and retinal development, we generated knock-in mice and compared their phenotypes to the orJ mouse. Molecular and genetic analyses enabled us to identify the transcriptional circuits driving the phenotypes caused by each mutation. Our data support the model that the proper execution of mammalian eye organogenesis and retinal development is built upon high affinity DNA binding by Vsx2, which is dependent on both the homeodomain and CVC domain. We also provide evidence suggesting that Vsx2 regulates one of its key targets, microphthalmia-associated transcription factor (Mitf) by two mechanisms; direct transcriptional repression and protein-protein interaction. Both mechanisms may be employed to prevent activation of aberrant gene expression programs that interfere with the execution of the developmental program in retinal progenitor cells (RPCs).

Results

The R200Q and R227W mutations alter the DNA binding affinity of VSX2 protein, but not its ability to repress transcription

We compared the DNA binding properties of in vitro translated VSX2 and the VSX2[R200Q] and VSX2[R227W] variants using an oligonucleotide containing a high affinity Vsx2 binding site by electrophoretic mobility shift assays (EMSA). Consistent with previous studies [28,32], robust DNA binding was observed for VSX2 whereas binding was not detected with VSX2[R200Q] (Figure 1B, left panel). VSX2[R227W] binding was detectable but weak, and is more visible with a longer exposure time (Figure 1B, top right panel). The reduced DNA binding properties were not due to variations in protein expression (Figure 1B, bottom right panel) or to alterations in the helical organization of the homeodomain as assessed with secondary structure prediction software (http://us.expasy.org; data not shown). These observations indicate that the arginines at positions 200 and 227 are required for high affinity DNA binding.

Of the known candidate targets of Vsx2-mediated transcriptional regulation [32,36–38], the basic helix-loop-helix/leucine zipper (bhlh/vg) gene Mitf is of high importance because its increased expression in the orJ retina contributes to the mutant phenotype [39–42]. Regulation of the Mitf locus is complex. Nine promoters have been identified and each produces an RNA transcript with a distinct first exon, several with limited protein-coding information. As a result, multiple Mitf isoforms on the RNA and protein levels are possible, although most if not all isoforms contain the domains and motifs needed for transcription factor activity (see Figure 1 in [39] for a detailed illustration of the mouse Mitf locus and gene products). Depending on the cell type, Mitf
Figure 1. DNA binding and transcriptional activities of VSX2 and the VSX2[R200Q] and VSX2[R227W] variants. (A) ClustalW alignment of the homeodomain and adjacent 60 amino acids in select VSX orthologs and the most similar non-VSX proteins in mice. Only the VSX sequences have a discernable CVC domain. The positions of the orJ, R200Q, and R227W mutations are shown. (B) Left panel: EMSA with in vitro translated VSX2, VSX2[R200Q], and VSX2[R227W] proteins and [32P]-labeled P3 oligo (see Table S1 for sequence). Top right panel: Extended exposure reveals weak binding by VSX2[R227W]. Bottom right panel: Western blot of in vitro translated proteins with VSX2 antibody (Lys, control lysate; -, P3 probe only). (C) Schematic shows five putative Vxs2 binding sites (Hx-6 – Hx-10) in the proximal promoter region (0.3 kb) of D-Mitf. Carats and dashed line marks the region of PCR amplification in the ChIP assay shown below schematic (primer set 13; Table S1). Arrowhead points to sequence-verified ChIP product. (D) Luciferase assays in P0 primary retinal cells transfected with the indicated expression vectors (x-axis) and 2.2 kb of the D-Mitf promoter region (pGL3P-Dmitf). (E) The Hx-9 site was mutated in pGL3P-mDmitf to eliminate DNA binding at that site. Reporter assays were normalized to empty vector controls (white bars). (F) CAT assays in HEK293 cells transfected with the X4G2CAT reporter and VSX2 variants fused to the LexA DNA binding domain. Gal4-Hsf1 was included to stimulate high basal reporter activity [34]. ** P ≤ 0.01; *** P ≤ 0.001.

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isofoms are expressed in different combinations, indicating that promoter utilization is context-dependent [43]. D-Mif is one of the isofoms upregulated in the oJ retina and has at least 10 putative Vsx2 binding sites within 1 kb upstream of its transcriptional start site (Hx-1 – Hx-10; [39]). Chromatin immunoprecipitation (ChIP) assays revealed Vsx2 binding in the vicinity of the Hx-1 – Hx-3 sites, approximately 0.8 kb upstream of the D-Mif transcriptional start site [39]. We found that Vsx2 bound to chromatin in the vicinity of the Hx-6 – Hx-10 sites, less than 0.3 kb upstream D-Mif transcriptional start site (Figure 1C). Based on these findings, the D-Mif promoter is likely to be a direct target of Vsx2-mediated transcriptional repression.

To test this further, transcriptional reporter assays were done with a construct containing ~2.2 kb of the D-Mif promoter region and SV40 early promoter driving a luciferase reporter (pGL3-P-DMif) and constructs for expressing Vsx2, VSX2[R200Q] or VSX2[R227W] proteins in P0 wild-type retinal cells. Inclusion of the SV40 promoter was necessary for reliable basal activity reporter activity. Consistent with our results for the Vsx1 promoter [32], Vsx2 repressed reporter activity and repression mediated by VSX2[R200Q] was diminished (Figure 1D). Like Vsx2, VSX2[R227W] also repressed reporter activity and required the presence of the Hx-9 site (Figure 1D, 1E). Similar results were obtained in HEK293 cells (data not shown), indicating that repression of reporter activity was not dependent on additional factors exclusive to retinal cells. The SV40 promoter was not used in these and all other reporter assays in HEK293 cells because it was not required for basal reporter activity. To determine if the mutations interfered with repressor function in addition to DNA binding, the LexA DNA binding domain was fused to the Vsx2 variants and reporter activity was determined in HEK293 cells with X4G2CAT, a chloramphenicol acetyltransferase (CAT) reporter containing multimerized LexA binding sites [34]. In this assay, each of the LexA fusions repressed CAT reporter activity to similar extents (Figure 1F). Thus, the repression mediated by Vsx2 and VSX2[R227W] depended on DNA binding and the R200 and R227 residues were not required for repression activity.

Generation and characterization of Vsx2[R200Q] and Vsx2[R227W] knock-in mice

If DNA binding is essential for Vsx2 function, then the VSX2[R200Q] or VSX2[R227W] protein should retain some degree of Vsx2 function. If true, then the simplest predictions for mice with these mutations are that homozygous Vsx2[R200Q] mutants (R200Q) should phenocopy oJ mutants and homozygous Vsx2[R227W] mutants (R227W) should exhibit a less severe or hypomorphic phenotype. It is also possible that these alleles could exhibit dominant negative activity if Vsx2 activity requires dimerization, similar to DNA binding mutations in the homeodomain protein Pitx2, which are linked to Axenfeld-Rieger syndrome [44,45]. We tested these predictions by generating R200Q and R227W knock-in mice by homologous recombination using the ACN targeting vector, which removed all gene targeting elements when the alleles were transmitted through the male germline (Text S1; Figure S1; [46]). The only foreign DNA retained was a 34 base pair sequence containing a remnant loxp site in intron 3 (Figure S1). Germine transmission was achieved (Figure S1) and the mutants used for this study were established in the 129sv genetic background to minimize strain-dependent modifier effects [40,41,47,48].

