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The homeobox gene *CHX10/VSX2* regulates *RdCVF* promoter activity in the inner retina

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Abstract

Rod-derived Cone Viability Factor (RdCVF) is a trophic factor with therapeutic potential for the treatment of retinitis pigmentosa, a retinal disease that commonly results in blindness. RdCVF is encoded by Nucleoredoxin-like 1 (Nxn1), a gene homologous with the family of thioredoxins that participate in the defense against oxidative stress. RdCVF expression is lost after rod degeneration in the first phase of retinitis pigmentosa, and this loss has been implicated in the more clinically significant secondary cone degeneration that often occurs. Here we describe a study of the Nxn1 promoter using an approach that combines promoter and transcriptomic analysis. By transfection of selected candidate transcription factors, chosen based upon their expression pattern, we identified the homeodomain proteins CHX10/VSX2, VSX1, and PAX4, as well as the zinc finger protein SP3, as factors that can stimulate both the mouse and human Nxn1 promoter. In addition, CHX10/VSX2 binds to the Nxn1 promoter in vivo. Since CHX10/VSX2 is expressed predominantly in the inner retina, this finding motivated us to demonstrate that RdCVF is expressed in the inner as well as the outer retina. Interestingly, the loss of rods in the rd1 mouse, a model of retinitis pigmentosa, is associated with decreased expression of RdCVF by inner retinal cells as well as by rods. Based upon these results, we propose an alternative therapeutic strategy aimed at recapitulating RdCVF expression in the inner retina, where cell loss is not significant, to prevent secondary cone death and central vision loss in patients suffering from retinitis pigmentosa.

MESH Keywords Animals ; Eye Proteins ; genetics ; metabolism ; Gene Expression Regulation ; Genes, Homeobox ; Homeodomain Proteins ; genetics ; metabolism ; Humans ; Mice ; Mice, Inbred BALB C ; Mice, Knockout ; Promoter Regions, Genetic ; Protein Binding ; Retina ; metabolism ; Retinitis Pigmentosa ; genetics ; metabolism ; Thioredoxins ; genetics ; metabolism ; Transcription Factors ; genetics ; metabolism

INTRODUCTION

Retinitis pigmentosa (RP) is an inherited photoreceptor degeneration leading to progressive visual loss that affects approximately 1 in 3,000 individuals worldwide (1). RP is characterized by an initial loss of night and peripheral vision caused by the degeneration of rod photoreceptors, and this is generally followed by the loss of central visual acuity due to secondary degeneration of cone photoreceptors (2–4). Forty loci responsible for RP have been mapped and 31 of the responsible genes have been identified (<http://www.sph.uth.tmc.edu/RetNet/>). Most of the mutations identified to date have been found in genes encoding proteins that are expressed specifically or preferentially by rods (2, 5–7), and they generally act by triggering photoreceptor death by apoptosis (8, 9).

The rd1 mouse model of RP carries a recessive mutation in the beta subunit of rod phosphodiesterase (Pde6b). The mutation leads first to the rapid degeneration of rods, and this is followed by the death of cones (6, 10–12). The fact that the Pde6b gene is expressed solely by rods implies that the cones degenerate through a non-cell autonomous mechanism. Clinically, in humans, the secondary loss of cones is the event that represents the major visual handicap, since cones are necessary for color and high acuity vision.

To study the mechanisms underlying the secondary cone death, we transplanted the photoreceptor layer from normal mice into the eyes of rd1 mice just after rod loss and showed that these grafts resulted in the partial rescue of the host animals' cones (13, 14). In vitro, the trophic effect was shown to be mediated by protein(s) secreted by rods (13, 14). A cone viability activity was previously described (15), but it was the identification of rod-derived cone viability factor (RdCVF), a novel trophic factor specifically expressed by rods that was identified by expression cloning, that led to a therapeutic strategy aimed at preventing cone death and central vision loss in patients suffering from RP (16). This strategy, in theory, should apply regardless of the nature of the mutation causing the primary rod degeneration (1). In this model, the death of rods in the first phase of the disease results in the loss of expression of RdCVF, which in turn leads to cone death through a mechanism related to the loss of trophic support (17). Consistent with this model is the intriguing finding that the sequences of RdCVF and its paralogue RdCVF2 (18) are homologous to the family of thioredoxins (19, 20), proteins involved in the defense mechanisms against oxidative stress (21–23). RdCVF and RdCVF2 correspond to the unspliced products of the nucleoredoxin-like genes Nxn1 (Txnl6) and Nxn2, respectively, and encode enzymatically inactive proteins with a truncation within the

thioredoxin fold (18), similar to TRX80 (24). Interestingly, the spliced products of the nucleoredoxin-like genes, the longer isoforms RdCVFL and RdCVF2L, which encode putative thioredoxin reductase enzymes, may provide a way to link photo-oxidative stress that affects cones particularly (25) to an adaptive trophic response (26).

We hypothesize that protection of cones in RP could be achieved by recapitulating normal RdCVF expression in patients whose rods have been lost. One way to accomplish this would be through expression of exogenous RdCVF, for example by viral vector delivery (27), encapsulated cell technology (28, 29), or by protein injection (30). Another approach would be to increase retinal levels of RdCVF by modulating expression of the endogenous gene. The restricted expression of RdCVF to the retina (16) (see also Txnl6 at <http://biogps.gnf.org>) suggested that it might be possible to upregulate its promoter without significantly affecting expression in other tissues. With this goal in mind, we have undertaken an effort to define the Nxn1 promoter and the transcription factors that regulate its activity. We have taken advantage of the opportunities provided by analysis of the transcriptome of the outer retina to develop a bioinformatic approach to identify candidate transcription factors (TFs) that could regulate Nxn1 expression. This analysis identified 44 candidate TFs, and these were screened in a transient transfection assay for the ability to transactivate the Nxn1 promoter. We demonstrate here that the homeoproteins CHX10/VSX2, VSX1 and PAX4, and the zinc finger protein SP3 can activate both the mouse and human RdCVF promoters. The identification of CHX10/VSX2, VSX1, which have been reported elsewhere to be involved in bipolar cells differentiation (31, 32), led us to reexamine the expression of RdCVF in the retina. This reexamination demonstrated that RdCVF is not only expressed by photoreceptors as previously thought, but is also expressed in the inner retina, most likely by bipolar cells.

RESULTS

Selection of transcription factors candidates

In order to identify transcription factors (TFs) that were candidate regulators of the nucleoredoxin-like 1 (Nxn1) gene, two successive incremented filters were applied. First, the Transfac® database was used to identify transcription factors that are predicted to bind to the 4.2 kb region upstream of the mouse Nxn1 gene. This analysis yielded 133 predicted TFs (Supplementary Table 1). The second filter, which retained from this set only those TFs expressed in the photoreceptor-containing outer region of the retina (402 TFs), reduced the candidate list to 44 TFs (Table 1). This selection utilized Affymetrix-based transcriptome analysis of the outer retina of 35 day old mice. The expression profiles of this subset of 44 TF candidates can be classified into two patterns based upon relative expression in the photoreceptor region of the retina vs. the whole (neural) retina: 27 TFs have an outer retina/neural retina ratio above 1, indicating preferential expression in the photoreceptor layer (e.g. Crx) and 32 TFs whose retina/brain ratio is higher than 1, potentially indicating involvement in a function that is particularly important in the retina (e.g. Chx10/Vsx2). As deduced from the pattern of expressed sequence tags (ESTs) available in the public databases, some of the 44 candidate factors are preferentially expressed in the retina, while others are more widely expressed (Table 1).

