

**THE HOMEBOX GENE CHX10/VSX2 REGULATES RDCVF PROMOTER
ACTIVITY IN THE INNER RETINA**

**Sacha Reichman¹, Ravi Kiran Reddy Kalathur², Sophie Lambard¹, Najate Ait-Ali¹,
Yanjiang Yang³, Aurélie Lardenois², Raymond Ripp² Olivier Poch², Donald J. Zack^{1,3},
José-Alain Sahel¹ and Thierry Léveillard^{*1}.**

¹Department of Genetics, Institut de la Vision, INSERM Université Pierre et Marie Curie-Paris6, UMR-S 968, Paris, F-75012 France, ²Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), 67404 Illkirch CEDEX France, ³Wilmer Ophthalmological Institute, Johns Hopkins University School of Medicine, 600 N. Wolfe St. Baltimore, MD 21287 USA.

*Corresponding author: Thierry Léveillard, Department of Genetics, Institut de la Vision, INSERM, UPMC Univ Paris 06, UMR-S 968, CNRS 7210, Paris, F-75012 France. Tel : 33 1 53 46 25 48, Fax : 33 1 53 46 25 02, Email : thierry.leveillard@inserm.fr

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 (Nxn1l)*, 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 *Nxn1l* 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 *Nxn1l* promoter. In addition, CHX10/VSX2 binds to the *Nxn1l* 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.

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* (*Txn16*) 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 thioloxydoreductase 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 *Txn16* 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 *Nxn11* 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 (*Nxn11*) 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 *Nxn11* 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 *Nxn11* promoter

Although the mouse and human *Nxn11* 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' *Nxn11* 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 μ g) 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 dependant manner (Fig. 1A). At the highest transfected dose (0.9 μ g), *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 *NXNLI* 5' upstream region. *Chx10/Vsx2* demonstrated 10.9 +/- 0.9 fold activation ($p < 0.001$) at the highest dose (0.9 μ g), *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 *Nxn11* promoters.

Since our bioinformatics analysis of the *Nxn11* 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 *Nxn11* 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 *Nxn11* 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 *Nxn11* expression or reflected background signal or perhaps cross-hybridization with another gene, we tested retinal sections from a *Nxn11* null mouse (Fig. 3A) (Cronin et al., in revision). The absence of signal in both the ONL and INL of the *Nxn11*^{-/-} 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 *Nxn11*^{+/+} 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 *Nxn11*^{-/-} 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 *Nxn11* 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 *Nxn11*, *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 *Nxn11* 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 *Nxn11* 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 *Nxn11* 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 synergistic effect observed here. Although *Usf1* can activate the human *Nxn11* Promoter, its co-transfection with *Chx10/Vsx2* led to a reduction of activity with both the mouse and human *Nxn11* promoter constructs.

DISCUSSION

A bioinformatic approach to study the *Nxn11* 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 *Nxn11* gene. As an alternative approach, since the absence of phylogenetic conservation of the *Nxn11* 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 *Nxn11* gene should be expressed in the outer retina. We further reasoned that those TFs that could transactivate both mouse and human *Nxn11* 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 *Nxn11* 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 *Nxn11* expression.

***Chx10/Vsx2* regulates the activity of the *Nxn11* 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 *Nxn11* 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 *Nxn11* 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 *Nxn11* 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 *Nxn11* 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 *Nxn11* 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 *Nxn11* 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 *Nxn11* expression is rod-dependant, and suggests that cell-to-cell communication between photoreceptor cells and bipolar cells is needed to maintain *Nxn11* 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*^{-/-} mice. *Nxn1*^{-/-} 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 *attR* sites flanking the *ccdB* 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 *attB* sites were incorporated into the forward (*attB* site 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3') and the reverse (*attB* 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 *Nxn11* 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 1ng 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 1x 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'-ATGGCATCTCTCTTCTCTGGACG-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 1x TSEI (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), 4x TSEII (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl), 1x buffer III (0.25 M LiCl, 1 % Nonidet P-40, 1 % deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0), and 3x 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 de 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, 35x [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, 5x FastStart LCDNA master SYBER (Roche) and amplified for 1x 95°C 10 min, 35x [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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CONFLICTS OF INTEREST