As in humans, R200Q and R227W mice were microphthalmic, which was apparent by E11.5 and became progressively more severe as development continued (Figure 2A–2L). Relative eye size in oJ and R200Q mutants were similar, but surprisingly, R227W mutants exhibited smaller eyes at E14.5 and beyond (Figure 2E–2L). In contrast to the lack of VSX2 protein in oJ RPCs, both knock-in mutants expressed Vsx2 protein in a manner similar to wild-type, suggesting that changes in expression or nuclear localization were not causing the phenotypes (Figure 2M–2P, Figure S2A). Consistent with the EMSA data, ChIP assays with E12.5 retinal lysates showed that VSX2[R200Q] protein was not detectable at the D-Mif promoter whereas VSX2[R227W] was bound although to a lesser extent than Vsx2 (Figure 2Q).

Histological analysis revealed that mutant eyes had a smaller lens, thickened retinal pigment epithelium (RPE), and a thinner retina compared to wild-type (Figure 3A–3H). Whereas the oJ and R200Q eyes were similar in appearance, R227W eyes were more severely affected as indicated by an even smaller lens, thinner retina, and an infiltration of mesenchymal cells into the vitreal chamber (Figure 3H, asterisks). The smaller retina in the oJ mouse is correlated with a reduction in RPC proliferation [30,47,49–53] and this was likely the case in the R200Q and R227W retinas as both mutants showed reduced phosphorylated Histone H3 expression (Figure S2B). Consistent with this, VSX2[R200Q] or Vsx2[R227W] overexpression in cultured oJ retinal cells was not sufficient to promote proliferation (Figure S2C).

The onset of neurogenesis occurs by E11.5 in the central retina and spreads as a wave toward the peripheral retina. This was delayed by 1–2 days in oJ and R200Q retinas and was not observed at all in the embryonic R227W retina (Figure 3I–3P; Figure S3; [47,52]). The lack of detectable neurogenesis suggested that R227W RPCs underwent a fundamental change in their developmental potential that differed from the other mutants.

Optic cup morphogenesis initiates at approximately E9.5, soon after onset of Vsx2 expression [17,54]. The Vsx2 expression domain marks the interior layer of the optic cup and gives rise to the retina. Under normal conditions, melanogenic pigmentation does not occur in the retina. In contrast, pigmentation and thinning of the epithelium is pronounced in the peripheral retina of oJ mice, and lineage analysis suggests these pigmented cells arise from retinal-specified progenitor cells [40]. Pigmentation of the central oJ retina is rare in the 129svj genetic background, but is fairly prevalent in mixed 129svj/C57Bl6 mice owing to uncharacterized genetic modifiers [40,41]. Similar background-dependent effects were observed for the R200Q retina (data not shown). Surprisingly, pigmentation was much more extensive in the R227W retina of mice with the 129sv genetic background (Figure 3Q–3T). This occurred in a progressive manner, which started in the peripheral retina at E14.5 and extended centrally to occupy most or all of the retina by E17.5 (Figure 3H, 3I, 3M, 3N). Vsx2[R227W] protein was detected in some pigmented cells at E17.5, which indicated that as in the oJ retina, the ectopically pigmented cells arose from RPCs (Figure 3U–3W). In contrast, Vsx2[R200Q] protein remained expressed in RPCs at E17.5 (data not shown). These data suggest that the pigmentation of the R227W retina was a direct consequence of the mutant allele.

In agreement with our in vitro data, the R200Q and oJ phenotypes were similar and support the hypothesis that homeodomain-dependent DNA binding is critical for Vsx2 function. That the R227W phenotype was more severe was unexpected since the VSX2[R227W] protein retained properties associated with transcriptional regulation. Our in vitro and in vivo data are consistent in that they indicate the VSX2[R227W] protein was functional, but they differ because the in vivo phenotype indicated that the R227W allele was not hypomorphic. One possibility for this discrepancy is that the VSX2[R227W] protein acquired a novel activity not revealed by the in vitro assays. If this
were true, then the R227W allele should be dominant or semi-dominant. This was not the case, however, since eye size, circumference, and retinal histology in R227W/+ mice were indistinguishable between wild-type, orJ/+ or R200Q/+ mice (Figure 4A–4L). Furthermore, hemizygous R227W/+ mice exhibited an intermediate phenotype compared to the orJ and R200Q mutants. (I–L) Dissected E17.5 eyes (right eyes rotated 90°) show similar reductions in eye size in orJ and R200Q homozygotes whereas the reduction in eye size of R227W homozygotes was the most severe. (M–P) VSX2 immunohistochemistry in E12.5 retinas. VSX2 protein was not detected in the orJ retina, confirming it as an expression null. VSX2[R200Q] and VSX2[R227W] were expressed similarly to VSX2[wt], although to a reduced extent in peripheral retina. Dashed lines bound retinas. (Q) ChIP assays with VSX2 antibody reacted with E12.5 native chromatin lysates from wild-type, R200Q, and R227W retinas and amplified using D-Mitf primer set 13 (Table S1). Arrowhead denotes amplification product. Graph shows quantification results of ChIP-qPCR. Scale bars: 0.5 mm (E11.5); 5 mm (E14.5); 1 mm (E17.5).

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Figure 2. The R200Q and R227W mutations cause non-syndromic congenital microphthalmia. (A–D) Mice homozygous for the orJ, R200Q, and R227W alleles had smaller eyes than wild-type by E11.5. (E–H) At E14.5, overall embryonic development was unaffected in the mutants, but the failure of the mutant eyes to keep pace with the growth of the wild-type eye was evident. Eye growth in the R227W mutant also failed to keep pace with the orJ and R200Q mutants. (I–L) Dissected E17.5 eyes (right eyes rotated 90°) show similar reductions in eye size in orJ and R200Q homozygotes whereas the reduction in eye size of R227W homozygotes was the most severe. (M–P) VSX2 immunohistochemistry in E12.5 retinas. VSX2 protein was not detected in the orJ retina, confirming it as an expression null. VSX2[R200Q] and VSX2[R227W] were expressed similarly to VSX2[wt], although to a reduced extent in peripheral retina. Dashed lines bound retinas. (Q) ChIP assays with VSX2 antibody reacted with E12.5 native chromatin lysates from wild-type, R200Q, and R227W retinas and amplified using D-Mitf primer set 13 (Table S1). Arrowhead denotes amplification product. Graph shows quantification results of ChIP-qPCR. Scale bars: 0.5 mm (E11.5); 5 mm (E14.5); 1 mm (E17.5).

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were true, then the R227W allele should be dominant or semi-dominant. This was not the case, however, since eye size, circumference, and retinal histology in R227W/+ mice were indistinguishable between wild-type, orJ/+ or R200Q/+ mice (Figure 4A–4L). Furthermore, hemizygous R227W/+ mice exhibited an intermediate phenotype compared to the orJ and R227W homozygotes (Figure 4J–4S), which suggested that the failure of the R227W allele to compete with the wild-type allele was not due to reduced expression of the mutant protein. Finally, overexpression of VSX2, VSX2[R200Q], or VSX2[R227W] in newborn wild-type retinal primary cells had minimal effects on proliferation (Figure S2D). These data revealed that even if the VSX2[R227W] protein acquired a novel activity, it is not sufficient to interfere with wild-type Vsx2 function. Thus, even though the R227W phenotype surpasses the null in severity, the allele displayed recessive behavior, consistent with what is observed in humans. Additionally, the recessive nature of the R200Q and R227W alleles suggest that Vsx2 does not require dimerization for function.