Screening of candidate transcription factors for their activity to transactivate the Nxn1 promoter

Although the mouse and human Nxn1 5' upstream regions do not show any obvious sequence conservation (Supplementary Fig. 1), which we found to be a surprising finding given their similar expression patterns, we nevertheless screened the 44 candidate TFs on both mouse and human promoter constructs because we felt that the finding of common activity between the two species would likely indicate functionally important conservation that might not be evident from simple linear sequence comparison. For the transactivation assays, 4.2 kb and 2.1 kb of the mouse and human 5' Nxn1 upstream regions, respectively, were cloned upstream of the luciferase reporter plasmid pGL4. The 44 candidate TFs were tested by transient transfection of HEK 293 cells with increasing dose (0.3, 0.6 or 0.9 µg) of the respective expression plasmid, and reporter activity was tested by dual luciferase assay 48 hours post-transfection. Chx10/Vsx2, Gtf2i, Rora, Pax4, Yy1 and Sp3 were all found to activate the mouse promoter in a dose dependent manner (Fig. 1A). At the highest transfected dose (0.9 µg), Chx10/Vsx2 demonstrated 3.3 ± 0.2 fold activation (p < 0.001), Gtf2i 2.1 ± 0.1 (p < 0.001); Rora 3.3 ± 0.2 (p < 0.001), Pax4 8.0 ± 0.4 (p < 0.001), Yy1 1.5 ± 0.2 (p < 0.001) and Sp3 demonstrated 10.5 ± 0.5 (p < 0.001) fold activation. We also noticed that some TFs, as Cux1 and Max reduce the activity of the promoter in a dose-dependent manner. The potential role of homeogenes as Chx10/Vsx2 and Pax4 in the regulation of RdCVF expression was further highlighted when the 44 TFs were assayed on the human NXNL1 5' upstream region. Chx10/Vsx2 demonstrated 10.9 ± 0.9 fold activation (p < 0.001) at the highest dose (0.9 µg), Pax4 11.4 ± 0.6 (p < 0.001), Crx 3.7 ± 0.2 (p < 0.001), TLX2 4.2 ± 0.2 (p < 0.001), Sp3 6.9 ± 0.2 (p < 0.001), SP1 1.8 ± 0.1 (p < 0.001), Usf1 3.6 ± 0.1 (p < 0.001), Gtf2i 4.0 ± 0.2 (p < 0.001), Pcaf/Kat2b 2.5 ± 0.2 (p < 0.001), POU2F1 4.3 ± 0.1 (p < 0.001), and Yy1 2.0 ± 0.1 (p < 0.001) fold activation (Fig. 1B).

Activity of other homeodomain proteins on the mouse and human Nxn1 promoters

Since our bioinformatics analysis of the Nxn1 promoter could have missed some important regulators, and because of the high frequency of homeodomain (HD) factors identified in our transient transfection analysis (Fig. 1) together with the known importance of HD proteins as important regulators of retinal gene expression (33), we evaluated the ability of additional HD proteins expressed in the retina (RAX, IRX6, VAX2, VSX1, SIX6 and OTX2) for their ability to activate the Nxn1 promoter. On the mouse promoter, of the total of 8 HD factors tested, 4 showed dose-dependent activation activity (Fig. 2A). At the highest dose, Chx10/Vsx2 activated the promoter

3.6 +/- 0.3 (p < 0.001) fold, Irx6 4.3 +/- 0.2 fold (p < 0.001), Vsx1 5.4 +/- 0.4 fold (p < 0.001), and Six6 2.0 +/- 0.1 fold (p < 0.01). On the human promoter, 7 of the 8 HDs tested showed dose-dependent activation (Fig. 2B). At the highest dose, Chx10/Vsx2 activated the promoter 11.8 +/- 1.4 fold (p < 0.001), Irx6 3.0 +/- 0.1 fold (p < 0.01), Vax2 4.8 +/- 0.2 fold (p < 0.001), Vsx1 11.6 +/- 1.4 fold (p < 0.001), Six6 3.3 +/- 0.1 fold (p < 0.01), Otx2 5.1 +/- 0.1 fold (p < 0.001), and Crx 3.6 +/- 0.3 fold (p < 0.001). Chx10/Vsx2, Vsx1, Six6, and Irx6 regulated both the mouse and human constructs. These results highlight the potential importance of HD proteins as regulators of RdCVF expression. Furthermore, the fact that only a subset of the tested HD proteins were able to activate the RdCVF promoter, even though they all recognize a common TAAT core sequence, demonstrates substantial specificity in the reporter assay used in our analysis.

RdCVF is expressed by cells in the inner retina

From our combined studies, the TFs that showed activity with both the mouse and human promoters were Chx10/Vsx2, Vsx1, Pax4, Sp3, Six6, and Irx6. As noted above, we felt that these factors that were active on both the mouse and human Nxn1 promoter constructs were the strongest candidates to be biologically important regulators of RdCVF expression in vivo. We were therefore surprised to see Chx10/Vsx2 and Vsx1 in this group because both have been reported to be expressed in the inner nuclear layer (INL) of the retina but not in photoreceptors, and RdCVF was thought to be predominantly expressed in photoreceptors, although a possible signal was present in the INL (16). To resolve this uncertainty, we reexamined the expression pattern of RdCVF by in situ hybridization. Using an exon 1 riboprobe that should detect mRNA for both RdCVF and RdCVFL, we observed expression of RdCVF in the outer nuclear layer (ONL) of wild type mice, which is consistent with what was observed previously (16). However, we also detected a weaker but reproducibly present in situ signal in the INL. As a control to test whether this INL signal was truly due to Nxn1 expression or reflected background signal or perhaps cross-hybridization with another gene, we tested retinal sections from a Nxn1 null mouse (Fig. 3A) (Cronin et al., in revision). The absence of signal in both the ONL and INL of the Nxn1^{-/-} retina demonstrates that the observed signal in the wild type retina is real, indicating that in addition to the ONL, RdCVF is also expressed in the INL of the mouse retina.

According to our previous observations (16), RdCVF is expressed in a rod-dependant manner since it is easily detectable in the retina of PN8 rd1 mice, but it is not detectable at PN35, after rod degeneration (Fig. 3B). The loss of ONL expression of RdCVF seems explained by the loss of rods. However, the loss of RdCVF expression observed in the INL seemed surprising since there is no obvious loss of INL cells. Since Chx10/Vsx2 is involved in the differentiation of rod-bipolar cells, we examined the rod-bipolar cells in the retina at PN35 using protein kinase C α (PKC α , red) as a marker (34). We did not observe any difference in PKC α labeling between rd1 mice at PN8 and PN35 (Fig. 3B) or between the Nxn1^{+/+} and ^{-/-} retinas (Fig. 3A). These results suggest that the INL cells that were producing RdCVF are still present, but have turned off their expression of the gene.

Increased expression of Chx10/Vsx2 in the inner retina of rd1 mice

The expression profile of Chx10/Vsx2 was also studied by in situ hybridization in wild-type (wt) and mutant mice. The expression of Chx10/Vsx2 was localized in the INL of wt mice, consistent with a report (35), and was found to be expressed at a similar level in the retina of Nxn1^{-/-} mice (Fig. 4A). In the rd1 retina, we observed that expression of Chx10/Vsx2 was down regulated between PN8 and PN35 (Fig. 4B), as reported in the wild type mouse (36). However, at PN35, the expression of Chx10/Vsx2 is higher in rd1 compared to wt retina (Fig. 4B), an observation that we confirmed by real time PCR (Fig. 4C). One trivial technical explanation for the observed increased expression of Chx10/Vsx2 is that the lack of ONL cells at P35 causes the INL cells to be a higher proportion of the remaining total retinal cells, and hence RNA from bipolar cells, for example, would appear to represent a higher fraction of total retinal RNA. To take into account this potential "artifact", we analyzed the expression of Nrp2, a gene specifically expressed by ganglion cells (37) (Supplementary Fig. 2), since enrichment of ganglion cell RNA should parallel that of INL cells. The expression of Nrp2 was indeed increased in wt compared to rd1 retina (1.7 +/- 0.1 fold), in a proportion that corresponded to the loss of rod photoreceptors in the rd1 retina. Using this value for normalization, the observed 3.8 +/- 0.2 fold increase in Chx10/Vsx2 expression would represent an actual increase of 2.4 fold +/- 0.1, indicating that there is indeed a real increase in Chx10/Vsx2 expression at the cellular level in the rd1 retina after rod degeneration.