No

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FIGURE LEGENDS

Figure 1. Transcription factors activity on both mouse and human *Nxnll* promoter constructs. (A) Assay with the murine 4.2 kb *Nxnll* 5' upstream region. (B) Assay with the human 2.1 kb *NXNLI* 5' upstream region. The TFs were tested by transient transfection of 0.3, 0.6 and 0.9 μ g of plasmid coding for each candidate TFs 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 2. Activity of retinally-expressed homeogenes on mouse and human *Nxnll* promoters. constructs (A) Activity of homeogenes on mouse *Nxnll* promoter, and (B) on human *NXNLI* 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. *Nxnll* is expressed in the outer and inner retina. (A) *In situ* hybridization and immunolocalization of PKC α (red) on *Nxnll*^{+/+} or *Nxnll*^{-/-} retinal sections from mice at PN35. (B) *In situ* hybridization and immunolocalization of PKC α (red) on *rdl* 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 *Nxnll*^{+/+}, *Nxnll*^{-/-} and *rdl* mouse retinas at postnatal 8 and 35. (A) *In situ* hybridization on *Nxnll*^{+/+} or *Nxnll*^{-/-} retinas at PN35. (B) *In situ* hybridization on *rdl* mice at PN35 or PN8 and wt at PN35, aligned at the INL level. (C) Relative gene expression level in wild type mice and *rdl* 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 *Nxnll* 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 *Nxnll* promoter. Activity of the *Chx10/Vsx2* with *Vsx1*, *Pax4*, *TLX2*, *Usf1*, *Sp3* and *Yy1* TFs on the (A) mouse and (B) on human *Nxnll* 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. 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. 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.

TABLES

Table 1

Gene ID NCBI	Gene Name	Gene Symbol	Probe Set ID	Signal Intensity (RMA)				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 (S. pombe)	Cdc5l	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 (Drosophila)	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	

Table 2

Gene ID NCBI	Gene Title	Gene Symbol	Probe Set ID	Signal Intensity (RMA)				Tissue			Clone Number
				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 (Drosophila)	Otx2						●		LA0AAA28YF06	