The data presented thus far best fits the genotype-phenotype correlation shown in Figure 4T. In wild-type mice, the RPC program driving retinal development progressed normally and
correlated with unhindered DNA binding activity by Vsx2. In orJ and R200Q mice, the RPC program progressed in a suboptimal manner and the RPCs were biased, but not necessarily committed, to expressing a pigmentation program. The orJ and R200Q alleles were recessive and lacked Vsx2-dependent DNA binding activity. In the case of orJ, this is because the protein was not present, and in the case of R200Q, because the DNA binding activity was specifically disrupted. In R227W mice, the RPC program initiated, but ultimately failed and was followed by robust expression of a pigmentation program. As with orJ and R200Q, the R227W allele

Figure 3. Ocular histology and neurogenesis in Vsx2 mutants. (A–H) Merged images of cryosections showing DAPI staining (blue) and melanogenic pigmentation (white) for each of the indicated genotypes and ages. Arrowheads in H point to aberrant pigmentation in peripheral retina asterisk denotes ectopic pericellular mesenchyme (POM) in vitreal cavity. (I–P) Expression patterns of the neuronal differentiation marker class III β-Tubulin (TUBB3). Neurogenesis lagged behind wild-type and to a similar extent in the orJ and R200Q retinas, but did not initiate in the R227W retina. (Q–T) Merged images of cryosections showing DAPI staining (blue) and melanogenic pigmentation (white) for each of the indicated genotypes at E17.5. The R227W retina was aberrantly pigmented, either partially (T(a)) or completely (T(b)). Arrowheads in T(a) and T(b) point to aberrant pigmentation in peripheral retina, arrows to central retinal regions, and asterisks to ectopic pigmentation in vitreal cavity. (U–W) Pigmented cells expressing VSX2[R227W] were detected in pigmented retinal region. L, lens; RPE, retinal pigment epithelium. Scale bars: 100 μm (A–T), 20 μm (U–W). doi:10.1371/journal.pgen.1002924.g003
Figure 4. The R200Q and R227W alleles and proteins do not exhibit dominant behavior. (A) Wild-type, orJ/+, R200Q/+, and R227W/+ eyes were indistinguishable at P0. No significant differences in eye circumferences were detected. (B–E) Merged images of cryosections showing DAPI staining (blue) and melanogenic pigmentation (white) for each of the indicated genotypes at P0. (F–I) Expression of the retinal ganglion cell marker POU4F2 in wild-type or Vsx2 heterozygous retinas. (J) Eye circumference of orJ/R227W heterozygotes was intermediate to orJ and R227W homozygotes. (K–S) Expression of VSX2, CCND1, and TUBB3 in orJ, orJ/R227W, and R227W retinas at P0. VSX2 was detected in the orJ/R227W retina only. CCND1 and TUBB3 were detected in orJ or orJ/R227W retinas but not in R227W pigmented retina. (T) Genotype-phenotype correlation of Vsx2 alleles arranged by retinal phenotype. * P<0.05 Scale bars: 1 mm (A, J); 100 μm (B–I, K–S).

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was recessive, but the protein retained DNA binding activity, albeit weakened compared to wild-type.

Multiple regulatory changes at the Mitf locus are associated with pigmentation in the R227W retina

Mitf is initially expressed throughout the optic neuroepithelium at the optic vesicle stage and is downregulated in the presumptive retinal domain soon after Vsx2 is expressed [54–56]. Since Mitf is a key regulator of the genetic pathways that drive melanogenic pigmentation, we suspected that Mitf expression levels would correlate with the degree of pigmentation in the mutants. In orJ and R200Q mice, MITF expression at E12.5 was modestly elevated in the central retina and highest in the peripheral retina where pigmentation was most prevalent (Figure 5A–5C). In contrast, MITF was highly expressed throughout the R227W retina (Figure 5D). We also examined the expression of the orthodenticle-related homeodomain proteins OTX1 and OTX2 (OTX) since they are also required for the pigmentation program in the RPE and can directly regulate Mitf expression [57–60]. Similar to MITF, OTX expression was modestly increased in the orJ and R200Q central retinas but was highly expressed throughout the R227W retina with the most notable increases in the periphery (Figure 5E, 5H; negative control staining shown in Figure S6C). Interestingly, VSX2 was downregulated in the peripheral retina, which correlated with the highest expression levels of MITF and OTX (Figure 5D, 5H) and where pigmentation was most apparent at E14.5 (Figure 3H), which suggested a change in fate for these cells. The scattered OTX expression in the wild-type retina is linked to the production of postmitotic Otx2+ cone photoreceptor precursors [61], which were not observed in the mutants, consistent with the delay or absence of neurogenesis.

To determine if changes in mRNA levels could account for the differences in MITF and OTX expression, we performed quantitative real-time reverse-transcription PCR (qRT-PCR) on total RNA lysates from E12.5 retinas. Using primers that recognize all Mitf isoforms (pan-Mitf), we found that compared to the orJ retina, wild-type had significantly less expression, R200Q was similar, and R227W had significantly more expression (Figure 5I), all of which is consistent with the immunohistochemical data. We also used gene specific primers for Otx1 and Otx2 to determine their relative expression levels in each genotype (Figure 5J). Compared to orJ, Otx1 expression was lower in wild-type and R200Q and higher in the R227W retina. All of the mutants expressed Otx2 at lower levels than wild-type. These data indicated that the increase in OTX protein expression was specific to Otx1. Furthermore, the low level of Otx1 in the R200Q retina revealed a difference with the orJ retina, which was unexpected in light of the overall similarities in the cellular and tissue phenotypes of the two mutants.

The A, D, H, and J isoforms of Mitf are expressed in the embryonic retinal pigment epithelium (RPE) with A, D, and H being the most abundant [39]. These isoforms are also detected in the retina at much reduced levels with the exception of A, which is expressed at a level comparable to the RPE [39]. We performed qRT-PCR of E12.5 retinal RNA to determine the isoform-profile in the different genotypes (Figure 5K). We found that D and H were higher in the orJ retina than in wild-type, consistent with previous findings [39]. We also observed a significant increase in J, an upward trend in B, and no change in A. In general, the isoform expression profile in the R200Q retina was similar to orJ. In the R227W retina, however, only A and H exhibited higher expression compared to orJ and R200Q. These data suggested the increased Mitf expression in the R227W retina was due to novel changes in the transcriptional regulation of the A and H isoforms.

Periocular mesenchyme (POM) promotes A-Mitf expression, but H-Mitf is the isoform that most likely accounts for the unique increase in the overall Mitf level in the R227W retina

The POM is composed of neural crest and mesoderm-derived progenitors that express Pitx2 and contribute to the formation of ocular structures such as the sclera, choroid, and cornea [62–64]. In chick, the POM contains an Activin-like factor that promotes Mitf expression in the nascent RPE domain of the optic vesicle [65]. Interestingly, the most profound changes with respect to tissue morphology, pigmentation, and upregulation of Mitf and Otx1 expression corresponded to regions of the neural retina that were juxtaposed to POM that invaded the vitreal chamber from the retinal peripheral margin after E10.5 (Figure 6A–6F). This was most pronounced in the R227W retina in which the entire vitreal chamber fills with PITX2 positive cells by E17.5 (Figure S5A). This was highly unusual since very few mesenchymal cells normally migrate into the vitreal cavity [63].