Chx10/Vsx2 binds the Nxn1 promoter in vivo

The above data is consistent with the possibility that Chx10/Vsx2 could regulate RdCVF expression in INL cells in vivo. In order to assess this hypothesis, we performed chromatin immunoprecipitation (ChIP) with mouse retina and anti-CHX10/VSX2 antibodies. The immunoprecipitate was used as a template for PCR with primers specific to the Nxn1, Vsx1 and β -globin promoter regions (negative control). As a positive control, we used the known interaction between CHX10/VSX2 protein and Vsx1 promoter (38). Compared to the no antibody control, amplification of the Nxn1 promoter region was significantly enhanced by the presence of the anti-CHX10/VSX2 antibody (Fig. 5). This shows that in the retina in vivo, a fraction of CHX10/VSX2 is bound to the Nxn1 promoter, supporting a model in which the activation of expression of RdCVF by CHX10/VSX2 is mediated by binding to an element within the promoter.

Synergistic activation of the Nxn1 promoters

To explore possible interactions and cooperativity between Chx10/Vsx2 and other selected TFs, we tested Chx10/Vsx2 activity individually or in combination with a subset of TFs (Fig. 6A and B). On the mouse promoter, the results showed that significant enhancement of transcriptional activation could be obtained upon co-expression of Chx10/Vsx2 and Vsx1 (+27.9% more than a simple additive effect), Chx10/Vsx2 -Pax4 (+25.8%), Chx10/Vsx2 -TLX2 (+46.2%). On the human promoter, the synergistic activity was observed for the same couple of TFs: Chx10/Vsx2 -Vsx1 (+69.4%), Chx10/Vsx2 -Pax4 (+96.4%), Chx10/Vsx2 -TLX2 (+13.9%). The fact that 0.9 µg of each TF transfected individually (Fig. 1 and 2) does not activate the RdCVF 5' region to the same extent as the combination of 0.5 µg of Chx10/Vsx2 + 0.5 µg of the other TF (Fig. 6) supports the synergetic effect observed here. Although Usf1 can activate the human Nxn1 Promoter, its co-transfection with Chx10/Vsx2 led to a reduction of activity with both the mouse and human Nxn1 promoter constructs.

DISCUSSION

A bioinformatic approach to study the Nxn1 promoter

The methodologies employed in the study of gene regulation at the promoter level classically involve gene reporter assays using transient transfection of luciferase, or other, reporters aimed at mapping the promoter sequences necessary and sufficient to drive the activity of the reporter constructs. Thereafter, this mapping information is used to identify, by diverse approaches including one-hybrid screening, the transcription factors (TF) that bind to these DNA elements (39, 40). The development of functional genomic approaches, such as transcriptomics, and bioinformatics tools allowed us to employ an alternative strategy. The sequences of DNA that define the binding sites of transcription factors are generally quite degenerate, and as a result programs aimed at identifying putative TF binding sites within promoter regions are often imprecise and give variable results depending upon the binding thresholds that are chosen, and result in considerable false positive and false negative predictions. For example, use of the Transfac® database identified 13,242 putative DNA binding elements within the 4.2 kb sequence 5' upstream of the translation initiation site of the Nxn1 gene. As an alternative approach, since the absence of phylogenetic conservation of the Nxn1 promoter region prevented us from using sequence conservation as a method to aid in the identification of functionally important sites, we reasoned that the transcription factors regulating the Nxn1 gene should be expressed in the outer retina. We further reasoned that those TFs that could transactivate both mouse and human Nxn1 promoter constructs would be particularly strong candidates as biologically relevant regulators. We have defined the transcriptome of the outer retina of the mouse using Affymetrix GeneChip®-based microarray analysis, work that took advantage of the layered structure of the retina which allows the purification of the outer retina by sectioning of flat-mounted retinas using a vibratome (41). Signal intensity was used to filter among the transcription factors that possess a predicted DNA binding element in the Nxn1 promoter, i.e. those whose expression was detected over a given threshold were chosen (see Material and Methods). The threshold used corresponds to the value above which the expression is unambiguous. This led us to establish a list of 44 candidate transcription factors that were tested in a transient transfection reporter assay. This novel approach was successfully developed and led to the identification of three transcription factors, two homeoproteins (CHX10/VSX2 and PAX4) and one zinc finger (SP3) protein that could regulate Nxn1 expression.

Chx10/Vsx2 regulates the activity of the Nxn1 promoter

By screening the list of candidate transcription factors selected through these bioinformatics and expression criteria, we identified three factors (Pax4, Chx10/Vsx2 and Sp3) that in a transient transfection-based assay demonstrated reproducible activity on both the mouse and human Nxn1 promoters. This result was further substantiated by the observation that another Chx10-like factor, Vsx1, also demonstrates a similar activity, and by the finding of synergistic activation of Chx10/Vsx2 with Vsx1, Pax4 and TLX2 on both mouse and human Nxn1 promoters. The proposed biological role of Chx10/Vsx2 in the regulation of RdCVF expression *in vivo* is supported by chromatin immunoprecipitation studies (Fig. 5).

In our transcriptomic data, the TFs were selected through their expression in the outer retina. Thus, the identification of Pax4 and Sp3 seemed reasonable given that Pax4 is expressed by photoreceptor cells (42) and the Sp3-related factor Sp4 regulates the photoreceptor gene Pde6b (43). However, given their exclusive expression in the inner retina (31, 32, 44, 45), the presence of Chx10/Vsx2 and Vsx1 in the outer retina preparation was unexpected and most likely results from a contamination during vibratome sectioning. Considering that RdCVF was originally described to be expressed by rod photoreceptors, its regulation by TFs normally present in the inner part of the retina was surprising. The data presented in this paper show that RdCVF is expressed in inner retinal cells in addition to photoreceptors (Fig. 3A). The exon 1 probe used detects mRNA encoding both the short and the long isoforms of RdCVF. Additionally, the two other homeoproteins, SIX6 and OTX2, that activate the Nxn1 promoter (Fig. 2), are expressed in the INL (46, 47), further supporting the role of these homeodomain proteins in the expression of RdCVF in the INL. The Nxn1 signal revealed by *in situ* hybridization most likely arises from bipolar cells since we have previously demonstrated by RT-PCR, using freshly dissociated cells, that Müller glial cells do not express RdCVF (16). While the role of the expression of RdCVF by bipolar cells is unclear, it is perhaps significant that CRX, a transcription factor expressed in photoreceptors that controls photoreceptor differentiation (48), is also expressed in the INL (36, 49). Additionally, since the Nxn1 gene encodes secreted proteins, its expression by bipolar cells may be related to the trophic support of

neighboring cells. The data presented here are consistent with what we have previously reported in 2004, with a lower Nxn1 signal in the inner retina (16).

An alternative strategy for preventing central vision loss

In the rd1 mouse, a model of retinitis pigmentosa, the expression by rods of the trophic factor RdCVF is lost through their degeneration, but the reduction in its expression by bipolar cells results from a downregulation of its expression (Fig. 3B). This phenomenon does not result from a decrease in Chx10/Vsx2 expression (Fig. 4B, C), nor of Vsx1 (data not shown). This observation indicates that Nxn1 expression is rod-dependant, and suggests that cell-to-cell communication between photoreceptor cells and bipolar cells is needed to maintain Nxn1 expression. As shown in figure 4B and C, it is possible that the increase of Chx10/Vsx2 expression by degenerating retina could represent a compensatory response for decreased photoreceptor-derived RdCVF, perhaps mediated by increasing the factors responsible for its expression by bipolar cells. This creates by extension an alternative therapeutic strategy aimed at recapitulation of RdCVF expression in the retina after rod degeneration by increasing its expression by bipolar cells. Such increased expression by bipolar cells could potentially sustain cones and thereby maintain central vision. This strategy is further substantiated by the finding that the injection of RdCVF protein in the P23H transgenic rat, a dominant model of rod-cone degeneration, not only protects the cones but preserves the cone ERG to an even greater extent (30). What could be the reason for the loss of expression of RdCVF by bipolar cells in the degenerating retina and what could be the best means to drive its expression after rod loss are matters for future research.