Figure 1

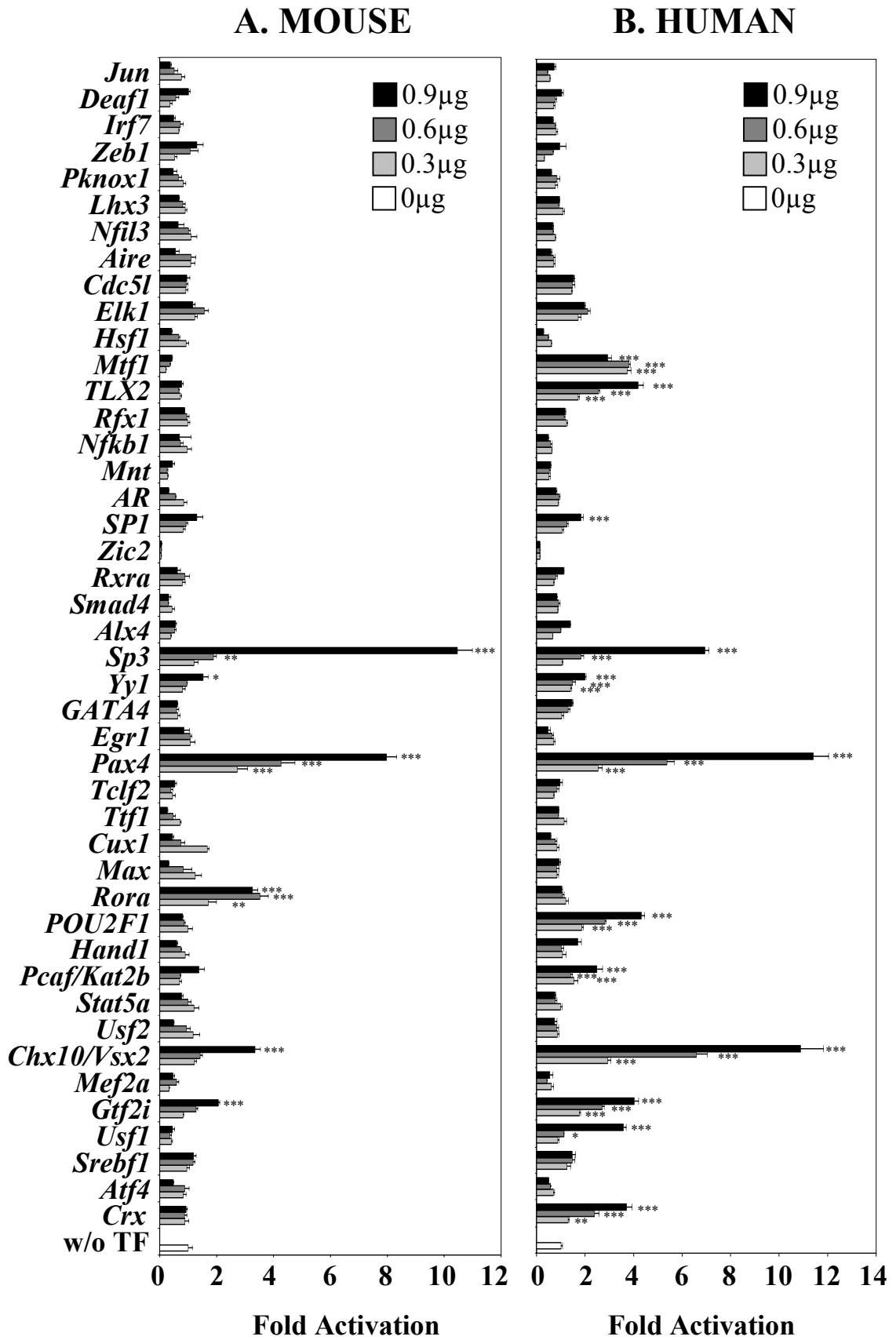


Figure 2