To determine whether the POM was responsible for the elevated expression levels of the A- or H-Mitf isoforms in the R227W retina, we cultured retinal explants from E10.5 orJ and R227W embryos with the lens attached but POM and RPE were removed for 48 hr and measured transcript levels. Infiltration of POM into the vitreal chamber was not detectable at E10.5 and we found no evidence of POM invasion after 48 hr in culture (data not shown). Under these conditions, A-Mitf was unchanged between the two mutants whereas H-Mitf was significantly higher in the R227W mutant (Figure 6G). To further test the influence of POM, we cultured E10.5 R227W explants containing whole retina and lens in the presence or absence of POM in four different conditions: POM removed (- POM); POM removed with vitreal space physically exposed (mock); retention of POM at the anterior pole (+POM); or POM implanted into the vitreal chamber (POM imp). The POM implant was the only condition in which A-Mitf expression was enhanced further (Figure 6H, left graph). Although the increase in H-Mitf expression was significant in the presence of POM (Figure 6H, middle graph), the magnitude of the change was small (note the difference in scale of the y-axes for the middle graphs in Figure 6G and 6H). The effects of the POM implant on the D- and J- isoforms were not significant (Figure 5B). These data suggested that the elevated level of A-Mitf was under the influence of signals from the POM whereas H-Mitf expression was largely independent of POM-derived signals. Since the high level of pan-Mitf expression also did not depend on the presence of POM (Figure 6H, right graph), H-Mitf appears to be the mRNA isoform primarily responsible for the elevated pan-Mitf level in the R227W retina and its regulation is likely to be cell-autonomous.

Dominant-negative Mitf reveals a positive feedback loop for driving high Mitf expression and pigmentation in the R227W retina

To better understand the role of Mitf in microphthalmia and the retinal defects caused by the Vsx2 mutations, we generated compound mutants homozygous for the 15x2 alleles and heterozygous for the Mitf allele (mi/+). mi is a dominant negative allele and encodes a 3 bp deletion resulting in the loss of an arginine in the basic domain, which disrupts DNA binding in all Mitf isoforms [66,67]. Early eye development proceeds relatively well in mi/+ mice [68] and orJ; mi/+ mice showed substantial improvements in eye size, tissue histology, and retinal neurogenesis by birth (Figure 7A, 7D, 7G, 7J). As expected, the degree of improvement
of eye development in R200Q; mi/+ was similar to orJ; mi/+ (Figure 7B, 7E, 7H, 7K), but interestingly, eye size and retinal development in R227W; mi/+ mice also improved to a level comparable with the other mutants (Figure 7C, 7F, 7I, 7L). Positive effects were observed early in retinal development as evidenced by increased eye size, circumference, and restored neurogenesis (Figure 7M, 7N). These findings were surprising because of the more profound consequences of the R227W mutation on eye development and the elevated levels of Mitf and Otx1. Consistent with the high degree of phenotypic rescue, pan-Mitf and Otx1 expression levels were significantly reduced (Figure 7O). Importantly, pan-Mitf levels decreased disproportionately relative to the allele dosage. This was correlated with large reductions in all isoforms except B-Mitf (Table 1), which is already expressed at low levels [39]. This suggested that the enhanced Mitf expression in the R227W retina is due to positive feedback. This was unique to the R227W retina because Mitf levels were not decreased in the orJ; mi/+ retina (Figure 7O). Our observations thus far reveal the essential and complex role of Mitf misregulation in causing microphthalmia and in interfering with retinal development in the Vsx2 mutant backgrounds.

Genetic reduction of \( p27^{kip1} \) enhances eye size and restores neurogenesis in the R227W retina

We previously showed that genetic inactivation of the cyclin-dependent kinase inhibitor \( p27^{kip1} \) (\( p27; \) MGI symbol: Cdkn1b) significantly enhances eye size and retinal development in \( \text{orJ} \) mice [50]. Although we didn’t examine the effects of \( p27 \) removal on Mitf expression or retinal pigmentation in that study, we have yet to observe pigmentation in regions where retinal histogenesis was restored. This was also true for R200Q; \( p27 \) double mutants (data not shown). To determine if \( p27 \) also contributed to the alterations in eye development and the pigmentation potential of R227W RPCs, we generated R227W; \( p27 \) compound mutants. We analyzed retinas from R227W homozygous, \( p27 \) heterozygous (R227W; \( p27^{+/−} \)) mice because they expressed \( p27 \) at a level similar to that in R227W; mi/+ retinas (Figure 8A). Interestingly, partial reduction in \( p27 \) was sufficient to inhibit pigmentation and promote retinal histogenesis (Figure 8B–8D). As in the \( \text{orJ} \), the peripheral regions of the R227W; \( p27^{+/−} \) retina was not rescued to the same extent as the central region. These data revealed that \( p27 \) was a key factor in promoting the Vsx2 mutant phenotypes regardless of allele.

Figure 5. Phenotypic severity correlates with the expression levels of Mitf and Otx1. (A–D) MITF expression at E12.5 for the indicated genotypes. The R227W retina expressed MITF at much higher levels compared to the orJ and R200Q mutants. (D’) Merged images of VSX2[R227W] (red) and MITF (green) shows overlap in expression. The lack of VSX2[R227W] expression in the peripheral retina corresponded to the highest levels of MITF. (E–H) OTX expression at E12.5 for the indicated genotypes. OTX expression was highest in the R227W retina. (H’) Merged images of VSX2[R227W] (red) and OTX (green). Like MITF, OTX expression was highest in regions lacking VSX2[R227W]. MITF and OTX were also expressed in RPE (outside lower dashed lines). (I–K) Relative mRNA expression levels of pan-Mitf (I), Otx1 and Otx2 (J), and the D-, H-, A-, J-, and B-Mitf isoforms (K) in E12.5 retinas of the indicated genotypes as determined by qRT-PCR. Samples were normalized to the expression level for each transcript in the orJ retina (white bars). * \( P<0.05 \) Scale bar: 50 μm.

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Figure 6. Influence of POM on Mitf expression in the R227W retina. (A–F) PITX2 (red) and MITF (green) expression at E10.5 (A–C) and E12.5 (D–F). Limited PITX2⁺ cells were detected in the vitreal chamber (between retina and lens) of wild-type eyes. PITX2⁺ cells were not detected in the vitreal chamber of mutant eyes at E10.5, but were abundant by E12.5 and continuous with POM at the retinal periphery (arrowheads). MITF expression levels were modestly upregulated at E10.5 in the mutant retinas and were clearly elevated by E12.5. (G) Relative expression levels of A-Mitf, H-Mitf, and pan-Mitf in E10.5 whole retina and lens explants cultured for 48 hr. R227W expression levels were normalized to orJ. Invasion of POM into the vitreal chamber did not occur in these cultures (data not shown). (H) Relative expression levels of A-Mitf, H-Mitf, and pan-Mitf in physically manipulated E10.5 R227W whole retina and lens explants cultured for 48 hr after the following manipulations: “−POM” (retina and lens only); “mock”
Vsx2[R227W], p27, H-Mitf, D-Mitf, and Otx1 form a transcriptional positive feedback loop.

As with the R227W; mi/+ retina, pan-Mitf and Otx1 mRNA and protein expression levels dropped in the R227W; p27−/− retina (Figure 8E; Figure S6A, S6B; data not shown). There were notable differences, however, between the two rescue models (Table 1). In the R227W; p27−/− retina, the pan-Mitf level was intermediate to the R227W and R227W; mi/+ retinas. Among the Mitf isoforms, only D was reduced in the R227W; p27−/− retina (Figure 8F; Table 1), whereas all Mitf isoforms (except B) dropped well below half in the R227W; mi/+ retina (Table 1). Considering that H-Mitf was the isoform primarily responsible for the elevated increase in the pan-Mitf level in the R227W mutant over αJ and R200Q, these data show that H-Mitf was not sufficient to drive the R227W phenotype when D-Mitf and p27 levels dropped down. These data are consistent with a genetic pathway in which D-Mitf is downstream of p27, and p27 is downstream of H-Mitf.