MATERIALS AND METHODS

Animals

Care and handling of mice in these studies conformed to the rules established by the Association for Research in Vision and Ophthalmology. Retinal tissues were obtained from post natal (PN) 8 or 35 day C3H wt/wt, C3H rd1/rd1 (50), BALB/c wt/wt, or BALB/c Nxn1 1-/- mice. Nxn1 1-/- mice were created by homologous recombination of the entire exon 1, which encodes the short isoform of RdCVF and the N-terminal part of RdCVFL, on a pure BALB/c background (Cronin et al., in revision). Animals were sacrificed and eyes were removed for in situ hybridization as described.

Promoter Reporter Plasmids Construction

The sequence upstream to ATG of both mouse and human nucleoredoxin-like 1 genes were cloned into the luciferase reporter plasmid pGL4.17 (Promega) using blunt-end cloning at the EcoRV site for the mouse 4.136 kb (chr8:74090479+74094614 at UCSC genome browser) 5' sequence of and with the gateway technology (Invitrogen) for 2.133 kb (chr19:17432676-17434808 at UCSC genome browser) of the 5' human NXNL1 sequence. Mouse genomic DNA was amplified by PCR with Pfx High fidelity polymerase (Invitrogen) using conditions provided by the supplier (Primers: Reverse 5'-GGTAGCAGTATGCAAGGAGCTG-3'/Forward 5'-GAGCACACAGCTGAGAC CGCAGGTACCGC-3'). For the human promoter construct, the pGL4.17 vector was converted into a gateway destination vector by introducing a cassette containing the att R sites flanking the ccd B gene into the multiple cloning sites. A DNA fragment corresponding to the human 5' sequence was obtained by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) with human genomic DNA as template. Primers were designed to amplify the 5' upstream region of the NXNL1 gene, and the att B sites were incorporated into the forward (att B site 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3') and the reverse (att B site 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3') primers. The PCR reactions were subjected to a hot start of 5 min at 94°C prior to the addition of the polymerase followed by 7 cycles of amplification: denaturing for 25 sec at 94°C, annealing for 7 min at 66°C, 35 cycles amplification: denaturing for 25 sec at 94°C, annealing for 30 sec to 7 min at 60°C and a final extension at 66°C for 10 min (Primers: Reverse 5'-GGCGGTAAC CTGGGTTGGGTGCTGGGGAC-3'/Forward 5'-CCTTGAAGTAACTAGTAGA GTCCATGTGA-3'). Positive clones were identified by restriction profiling and verified by DNA sequencing. DNA used for transient transfection was prepared using Qiagen plasmid maxi-prep according to the manufacturer's protocol.

Expression plasmids for transcription factors

Twenty one to 44 TFs come from a normalized neural retinal library. This mouse library was constructed using mRNA purified from neural retina of C57BL/6@N mice at 8 and 35 days post-natal by Cesium Chloride ultracentrifugation (51), followed by oligodT purification on oligoTex (Qiagen). The normalization was performed by Invitrogen and the normalized libraries in pCMVSPORT6 were swapped altogether into pEXP-cDNA3.1 (5.6 kb), by Gateway technology (Invitrogen). Large scale sequencing of this library was performed at the Genoscope (www.genoscope.cns.fr/spip/Mus-musculus-degeneration-of.html) using the 5' Sequencing primer: 5'-TTAATACGACTCACTATAGGG-3'. Plasmids from neural retinal normalized library are identified by their identifying number in the Table 1. Fifteen to 44 expression vectors were obtained from commercial sources. Gtf2i, Pcaf/Kat2b, Hand1, Elk1, SP1, TLX2 and POU2F1 (Origene); Mtf1, Deaf1, Mef2a, Tcf12, Sp3, Alx4, Smad4 and Rora (Invitrogen). The remaining 8 expression plasmids were generously provided by Ttf1 (I. Grummt), GATA4 (C. Asselin and W. L. Miller), Lhx3 (I. Bach), AR (H. Gronemeyer), ZEB1 (T.

Brabletz), Mnt (R.N. Eisenman), Pax4 (A. Mansouri) and Zic2 (Y. Yang). All expression vectors are driven by a CMV promoter except for AR (SV40 promoter). All plasmids were constructed from mouse sequences except SP1, TLX2, POU2F1, AR and GATA4 derived from human sequence and ZEB1 from chicken.

In silico identification of potential regulatory elements

The analysis was performed on the Nxn1 5' genomic region of 4.2 kb upstream of the translation initiation site to predict transcription factor binding elements potentially involved in the regulation of the expression of this gene. The TRANSFAC® professional database release 9.1 (<http://www.biobase-international.com>) (52, 53) and the MATCH program (54, 55) were used with the profile containing the high quality matrices allowing the minimization of the false positive and false negative predictions.

RNA purification

The Cesium chloride (CsCl₂) method used here was based on the protocol described by Glisin et al (51). RNA integrity was assessed by denaturant gel electrophoresis, using a method adapted from published procedures (56–58).

Transcriptomic analysis

The neural retina was dissected as in (59) and the brain sample corresponds to the whole brain hemisphere including the cerebellum. The outer retinal cells of the C57/BL6@N at PN35 (n = 5) were isolated using vibratome sectioning as described previously (41). The RNA was purified by CsCl₂ ultracentrifugation as previously described and used to generate double-stranded cDNA, then transcribed in vitro to form biotin-labeled cRNA, fragmented and hybridized to the mouse Affymetrix U74v2 GeneChips® Array for 16 h at 45°C. Arrays were washed and stained using standard protocols (60). Scanned data (.DAT file) were captured by the Affymetrix GeneChip® Laboratory Information Management System that allows for the average intensities for all probes cells (.CEL file). Quality control performed using RReportGenerator (61) confirmed that all arrays used in the study were of good and consistent quality (available on request). To select the transcription factors, a filter was used to retain 402 TFs with a signal intensity value superior to 40 units in the sample prepared from the outer retina at PN40 after normalization by Robust Multi-array Average (RMA) (62).

Retinobase utilization

Retinobase provides efficient access to the global expression profiles of genes from different tissues (Outer Retina, Neural Retina, and Brain) of C57/BL6@N mouse at PN35 (62). After connection on the site <http://genoret.igbmc.fr/RetinoBase/TranscriptomicGraphManager.php> using either Internet explorer or Mozilla firefox enter the user name [guest] and password [star128], 2- In "Experiment" select Exp-34:subPG161-NeuraleRetina-OuterRetina-Brain-PN35, 3- In "related Arraytype" select the Mouse Genome U74ABv2, 4- In the new link, select "Search Probesets", click on "Term search", enter a keyword or when known, the code of the gene and press "Search", 5- In the table, select first the gene which possesses an RMA profile and then select the RMA column (alternatively dChip), 6- In the new link, select "Graphes" and change the "graph type" radar to histogram, 7- Press "Get graph" to validate and see the results.

Luciferase assay

Griptide 293 MSR cells (Invitrogen) were cultivated with DMEM (Gibco) with 10 % BSA (Gibco), 600 µg/ml geneticin and subcultured a minimum of 3 passages after thawing before used. For transient transfection assays cells were seeded to 80–90 % confluency in 96-wells plates, then after overnight incubation were transfected with Lipofectamine 2000 (Invitrogen) following manufacturer instructions. Before transfection, the medium was replaced by 100 µl of OPTI-MEM (Invitrogen) without serum. For all transfection, the total quantity of DNA and the volume of reagents and medium added to each well were equalized. For testing candidate transcription factors, a reaction mixture containing 1 µg of DNA in 100 µl (Opti MEM/lipofectamine 2000) corresponding to of 0.1 µg of luciferase reporter, 0.0 to 0.9 µg of plasmids encoding for candidate TF, 0.0 to 0.9 µg of pcDNA3.1+ plasmid (empty vector) and 1 ng of internal control pRL-TK (thymidine kinase promoter, Promega). We divided this transfection reaction in triplicates of 20 µl that were added to the cells in three wells. To assay for synergetic effect, a reaction mixture of 1.1 µg DNA in 100 µl (Opti MEM/lipofectamine 2000) containing 0.5 µg of individual transcription factor and 0.5 µg pcDNA3.1+ or, alternatively 0.5 µg TF#1 + 0.5 µg TF#2 was mixed with 0.1 µg of reporter plasmid and 1 ng of internal control plasmid pRL-TK. This reaction was used to perform triplicates. The cells were lysed with 45 µl of 1× passive lysis buffer (Promega) after 48 hours of culture. To measure luciferase activity using Dual Luciferase assay (Promega), 50 µl of firefly luciferase reagent and 50 µl of Renilla luciferase reagent were subsequently injected to 20 µl of cell lysate in each well and recorded using a luminometer (MicroLumat Plus LB 96V, Berthold) after 2 sec delay for 10 sec. Each experiment was repeated independently three times.