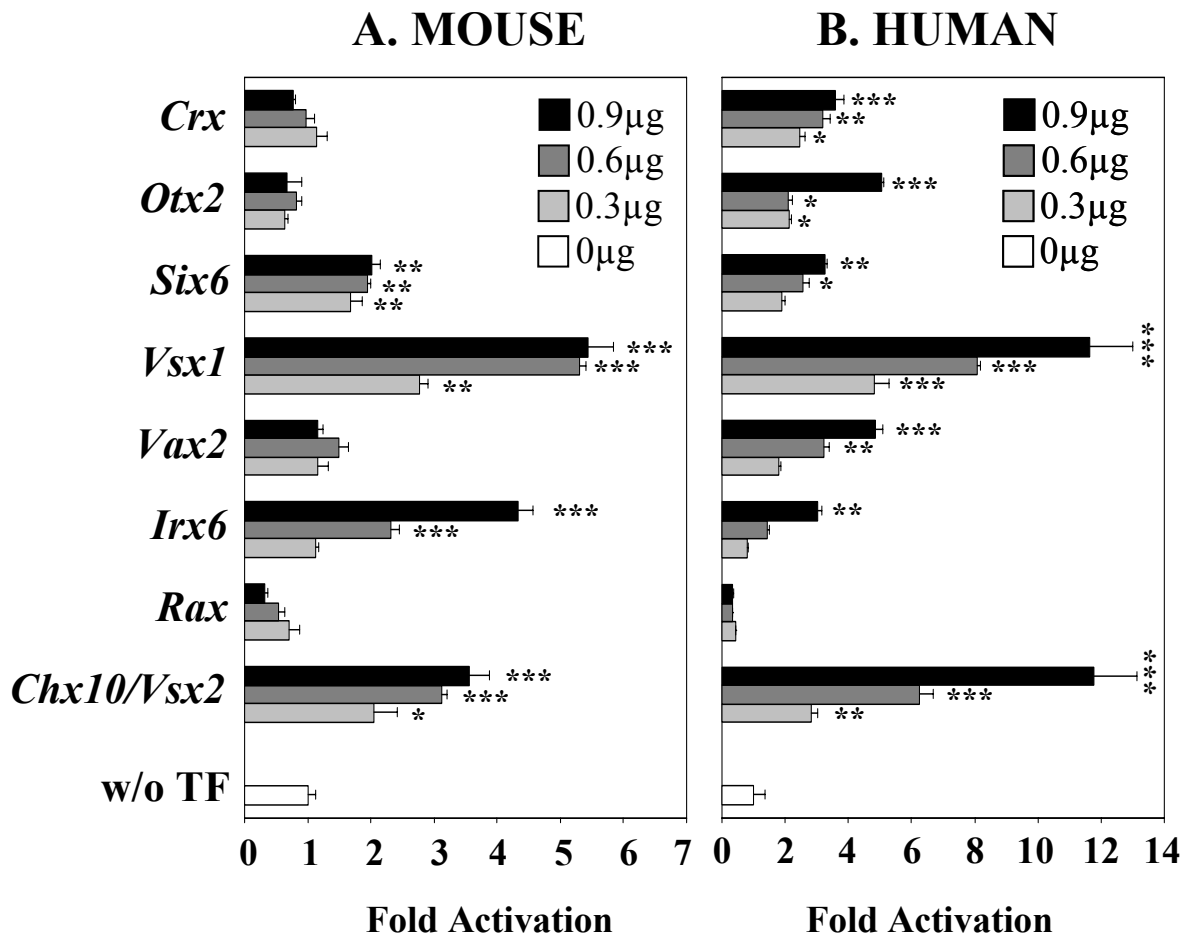


Figure 3

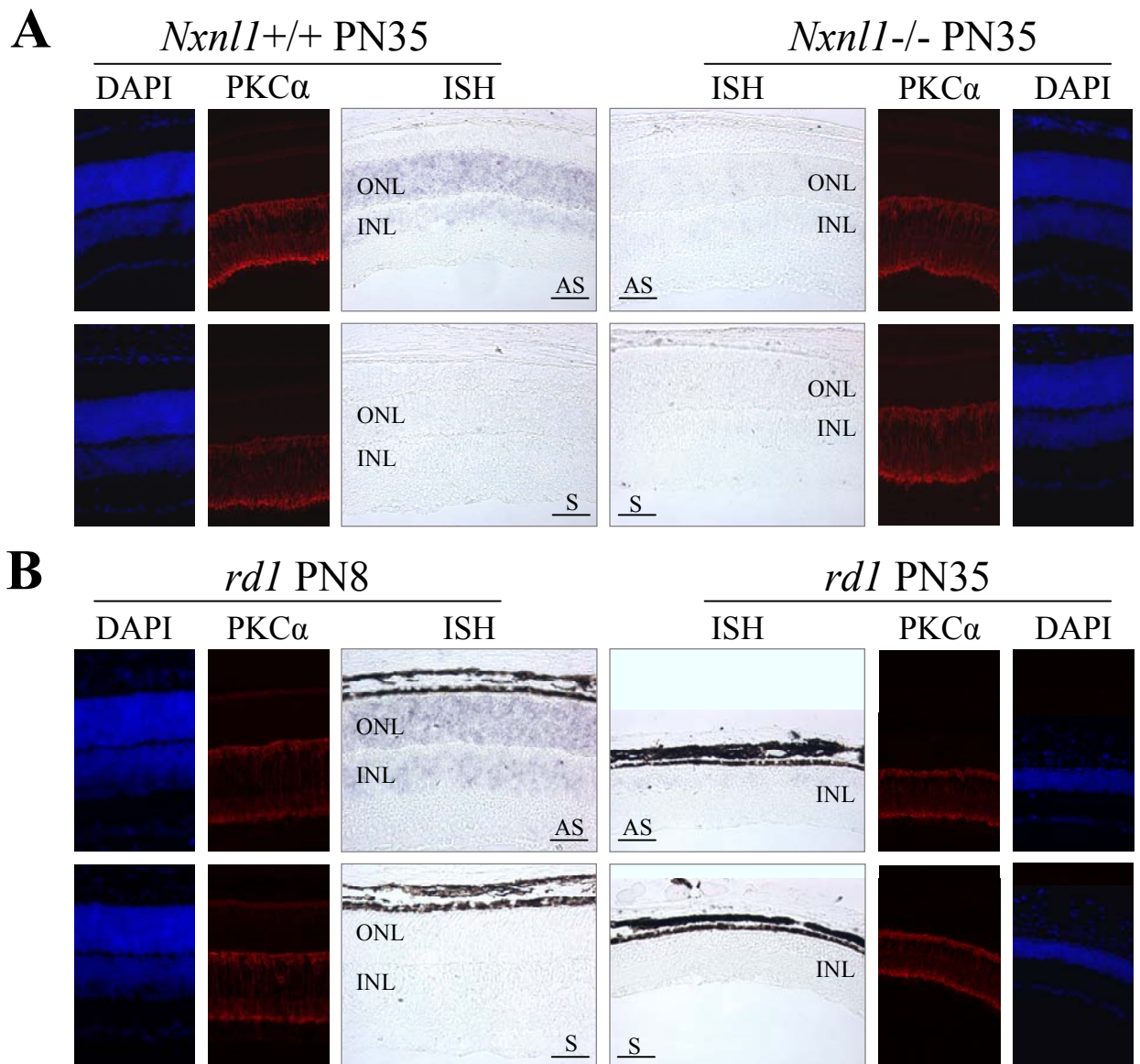