Since D-Mitf mRNA expression decreased when one allele of p27 was inactivated in the R227W retina, we asked whether this regulation could be direct. First, we overexpressed p27 with our D-Mitf luciferase reporter (pGL3B-DMitf) in HEK293 cells. In contrast to the repression observed with Vsx2 and Vsx2[R227W] (Figure 1D), p27 enhanced reporter activity (Figure 8G, left graph). That this reflects a specific interaction is supported by our observation that p27 did not significantly alter reporter activity when Luciferase was expressed under the control of αJ or R200Q (Figure 8G, right graph). Second, we performed ChIP assays with an antibody against p27 and chromatin lysates isolated from E12.5 R227W retinas using 13 primer sets that covered ~2.2 kb of 5′-intergenic sequence. PCR enrichment was observed with primer set 1 and primer set 13 (Figure 8H). These data suggest p27 acts directly to regulate D-Mitf transcription in R227W RPCs.

Since p27 is an important factor in promoting both the αJ and R227W phenotypes and yet the phenotypes differ in severity, we asked whether p27 is regulated differently in the two mutants. p27 transcript levels were equivalent in wild-type and αJ retinas at P0 [50] and its expression was not significantly different between wild-type, αJ, or R200Q at E12.5 (Figure 9A). In contrast, p27 mRNA and protein expression was higher in the R227W retina (Figure 9A; Figure S6D-S6G), with the highest expression in the peripheral retina (Figure 9B). This appeared to depend on DNA binding by Vsx2[R227W], the natural DNA binding activity by MITF, and the interaction between the two proteins.

While we now have explanations for the elevated levels of p27 and D-Mitf in the R227W mutant retina, how the H-Mitf level increased was still not clear. Although Vsx2 is associated with chromatin in the H-Mitf regulatory region [39], reporter activity in HEK293 cells transfected with pGL3B-DMitf was not different between cells expressing Vsx2 or its variants (Figure 9E, left graph). H-MITF was also not sufficient to enhance reporter activity, but rather caused moderate repression (Figure 9E, right graph). In contrast, Otx1 increased reporter activity and this was
Figure 7. The dominant negative allele Mitf<sup>mi</sup> restores retinal development in the Vsx2 mutants. (A–F) Merged images of cryosections showing DAPI staining (blue) and melanogenic pigmentation (white) in P0 orj, R200Q, and R227W mice that were Mitf wild-type (Mitf<sup>+/+</sup>; A–C) and mi heterozygous (Mitf<sup>mi/+</sup>; D–F). Insets show whole eyes. The retina in C was completely transformed into pigmented tissue (bounded by dashed line) and ectopic POM was partially pigmented (asterisk). Eye size and retinal histology were restored to a comparable degree in all Vsx2, mi compound mutants (D–F). Also notable in the R227W, mi compound mutant was the lack of POM in the vitreal chamber (asterisk in F). (G–L) TUBB3 staining at P0. In all cases, lamination patterns were restored in the compound mutants, indicating robust neurogenesis. Retinal tissue in I is bounded by the dashed lines. (M) The reduced eye size in the R227W mutant was partially rescued in the R227W; Mitf<sup>mi/+</sup> mutant at E12.5. (N) The expression of TUBB3 was detected in the R227W; Mitf<sup>mi/+</sup> retina at E13.5. (O) Mitf and Otx1 transcript levels were much lower in the R227W; Mitf<sup>mi/+</sup> retina (black bars) compared...
further enhanced by the presence of H-MITF (Figure 9E, right graph). This interaction was synergistic because the activity of OTX1 and H-MITF expressed together was greater than the sum of the activities of the factors expressed individually, and the P-value (0.004) from a two-way ANOVA supports this conclusion. Unexpectedly, the relative luciferase activity in cells co-expressing OTX1 and the H-MITF(Δn) was similar to the co-expression of OTX1 and wild-type H-MITF (Figure 9E, right graph). In this case, however, the effect was not synergistic (P = 0.09, two-way ANOVA), but rather reflected an additive effect since H-MITF(Δn) expressed alone also enhanced luciferase activity over the control (Figure 9E, right graph). These observations indicated that the synergistic effect of OTX1 and H-MIF on the H-MITF promoter was dependent on DNA binding by H-MIF. In sum, the uniquely elevated level of H-MIF in the R227W mutant was not likely due to the direct action of the VSX2[R227W] protein at the H-MITF promoter, but rather to the collaboration of OTX1 and H-MIF, and possibly other Mitf isoforms.

Discussion

We show that the homeodomain and CVC mutant proteins VSX2[R200Q] and VSX2[R227W] have diminished DNA binding capacities but retain other functions important for transcriptional activity such as nuclear localization and the ability to regulate transcription when fused to a heterologous DNA binding domain. As in humans, both mutations cause severe non-syndromic bilateral microphthalmia in mice thus confirming their pathogenicity. The recessive nature of these alleles in both species suggests that the alterations in the functional properties of VSX2 caused by each mutation are conserved. Our data argue that the homeodomain is dependent on the CVC domain for high affinity DNA binding highlighting the importance of non-homeodomain residues in mediating canonical homeodomain function. Furthermore, our data suggest that high affinity DNA binding by VSX2 is required to suppress the activation of transcriptional circuits that interfere with the retinal gene expression program in RPCs.

Table 1. Relative retinal mRNA levels in R227W and compound mutants at E12.5.

|          | R227W    | R227W; MitfΔn | R227W; p27Δn |
|----------|----------|---------------|---------------|
| pan-Mitf | 1.00±0.12| 0.15±0.08***  | 0.52±0.05***  |
| D-Mitf   | 1.00±0.07| 0.09±0.04**   | 0.27±0.05**   |
| A-Mitf   | 1.00±0.12| 0.14±0.06**   | 1.69±0.29**   |
| H-Mitf   | 1.00±0.11| 0.07±0.03**   | 0.78±0.23**   |
| B-Mitf   | 1.00±0.09| 0.80±0.40     | 0.92±0.20     |
| J-Mitf   | 1.00±0.20| 0.19±0.06**   | 1.12±0.29**   |
| Otx1     | 1.00±0.11| 0.37±0.13*    | 0.45±0.04**   |

**P<0.05; ***P<0.001 (compared to R227W).  
**P<0.01 (compared to pan-Mitf).

Homeodomain-dependent DNA binding is required for VSX2 function

Two ways in which a mutation can alter the DNA binding properties of a protein are by changing the binding site preference (gain of function) or by reducing overall DNA binding affinity (loss of function). Our data support the contention that the VSX2[R200Q] protein is DNA binding deficient: the VSX2[R200Q] mutant protein failed to bind its preferred sites in vitro and in vivo and the R200Q allele is recessive. Important to note, however, is that the Otx1 transcript level in the R200Q retina was not upregulated, as in the otf and R227W retinas. On this basis, the R200Q allele behaves as a strong hypomorph since its phenotype at the cell and tissue levels overlaps with the null phenotype, but with at least one difference at the molecular level. While the low level of Otx1 also suggests that the VSX2[R200Q] protein can still regulate some downstream factors, it is unlikely to accomplish this through direct DNA binding. R200 corresponds to residue 53 of the homeodomain and an arginine at this position (HD-R53) is among the most conserved residues in the homeodomain. HD-R53 forms hydrogen bonds with two phosphates in the DNA backbone and is not directly involved with sequence specificity. Rather it orients the recognition helix into the major groove such that other homeodomain residues can make base-specific contacts, thereby enabling stable and sequence-specific DNA binding [2,3,70,71]. The glutamine substitution likely disrupted the ability of the mutant protein to productively dock in the major groove of the DNA in its binding site, thereby greatly reducing or eliminating its ability to directly regulate downstream target genes. Thus, the lack of Otx1 upregulation in the R200Q retina could be the result of a more complex mechanism, such as by interfering with or altering the transcriptional regulation of an interacting partner such as Mitf.