In situ hybridization and immunostaining

In situ hybridization was performed as described in Chotteau-Lelièvre et al. (63). Mouse eyes were fixed overnight in 4 % paraformaldehyde in PBS at 4°C, incubate 1 hour in 10 % sucrose/PBS and incubate overnight in again 20 % sucrose/PBS at 4°C. The eyes

were orientated and flash frozen in O.C.T (Shandon Cryomatrix, Thermo) using liquid nitrogen steam. In situ hybridization analysis was performed with digoxigenin-labeled riboprobes on 8 µm cryostat sagittal section through the optic nerve. The Nxn11 probe was generated from pGEM-T plasmid (Promega) into which a 332 bp fragment of mouse Nxn11 exon 1 (Primers, 5'-ATGGCATCTCTCTCTCTGGACG-3'/5'-CCTCACCTCCTCAGTTCATCAT GG-3'). The plasmids were linearized with either SacII (sense probe) or SacI (antisense probe). Chx10/Vsx2 probes (Generously gift by Sandro Banfi, TIGEM) corresponding to the ORF of Chx10/Vsx2, was linearized with NotI (sense probe) or EcoRI (antisense probe). Purification of the riboprobes was performed by size exclusion on a G-50 column (ProbeQuant, Amersham).

Chromatin Immunoprecipitation

The Chromatin Immunoprecipitation (ChIP) was performed as described previously (64) (65). Briefly, retinas from PN35 BALB/c wild-type (wt) mice were frozen on liquid nitrogen and kept at -80°C until use. Retina was cross-linked with ice-cold 4 % formaldehyde in phosphate-buffered saline (PBS) for 30 min, rinsed in PBS, and sonicated in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0) plus protease inhibitors (Sigma) to an average DNA size of 1 kb (Vibra Cell, Sonics and Materials Inc., Danbury, CT). The sonicated sample was centrifuged at 15,000 g for 10 min at 4°C, the supernatant, precleared with G-sepharose beads at room temperature (RT), was aliquoted to 100 µl (equivalent to 1 whole retina) and diluted to 1 ml with dilution buffer (1 % Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.0). Each diluted sample was incubated for 1 h at RT with 5 µl of anti-CHX10/VSX2 N-terminal antibody (Exalpha Biologicals). Samples were centrifuged at 15,000 g for 10 min at 20°C, the supernatant mixed with 40 µl of protein G-Sepharose (Sigma), 200 µg of sonicated salmon sperm DNA and 2 mg of yeast tRNA (Invitrogen), and incubated for 1 hour at RT. Precipitates were washed sequentially for 10 min at RT in 1× TSEI (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), 4× TSEII (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl), 1× buffer III (0.25 M LiCl, 1 % Nonidet P-40, 1 % deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0), and 3× TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Samples were then eluted and cross-links reversed by overnight incubation at 65°C in 100 µl of elution buffer (1 % SDS, 0.1 M NaHCO₃) and DNA fragments were purified by phenol-chloroform and resuspended in 100 µl of TE' (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). PCR was used to amplify 2 µl of the final immunoprecipitated material. The Primers used was 5'-CTGGTGGTGATG AAGATCTCGGCC-3'/5'-GGGGAGCAG GTGCAGAGAATCTGAT- 3' for the Nxn11 promoter, 5'-GCCGAAATTTGGATTTA CGA-3'/5'-TGGATGAGTGGGGAGAAAT C-3' for the Vsx1 promoter and 5'-TTACTTGAGAGATCCTGACTCAACAATAA-3'/5'-TCAATAACTGCCTTCA GAGAATCG-3' for the β-Globin promoter. PCR reaction was performed in 25 µl for 94°C 3 min, 35× [94°C 30 sec-60°C 30 sec-72°C 1 min], 72°C 3 min.

Reverse transcription and real-time PCR

One µg of RNA was used for the retro transcription with Superscript II RNase H- (Invitrogen) following manufacturer instructions. The cDNA were purified by phenol-chloroform extraction and ethanol precipitation and dissolved in 50 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). For the real-time PCR (Light cycler, Roche), 2 µl of 1/10 cDNA were used in a 25 µl total volume containing H₂O, 2.5 µM of each primer, 5× FastStart LCDNA master SYBER (Roche) and amplified for 1× 95°C 10 min, 35× [2 sec at 95°C; 5 sec at 60°C, 10 sec at 72°C]. The melting temperature was measured after 2 min at 95°C by increasing the temperature from 65°C to 95°C at a rate of + 0.1° C/sec. The primers used was 5'-TGCTGGCCTCGGAACTGCTCTT-3'/5'-AGGACGCTGGATCGGGAGTATGTC-3' for the Chx10/Vsx2 and 5'-CCCCATCC CGAATCTCAATGAAGT-3'/5'-CAGGA CACGAAGTGAGAAGCCAGC-3' for the Neuropilin 2 (Nrp2).

Tissues Expression Profile bioinformatic analysis

The expression profiles on the candidate TFs in Table 1 and 2 were analyzed with data available at NCBI. The count of EST for each gene was calculated for all tissues reported in unigene (e.g. Chx10/Vsx2, <http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.4405>).

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Footnotes:

CONFLICTS OF INTEREST No

ABBREVIATIONS

ChIP : Chromatin Immunoprecipitation

HD : Homeodomain

INL : Inner Nuclear Layer

NR : Neural Retina

ONL : Outer Nuclear Layer

OR : Outer Retina

PN : Post Natal

RdCVF : Rod-derived Cone Viability Factor

RP : Retinitis Pigmentosa

TF : Transcription Factor

wt : wild-type

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Figure 1

Transcription factors activity on both mouse and human Nxn1 promoter constructs. (A) Assay with the murine 4.2 kb Nxn1 5' upstream region. (B) Assay with the human 2.1 kb NXNL1 5' upstream region. The TFs were tested by transient transfection of 0.3, 0.6 and 0.9 μg of plasmid coding for each candidate TFs and 0.1 μg of reporter plasmid in HEK 293 cells and assessed by dual luciferase assays. The results were normalized by using an internal control plasmid (Renilla) driven by the thymidin kinase promoter. Each experiment was repeated in triplicate. $p < 0.001$ (***) ; $p < 0.01$ (**); $p < 0.05$ (*).