Figure 4

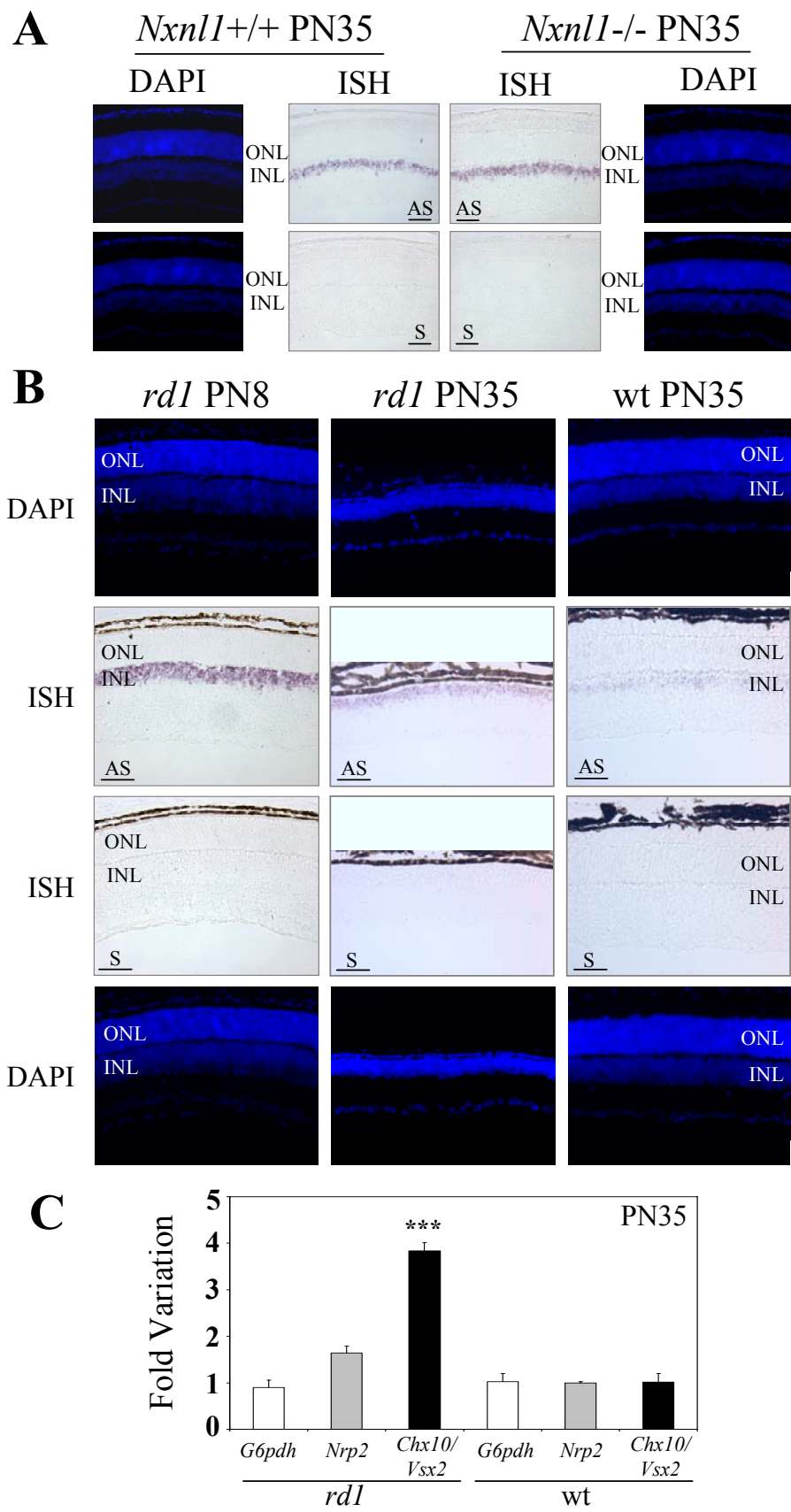


Figure 5