The CVC domain is required for high-affinity DNA binding by the homeodomain

Genetic data from humans, C. elegans, and now mouse confirm that the CVC domain is essential for the function of Vxs-class proteins. Our EMSA and ChIP data show that a single amino acid substitution in the CVC domain (R227W) weakened the DNA binding capacity of VSX2 to its preferred sites, indicating that optimal homeodomain function depends on the CVC domain. How the CVC domain assists the homeodomain is not clear. One possibility is that it recruits additional proteins required for DNA binding. Alternatively, the CVC domain may directly interact with the homeodomain to overcome a structural constraint on the homeodomain-DNA interaction, similar to that proposed for the C-terminal tail in PBX homeodomain proteins [12]. Structural limitations can be an intrinsic property of the homeodomain or the DNA binding site [14], but since the preferred binding sites of closely related homeodomain proteins such as Rx, Alx, and Arx overlap with the VSL proteins [10], and yet these proteins lack CVC domains, leads us to predict that any structural limitation will be endemic to VSX homeodomains.

The R227W allele is a recessive neomorph that activates a cryptic transcriptional circuit through its interaction with Mitf

Identifying the mechanisms underlying the mutant phenotypes revealed how R227W could surpass otf and R200Q in severity
During normal eye development, Mitf expression is activated in optic neuroepithelial cells (Mitf ONC) of the optic vesicle, presumably by signals that promote RPE specification and the pigmentation program [72]. This is followed by activation of Vsx2 in the presumptive retinal domain, which leads to repression of Mitf activity in RPCs (MitfRPC), effectively blocking the execution of the pigmentation program in these cells (Figure 10A). In orJ and R200Q mice, the ability to repress Mitf in RPCs is lost, leading to maintained Mitf activity and an increased probability that a pigmentation program will be activated. The primary differences are that in orJ mice, VSX2 protein is absent (Figure 10B) and in R200Q mice, VSX2[R200Q] protein is present but unable to bind DNA (Figure 10C). Like the orJ and R200Q mutants, Mitf expression also persists in early RPCs of the R227W mutant. Our *in vitro* reporter data indicates that the VSX2[R227W] protein can still repress D-Mitf, but the *in vivo* ChIP data suggests that its weak DNA binding may reduce or abrogate its repressive effects on the endogenous D-Mitf promoter. Ultimately, our data suggests the interaction between the VSX2[R227W] and Mitf proteins activates a positive feedback loop that significantly elevates the expression of p27, Otx1 and Mitf, leading to a robust pigmentation program and a blocked retinal program in R227W RPCs (Figure 10D). Our genetic data also suggests that p27 is a novel target specific to the R227W:MITF complex, even though Mitf is proposed to be a direct regulator of p27 expression in chick RPE [69].

Mechanistically, manifestation of the R227W phenotype likely occurs in two steps; the weakened DNA binding activity first acts as a partial loss-of-function that is sufficient to allow persistent Mitf expression. This is followed by the activation and maintenance of the feedback loop, which depends on the protein interaction between the VSX2[R227W] and MITF proteins. Since the MITF interaction is shared among the three VSX2 variants, it by definition is not a gain-of-function activity, which suggests there must be another activity unique to the VSX2[R227W] variant. This novel activity could be the result of the unique combination of the
Figure 9. Molecular interactions between the VSX2 variants and components of the pigmentation circuitry. (A) p27 mRNA expression in E12.5 retinas of the indicated genotypes as determined by qRT-PCR. Samples were normalized to orJ. Only the R227W retina was significantly different. (B) Luciferase activities from HEK293 cells transfected with the indicated expression vectors (x-axes) and 1.1 kb of the p27 promoter region (pGL3B-p27). Graph I: H-MITF repressed reporter activity in a DNA binding-dependent manner. Graph II: VSX2 and VSX2[R227W] enhanced reporter activity. Graph III: H-MITF combined with VSX2[R227W] elicited a specific and synergistic increase in reporter activity that depended on DNA binding as revealed by the abrogated activity of the VSX2[R200Q, R227W] double mutant (RQRW). Graph IV: Expression of the mi version of H-MITF had no effect on...
weak DNA binding caused by the \(R227W\) mutation and the interaction with MITF, ultimately leading to a change in transcriptional regulation of downstream targets, novel or otherwise. The VSX2\[^{[R227W]}\] protein may have acquired novel off-target binding properties allowing it regulate a suite of genes not normally regulated by Vsx2. While possible, this is unlikely on its own to explain the phenotype because acquisition of new binding site preferences is typically revealed as a dominant or semi-dominant gain of function phenotype, which was not observed. In addition to its effects on DNA binding, the \(R227W\) mutation may have induced a conformational change in the VSX2:MITF complex that alters how the complex interacts with DNA and regulates transcription. It is also possible that another interacting partner is involved and is present only in the \(R227W\) mutant. Distinguishing between these possibilities requires further genetic and molecular analyses.

Since the positive feedback loop is not found in the \(a\) or \(R200Q\) mutants, it is by definition a novel mechanism. Combined with the genetic properties then, the \(R227W\) allele best fits the classification of a recessive neomorph. Although predicted to exist over 80 years ago by H.J. Muller [73], reports of recessive neomorphic alleles are difficult to find. Recently, strong evidence for another atypical \(R227W\) was reported for the CXC domain-containing gene \(TSO1\) in Arabidopsis [74]. It is likely that more recessive neomorphs and antimorphs will be identified as targeted alleles are generated in genetic models that mimic disease-linked missense mutations found in natural populations. Their identification will require direct comparison to a corresponding null allele and may be more easily identified at loci that are highly susceptible to missense mutations and show a wide range of phenotypes.

A novel role for p27 as a regulator of transcription

In identifying the molecular mechanism driving the \(R227W\) phenotype, we uncovered evidence that p27 regulates transcription, specifically of the \(D-Mif\) isoform. This was unexpected because \(D-Mif\) levels are not dependent on p27 in the \(a\) retina (data not shown), indicating that this functional link is context-dependent. Consistent with this, partial genetic reduction in p27 expression in the \(R227W\) retina reduced \(D-Mif\) expression, which suggests that the elevated expression of p27 unmasked its transcriptional activity. It is not yet clear if p27 regulates transcription in the wild-type retina or if this is a common feature of p27 function. There are hints, however, that support these possibilities. p27 enhances reporter activity driven by \(Myelin Basic Protein\) promoter in an \(Sp1\)-dependent manner [75,76], p27 also binds to and stabilizes Neurogenin 2, a pro-neurogenic bHLH transcription factor [77]. In the retina, Cyclin D1 was recently shown to bind to chromatin and regulate genes such as Notch1 [78]. As Cyclin D1 and p27 interact genetically and biochemically [79,80], it is conceivable that misregulation of transcriptional targets could be a causative factor in the alterations in retinal development found in Cyclin D1 and p27 knockout mice [61,81–84].