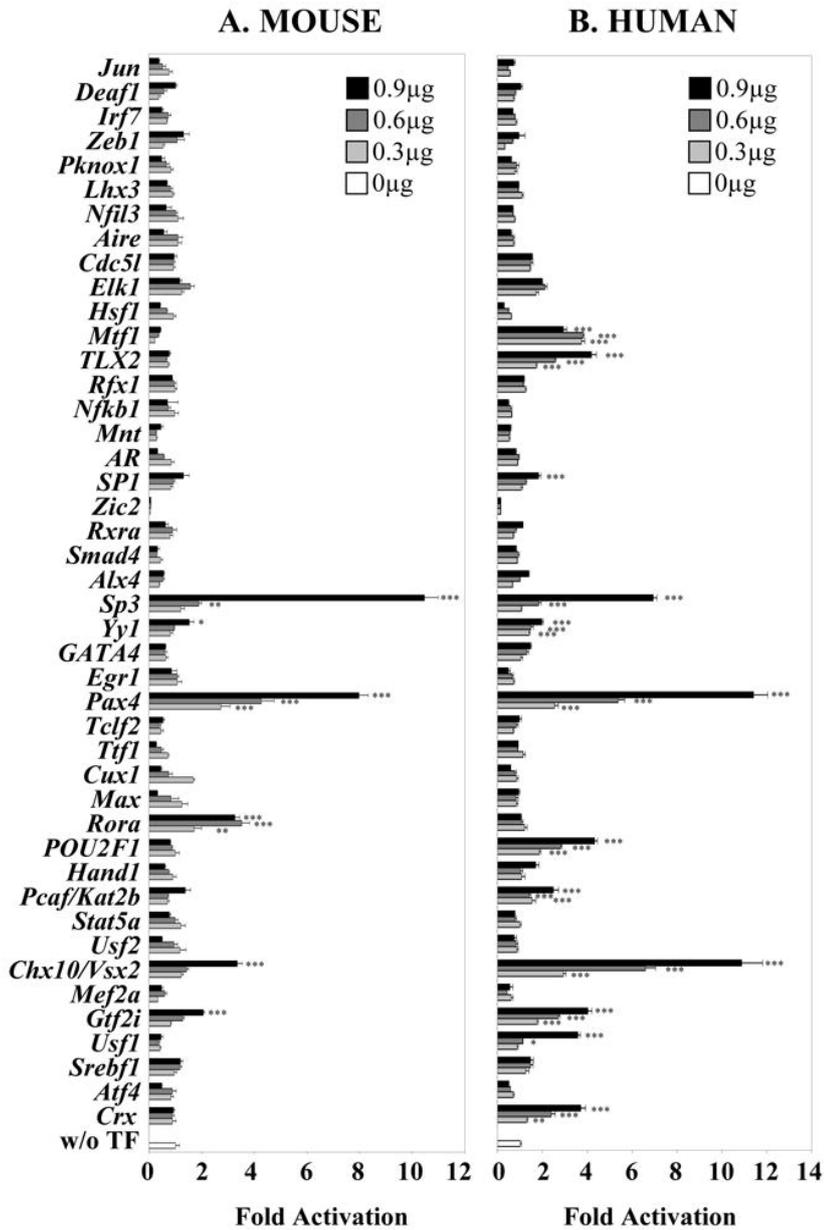
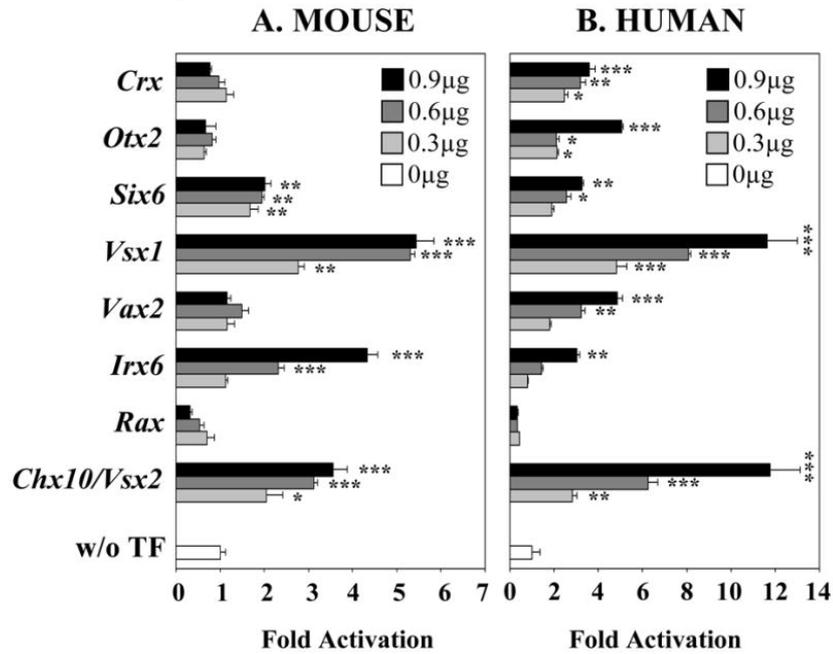


Figure 2

Activity of retinally-expressed homeogenes on mouse and human *Nxn1* promoters. constructs (A) Activity of homeogenes on mouse *Nxn1* promoter, and (B) on human *NXNL1* promoter. The HD TFs were tested by transient transfection of 0.3, 0.6 and 0.9 μ g of plasmid coding for each candidate HDs TF and 0.1 μ g of reporter plasmid in HEK 293 cells and assessed by dual luciferase assays. The results were normalized by using an internal control plasmid (Renilla) driven by the thymidin kinase promoter. Each experiment was repeated in triplicate. $p < 0.001$ (* **); $p < 0.01$ (**); $p < 0.05$ (*).

**Figure 3**

Nxn1 is expressed in the outer and inner retina. (A) In situ hybridization and immunolocalization of PKC α (red) on *Nxn1* $+/+$ or *Nxn1* $-/-$ retinal sections from mice at PN35. (B) In situ hybridization and immunolocalization of PKC α (red) on *rd1* mice at PN8 and PN35 aligned at the INL level. Nuclei stained with DAPI (blue). ONL, Outer Nuclear Layer; INL, Inner Nuclear Layer. AS, antisense and S, sense riboprobes. Bar scale 50 μ m.

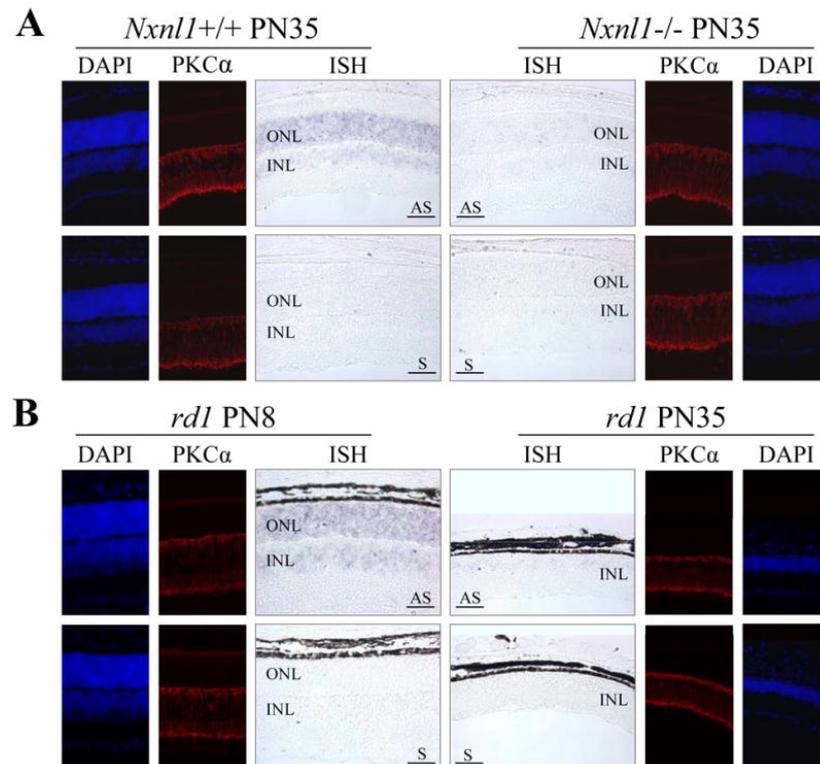


Figure 4

Chx10/Vsx2 expression profile on *Nxn1* +/+, *Nxn1* -/- and *rd1* mouse retinas at postnatal 8 and 35. (A) In situ hybridization on *Nxn1* +/+ or *Nxn1* -/- retinas at PN35. (B) In situ hybridization on *rd1* mice at PN35 or PN8 and wt at PN35, aligned at the INL level. (C) Relative gene expression level in wild type mice and *rd1* at PN35. *Nrp2*, neuropilin 2 (Mm.266341). Nuclei are stained with DAPI (blue). ONL, Outer Nuclear Layer; INL, Inner Nuclear Layer. AS, antisense and S, sense riboprobes. Bar scale 50 μ m. $p < 0.001$ (***)