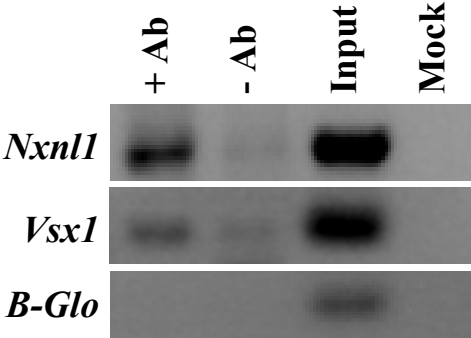
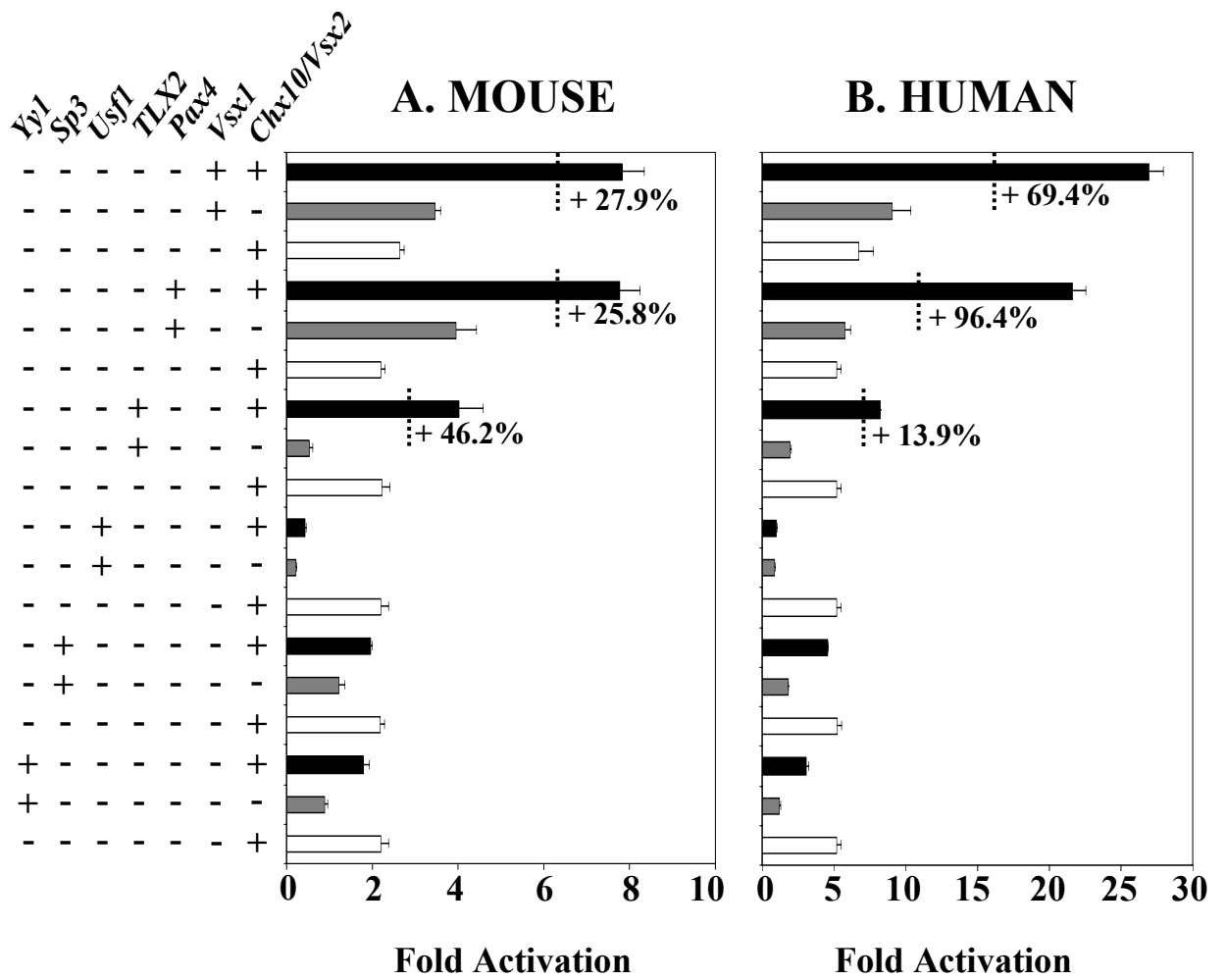