Vsx2 acts as a gatekeeper allowing the retinal progenitor program to proceed without interference from other gene expression programs

The neuroepithelial-derived components of the eye field develop through a process of progressive specification from the anterior neuroectoderm and Vsx2 expression is arguably the strongest indicator of retinal domain specification [72]. To date, it is the only transcription factor expressed exclusively and comprehensively in the retinal domain and its onset of expression immediately precedes the formation of the optic cup, which coincides with the earliest morphological changes that distinguish RPCs from the remainder of the optic neuroepithelium [17,54]. Since Vsx2 expression initiates well after eye field specification, it stands to reason that activation of the RPC program is an actively promoted process. Consistent with this, eye field specification occurs and optic vesicles form in the \(vnx2\) mutant, but Vsx2 is not expressed and the RPC program fails to initiate [54]. Rather, the more likely fate following eye field specification is pigmented epithelium. Mitf is expressed throughout the optic neuroepithelium prior to Vsx2 expression and in mice in which FGF or BMP7 signaling is disrupted, Mitf expression persists in the presumptive retinal domain, which correlates with the failure to express Vsx2 and a high probability of pigmentation where the retina would normally form [72,85,86].

Despite these findings, Vsx2 does not act as a master regulator to specify or activate the RPC program because the retinal domain and optic cup still form in all three Vxs2 mutants. Retinal development, although compromised, still occurs in the absence of Vxs2 protein (\(a\) mouse) and this is not due to genetic compensation or functional redundancy by Vxs1 [32]. Importantly, the Vxs2 gene is still expressed in the Vxs2 mutants, indicating that initiation of the RPC program is independent of Vxs2 function, or in the case of the \(R227W\) mutant, despite the neomorphic behavior caused by the mutant protein. Thus, a primary role of Vxs2 is not to initiate specification, but rather to allow the RPC program to proceed without interference from competing gene expression programs. A failure to block these programs may not convert RPCs to another well-defined fate \(per\) se, but they hinder the RPC program and lead to, in some contexts, to aberrant differentiation as revealed by the pigmenta-tion phenotype.

Antagonizing Mitf activity through transcriptional repression is a key function for Vxs2 in this process. While a likely mechanism for D-Mif regulation [this study; [39]] other Mitf isoforms are also misexpressed in the Vxs2 mutant retinas and our data suggest that the H and A isoforms are not transcriptionally regulated by Vxs2. Supporting this last point is that A-Mitf is expressed in the wild-type retina during development [39]. Although A-Mitf may be
expressed below a threshold needed to disrupt the RPC program, it is noteworthy that in vivo overexpression of Mitf failed to interfere with retinal development as long as Vsx2 was expressed [41]. This suggests that an additional Vsx2-dependent mechanism exists for antagonizing Mitf activity and we propose that it requires VSX2 binding to MITF, preventing MITF from regulating genes important for pigmentation. This mechanism could be important during the initial specification of the retinal domain, when Vsx2 has not had enough time to efficiently downregulate Mitf activity through transcriptional repression (Figure 11A), and during retinal histogenesis for Mitf isoforms that are not transcriptionally repressed by Vsx2 (Figure 11B). In effect, this enables Vsx2 to block Mitf from interfering with the execution of the RPC program by regulating its activity at two levels. Interestingly, the functional significance of the protein interaction between Vsx2 and Mitf may also depend on high affinity DNA binding by Vsx2, which was suggested by the behavior of the VSX2[R200Q] and VSX2[R227W] proteins; both interacted with MITF but were unable to prevent Mitf-dependent alterations in retinal development. In the case of VSX2[R227W], the interaction likely contributed to the more severe Mitf-dependent effects.

In sum, this study provides an explanation for the stable association of the homeodomain and CVC domain in the VSX proteins through evolution. Our mutational analysis suggests that an essential function of the CVC domain is to assist the homeodomain in achieving high affinity binding to its preferred sites. Although the specific downstream targets of the VSX proteins may vary in vertebrates and invertebrates, the molecular requirement for the CVC domain in allowing high affinity DNA binding by VSX homeodomains was likely to have been established much earlier in animal evolution than the innovation of the vertebrate eye. In the case of Vsx2, the stability of the homeodomain-CVC domain arrangement may have provided a platform for Vsx2 or its archetype to acquire the ability to efficiently regulate a complex locus such as Mitf, a key step in mammalian eye organogenesis and retinal development. Finally, our study shows that the generation of a small, but targeted allelic series of mutations in mice has the potential to reveal insight into protein function and human disease etiology.

**Materials and Methods**

**Ethics statement**

Procedures involving mice were approved by the Institutional Animal Care and Use Committee at the University of Utah and conformed to the standards outlined in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Mice**

Vsx2<sup>129/SvJ</sup> (129/SvJ genetic background) and Mitf<sup>−/−</sup> (B6C3Fe genetic background) mice were obtained from the Jackson Laboratories (Bar Harbor, ME). p27 knockout mice (129/C57Bl6 hybrid genetic background) were provided by Drs. Matthew Fero and James Roberts (Fred Hutchinson Cancer Center, Seattle, WA). Vsx2<sup>R200Q</sup> chimeric mice were generated at inGenious Targeting Laboratory (Stony Brook, NY) and Vsx2<sup>R227W</sup> chimeric mice were generated at the University of Utah Gene Targeting and Transgenic Core Facility (See Text S1 and Figure S1 for details on gene targeting strategy). Germine transmission into the 129/SvJ genetic background was achieved by breeding chimeric mice with mice from the <i>or</i> strain. All alleles were identified by PCR genotyping (primers listed in Table S1). For timed matings, females were considered to be at 0.5 days of gestation (E0.5) at noon on the day a vaginal plug was detected.

**Proliferation assays**

P0 retinal cells from wild-type or <i>or</i> mice were dissociated with trypsin and triturated and cultured in DMEM/F12 medium with...
1% fetal bovine serum (FBS), growth supplements, and a penicillin/streptomycin mix (Invitrogen) [87]. This age was selected for these and other culture experiments in order to maximize the cell yield on the days the cultures were established. Cells were transfected one day after plating (cell confluence, 80%) with Lipofectamine and Plus Reagent (Invitrogen). Plasmids (Table S2) were transfected in equal amounts (0.6 μg per well in a 24-well plate, 0.2 μg per well in a 96-well plate). BrdU (10 μg/ml) was added for the last 4.5 hours of the culture period, starting at 44 hr post-transfection. Cultures were fixed with 4% PFA and stained with GFP, VSX2 and BrdU antibodies (Table S3). The percentages of BrdU+ cells in the VSX2+ or GFP+ cell populations were determined. A minimum of three independent trials was performed. Data were graphed as mean±SE. Statistical comparisons were done with the Kruskal-Wallis test. For simplicity, only statistical comparisons of the test conditions (black bars) versus the control (white bar) are shown.

Luciferase assays

Dissociated P0 retinal cells were plated onto poly-D-lysine and 15 μg/ml laminin in the same medium as described above. HEK293 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). In both cases, cells were transfected one day after plating (cell confluence ~80%) with Lipofectamine and Plus Reagent. Plasmids (Table S2) were transfected in equal amounts (0.6 μg per well in a 24-well plate, 0.2 μg per well in a 96-well plate). BrdU (10 μg/ml) was added for the last 4.5 hours of the culture period, starting at 44 hr post-transfection. Cultures were fixed with 4% PFA and stained with GFP, VSX2 and BrdU antibodies (Table S3). The percentages of BrdU+ cells in the VSX2+ or GFP+ cell populations were determined. A minimum of three independent trials was performed. Data were graphed as mean±SE. Statistical comparisons were done with the Kruskal-Wallis test. For simplicity, only statistical comparisons of the test conditions (black bars) versus the control (white bar) are shown.