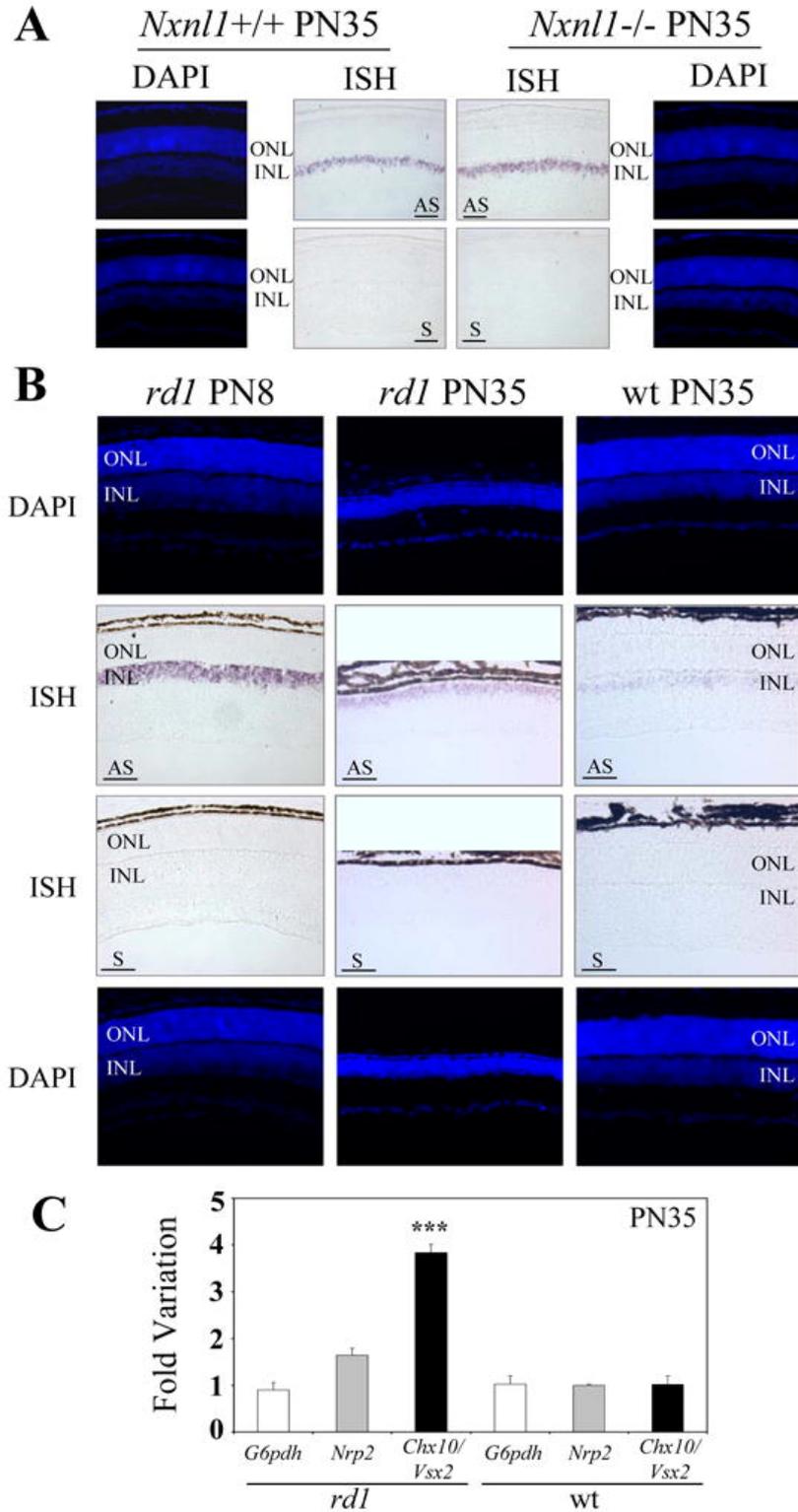
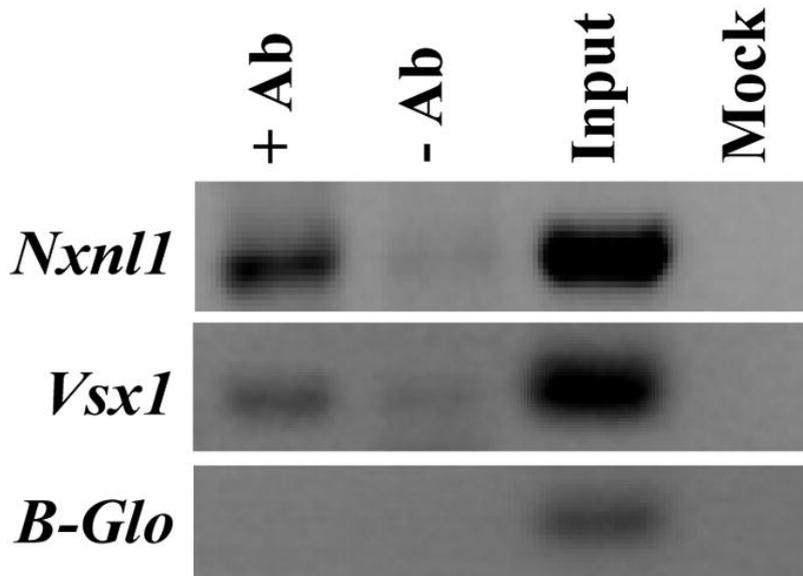


Figure 5

CHX10/VSX2 binds to the *Nxn1* promoter in vivo shown by chromosome immunoprecipitation assay (ChIP). ChIP was done with wild-type mouse retina. +Ab, anti-CHX10/VSX2 antibodies. -Ab: no antibody, Input: mouse genomic DNA. Mock: without mouse genomic DNA. *Vsx1* and β -Globin (β -Glo) promoters amplification were used as positive and negative controls, respectively.

**Figure 6**

Chx10/Vsx2 acts with other transcription factors to synergistically activate the *Nxn1* promoter. Activity of the Chx10/Vsx2 with *Vsx1*, Pax4, TLX2, Usf1, Sp3 and Yy1 TFs on the (A) mouse and (B) on human *Nxn1* promoter. Activity tested by transient transfection in HEK 293 cells with dual luciferase assay. 0.5 μ g of each TF was tested individually or paired with 0.5 μ g of Chx10/Vsx2 in cotransfection assays with 0.1 μ g of reporter plasmid. The results are expressed as percentage of synergistic activation over the sum of both TFs when tested individually. The results were normalized by internal control (Renilla) driven by the thymidine kinase promoter. Each experiment was repeated in triplicate.

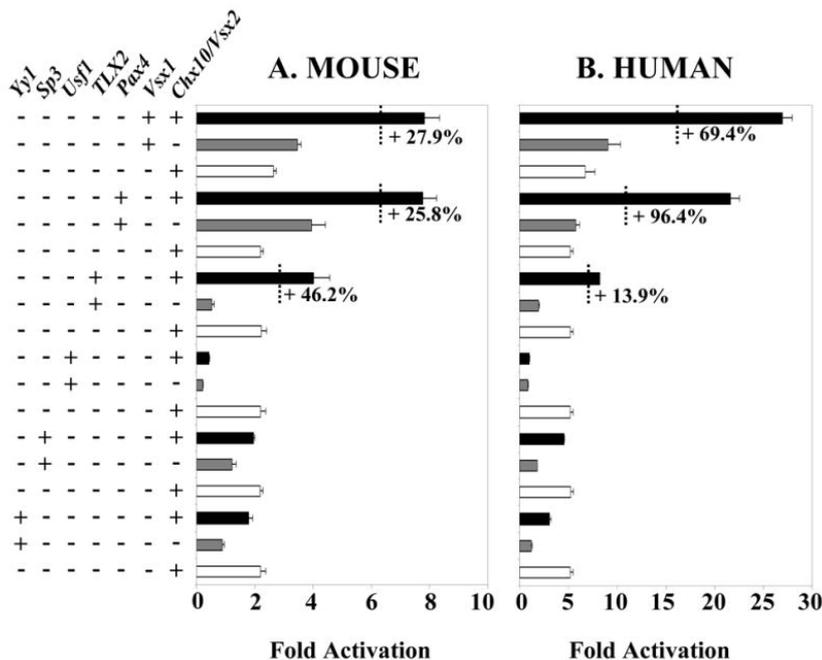


Table 1

Expression level of the transcription factors selected in the study.