CAT assays

HEK293 were cultured as described for Luciferase assays. 5 μg of LexA-Vsx2, –R200Q, –R227W or an equimolar amount of LexA were co-transfected with 0.5 μg of Gal4-HSF1 and 0.5 μg of X4G2CAT in 60-mm dishes at ~50% confluency with Lipofectamine and Plus reagent. CAT assays were performed 24 h post-transfection. The amount of lysate used for CAT analysis was determined by quantifications of western blots of Gal4-HSF1 in ImageJ (http://imagej.nih.gov/ij/). The CAT activity was counted in an LS 6000IC liquid scintillation system (Beckman, Fullerton, CA) 60 min after the addition of [3H]acetyl-CoA (Sigma-Aldrich, St. Louis, MO). Relative CAT activity was calculated by using control condition CAT activity as a normalization factor for each independent trial. Each trial was performed in duplicate and three independent trials were performed. Data were graphed as mean±SE. Statistical comparisons were done with Fisher’s Least Significance Difference (LSD) test and P-value ranges are shown only for test conditions (black bars) versus control (white bar).

Explant cultures

Ocular tissues were dissected from E10.5 embryos in HBSS. To implant POM, a local separation of the retina and lens was made.
with a 32G needle and POM was placed into the separated region. Explants were grown in the culture medium described for dissociated retinal cells. At the end of the culture period (2 days), explants were processed for immunohistochemistry or retinas were dissected free of other tissues and prepared for qRT-PCR.

**Immunohistochemistry**

Heads, eyes, or retinas were fixed with 4% PFA and cryopreserved [61]. Sections were cut at a thickness of 12 μm. Primary antibodies are listed in Table S3. Primary antibodies were followed with species-specific secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen) or Alexa Fluor 568 (Molecular Probes). Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

**Visualization of pigmentation in combination with fluorescence-based images**

Images of DAPI stained sections were captured with epi-fluorescence illumination followed by bright-field illumination (BF). BF images were adjusted in Photoshop (Adobe, San Jose, CA) to high contrast and pixel values were converted to their inverse using the *invert* image function. DAPI images were made transparent with the *screen* layer setting and the images were merged.

**Eye circumference measurements**

Eye circumference was assessed in image J and graphed as mean±SE. Statistical comparisons were done with the Kruskal-Wallis test.

**Quantitative real-time RT–PCR (qRT–PCR)**

Unless noted, retinal tissue was dissected from the surrounding RPE, POM, and lens following treatment with 40 μg/ml dispase (Sigma-Aldrich) in HBSS for 5 min at room temperature. Total RNA was isolated with the RNeasy Mini or Micro Kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). cDNAs were prepared with the Superscript RT III Kit (Invitrogen) using 20 ng of total RNA and purified with the PCR purification kit (Qiagen). cDNA corresponding to 2 ng of total RNA was used for each PCR reaction.

PCR was done on an ABI 7300 Real-Time PCR System with Power SYBR green PCR Master Mix and using the Relative Standard Curve Method for detection and measurement (Applied Biosystems, Invitrogen). Optimization was performed with serial dilutions of cDNA prepared from *afl* retina. Gapdh was used as the endogenous control mRNA for all samples. RNA preparations from *afl* or R227W retinas were used as calibrator samples. PCR product specificity was confirmed by gel electrophoresis and sequencing. Primer sets are listed in Table S1. Quantitative real-time PCR (qPCR) using the Relative Standard Curve Method was performed to assess the relative strengths of protein:chromatin interactions in each genotype. Results were expressed as the percentage enrichment normalized to the equivalent starting material (input). Statistical comparisons were done with the Kruskal-Wallis test and P-value ranges are shown for test conditions (black bars) versus IgG controls (white bars) in each genotype.

**Supporting Information**

Figure S1 Generation of R200Q and R227W mutant mice. (A) Sequence tracks of PCR products encompassing the R200Q or R227W mutations. Asterisks denote base substitutions. Template DNA was prepared from mouse-tail DNA (wild-type) and targeted ES clones (mutants). (B) Schematic representation of the targeting strategy. The R200Q and R227W mutations (asterisk) were introduced into exon 4 and the ACN cassette [46] was cloned into intron 3 of Vsx2. These elements were then placed into the pTK1TK2 targeting vector [88]. After homologous recombination, R200Q-ACN and R227W-ACN chimeric mice were generated. The ACN cassette was deleted by Cre recombination in the male germ line generating the R200Q and R227W alleles. Chimeric males were crossed with wild-type females to generate germ-line-transmitted R200Q and R227W heterozygous mice. B: BamHI, H: HindIII, N: NheI. (C) Southern blots of genomic DNA from electroporated ES cell clones containing homologously recombined R200Q-ACN and R227W-ACN insertions. HindIII digestion and 3′ flanking probe (shown in B) were used. The wild-type band is 6.7 kb and the correctly targeted band is 7.6 kb. (D) Genotyping of mutant mice. P1 and P2 primers shown in B (arrows) were used for PCR genotyping. As the mutant allele contains a 34 bp insertion, wild-type and mutant alleles give 258 bp and 292 bp bands, respectively. (TIF)
Figure S2  Proliferation changes associated with VSX2[R200Q] and VSX2[R227W] overexpression. (A) Micrographs showing expression of enhanced Green Fluorescent Protein fused to a nuclear localization signal (nlsGFP), VSX2, VSX2[R200Q], and VSX2[R227W] in transfected P0 αR7 retinal cells. (B) Phosphorylated histone H3 (pHH3) expression was reduced in the mutant retinae compared to wild-type. (C) Quantification of BrdU incorporation in P0 αR7 retinal cells overexpressing nlsGFP (control), VSX2, VSX2[R200Q] or VSX2[R227W]. (D) Quantification of BrdU incorporation in P0 wild-type retinal cells overexpressing nlsGFP (control), VSX2, VSX2[R200Q], or VSX2[R227W]. Cells were transfected at 24 hr following initial plating and cultured for an additional 48.0 hr. BrdU was added for the last 4.5 hr of the culture period. Only wild-type VSX2 enhanced proliferation over the control. * P<0.05; ** P<0.01 Scale bars: 100 μm. (TIF)

Figure S3  Marker expression in wild-type, αR7, R200Q, and R227W retinas at P0. (A–D) Neurofilament-M (NF-M) expression in wild-type, R200Q, or R227W/+ retina at E12.5. MITF expression in wild-type, R200Q, or R227W/+ retinas. p27 is also abundantly expressed in lens and periphery of the retina at this age. p27 staining to reveal staining in neuroblast layer. Bright staining at inner surface of central retina of wild-type is newly generated postmitotic precursors. Bright staining at the periphery of the R200Q retina is also observed occasionally in αR7 retina at this age. p27 is also abundantly expressed in lens and surrounding extraocular tissues. (TIF)

Figure S4  Marker expression in wild-type and R227W/+ retinas at P0. Expression patterns of TUBB3 (A,B), NF-M (C,D), and SOX2 (E,F) in wild-type and R227W/+ retinas were similar. Scale bars: 100 μm. (TIF)

Figure S5  Invasion of POM into the vitreal chamber in R227W eyes. (A) PITX2α cells filled the vitreal chamber in the E17.5 R227W eye. (B) The relative expression levels of D-Mitf and J-Mitf were not statistically different between control (−POM) and POM implanted whole retina and lens explant (POM imp) cultures (E10.5+2DIV). (TIF)

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