Gene ID NCBI	Gene Name	Gene Symbol	Probe Set ID	Signal Intensity (RMA)			OR/NR	Tissue		Clone Number
				OR	NR	Brain		OR/NR ≤ 5	> 5 ND	
1406	cone-rod homeobox containing gene	Crx	99900_at	1496.55	1028.57	138.36	1.45	•	LA0AAA31YM07	
11911	activating transcription factor 4	Atf4	100599_at	1162.04	834.98	526.77	1.39	•	LA0AAA82YD23	
20787	sterol regulatory element binding transcription factor 1	Srebf1	93264_at	603.15	620.27	344.61	0.97	•	3030000054066890	
22278	upstream transcription factor 1	Usf1	93655_at	509.79	502.43	565.12	1.01	•	LA0AAA18YG17	
14886	general transcription factor II I	Gtf2i	94295_at	490.59	657.20	443.33	0.75	•	O-MC203628	
17258	myocyte enhancer factor 2A	Mef2a	93852_at	467.42	256.30	334.67	1.82	•	I-4979487	
12677	visual system homeobox 2/C. elegans ceh-10 homeo domain containing homolog	Chx10/Vsx2	101127_at	422.56	676.39	174.46	0.62	•	LA0AAA60YM14	
22282	upstream transcription factor 2	Usf2	103013_at	403.89	381.70	336.32	1.06	•	LA0AAA14YJ06	
20850	signal transducer and activator of transcription 5A	Stat5a	100422_i_at	348.98	393.99	275.96	0.89	•	LA0AAA118YN05	
18519	p300/CBP-associated factor	Pcaf/Kat2b	104070_at	347.69	270.53	107.61	1.29	•	O-MC206206	
15110	heart and neural crest derivatives expressed transcript 1	Hand1	92766_at	298.25	272.02	275.05	1.10	•	O-MC201881	
18986	POU domain, class 2, transcription factor 1	Pou2f1	102894_g_at	287.05	198.01	105.69	1.45	•	O-SC321473	
19883	RAR-related orphan receptor alpha	Rora	101889_s_at	250.18	246.88	130.27	1.01	•	I-3592667	
17187	Max protein	Max	99095_at	249.53	168.31	128.92	1.48	•	LA0AAA9YP20	
13047	cut-like 1	Cutl1/Cux1	98073_at	244.28	247.43	245.71	0.99	•	LA0AAA19YG15	
22130	transcription termination factor 1	Ttf1	96435_at	242.04	200.37	165.17	1.21	•	pEGFP-C1-TTF1	
21406	transcription factor 12	Tcf12	98981_s_at	230.07	183.68	127.60	1.25	•	I-5345693	
18506	paired box gene 4	Pax4	99908_at	223.70	212.43	236.84	1.05	•	pCMV5-Pax4	
13653	early growth response 1	Egr1	98579_at	222.67	147.48	210.74	1.51	•	LA0AAA78YH20	
14463	GATA binding protein 4	Gata4	102713_at	212.37	206.01	222.56	1.03	•	pCDNA3-GATA4	
22632	YY1 transcription factor	Yy1	98767_at	212.22	207.68	170.07	1.02	•	LA0AAA124YE13	
20687	trans-acting transcription factor 3	Sp3	96192_at	207.29	155.13	103.29	1.34	•	I-5323205	
11695	aristaless 4	Alx4	101622_at	205.97	207.49	210.68	0.99		I-6506755	
17128	MAD homolog 4 (Drosophila)	Smad4	160440_at	193.88	170.61	112.18	1.14	•	I-6313280	
20181	retinoid X receptor alpha	Rxra	92235_g_at	178.40	153.92	167.35	1.16	•	LA0AAA26YE01	
22772	Zic finger protein of the cerebellum 2	Zic2	98844_at	172.96	194.99	186.02	0.89	•	pcDNA-Zic2	
20683	trans-acting transcription factor 1	Sp1	100032_at	163.35	123.95	100.51	1.32	•	O-SC101137	
11835	androgen receptor	Ar	92667_at	150.68	134.52	145.60	1.12	•	pSG5-Ar	
17428	max binding protein	Mnt	92300_at	149.80	176.99	154.56	0.85	•	pRc/CMV-Mnt	
18033	nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	Nfkb1	98427_s_at	149.05	166.08	155.34	0.90	•	LA0AAA79YO10	
19724	regulatory factor X, 1 (influences HLA class II expression)	Rfx1	99880_at	146.38	117.93	105.17	1.24	•	LA0AAA111YO22	
21909	T-cell leukemia, homeobox 2	Tlx2	97679_at	146.02	159.19	136.67	0.92	•	O-SC123839	
17764	metal response element binding transcription factor 1	Mtf1	100018_at	144.31	161.30	138.83	0.89	•	I-4210829	
15499	heat shock factor 1	Hsf1	100451_at	144.31	129.06	108.95	1.12	•	LA0AAA40YJ15	
13712	ELK1, member of ETS oncogene family	Elk1	96593_at	139.54	128.23	146.58	1.09	•	O-MC201893	

71702	cell division cycle 5-like (<i>S. pombe</i>)	Cdc51	97846_at	126.01	139.73	103.93	0.90	•	3030000053137993
11634	autoimmune regulator (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy)	Aire	97159_at	122.46	113.97	111.15	1.07	•	LA0AAA87YA17
18030	nuclear factor, interleukin 3, regulated	Nfil3	101805_f_at	119.72	121.57	117.12	0.98	•	LA0AAA21YP14
16871	LIM homeobox protein 3	Lhx3	102902_at	116.83	123.03	74.42	0.95	•	pCDNA3-Lhx3
18771	Pbx/knotted 1 homeobox	Pknox1	102257_at	109.04	104.19	94.44	1.05	•	LA0AAA33YP05
21417	zinc finger E-box binding homeobox 1	Zeb1	99052_at	106.50	127.10	151.06	0.84	•	pcl-Flag-Zeb1
54123	interferon regulatory factor 7	Irf7	104669_at	103.14	106.28	83.20	0.97	•	LA0AAA120YH12
54006	deformed epidermal autoregulatory factor 1 (<i>Drosophila</i>)	Deaf1	96171_at	101.56	105.33	99.68	0.96	•	I-5063995
16476	Jun oncogene	Jun	100130_at	99.73	87.21	105.48	1.14	•	LA0AAA20YJ01

OR, outer retina NR, neural retina. The tissue distribution of ESTs within the public database (≤ 5 tissues with eyes), large (> 5 tissues with eyes) or no determined (ND) of each TFs in mouse (•) tissues (according to UniGene, NCBI). Clone number refers to the sequenced library or to the plasmids used.

Table 2

Expression level of the Homeogenes selected in the study.

Gene ID NCBI	Gene Title	Gene Symbol	Probe Set ID	Signal Intensity (RMA)				Tissue		
				OR	NR	Brain	OR/NR	≤ 5	> 5	ND
19434	retina and anterior neural fold homeobox	Rax	97149_at	447.86	431.18	130.36	1.04	•		LA0AAA52YA10
64379	Iroquois related homeobox	Irx6						•		LA0AAA16YO24
24113	ventral anterior homeobox containing gene 2	Vax2	99817_at	263.54	259.22	204.13	1.02	•		LA0AAA102YH09
114889	visual system homeobox 1 homolog (zebrafish)	Vsx1						•		LA0AAA53YE15
20476	sine oculis-related homeobox 6 homolog	Six6	101797_at	62.90	56.44	57.46	1.11	•		LA0AAA107YP10
18424	orthodenticle homolog 2 (<i>Drosophila</i>)	Otx2						•		LA0AAA28YF06

OR, outer retina NR, neural retina. The tissue distribution of ESTs within the public database (≤ 5 tissues with eyes), large (> 5 tissues with eyes) or no determined (ND) of each HDs in mouse (•) tissues (according to UniGene, NCBI). Clone number refers to the sequenced library.