Figure 6



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

SUPPLEMENTARY MATERIAL

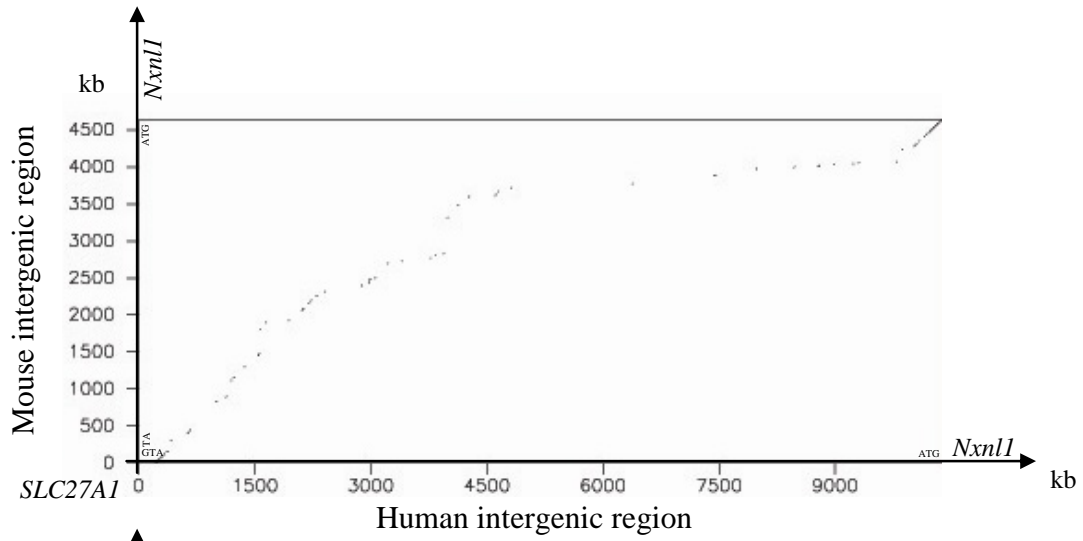
Supplementary Figure 1. DotPlot analysis of the 5' upstream region of Nucleoredoxin-like 1 genes. The DotPlot compares two sequences according to their homologies on this representation. Each point shows a sequence homology at the corresponding coordinates. (A) Human and mouse DotPlot. (B) Human and rat DotPlot. (C) Mouse and rat DotPlot. The aligned regions correspond to the intergenic sequences between the ATG of *Nxn11* gene and *SLC27A1*, a gene located in the 5' direction of *Nxn11* in each species. The analysis was done with the Software *dotpath* (EMBOSS, parameter: word size = 4)

Supplementary Figure 2. Localization of Neuropilin 2 by binding of its ligand the semaphoring III/F. (A) Western Blot determining the production the recombinants proteins Alkaline Phosphatase (AP-Tag) and Semaphorin III/F fused with the alkaline Phosphatase (AP-SemaIII/F), in Cos-1 Cells. The human *SemaIII/F* has been cloned in the APTag1, vector previously descript (68). (B) Binding Assay. The recombinants proteins were applied on C57BL6@N mouse retinal sections at PN35 using the AP-TAG™ Kit B (GenHunter Corp.). Only the ganglion cells were marked with AP-*SemaIII/F* protein, localizing its cell surface receptor the Neuropilin 2. ONL, Outer Nuclear Layer; INL, Inner Nuclear Layer; GCL, Ganglion cells Layer. Bar scale 50 μm.

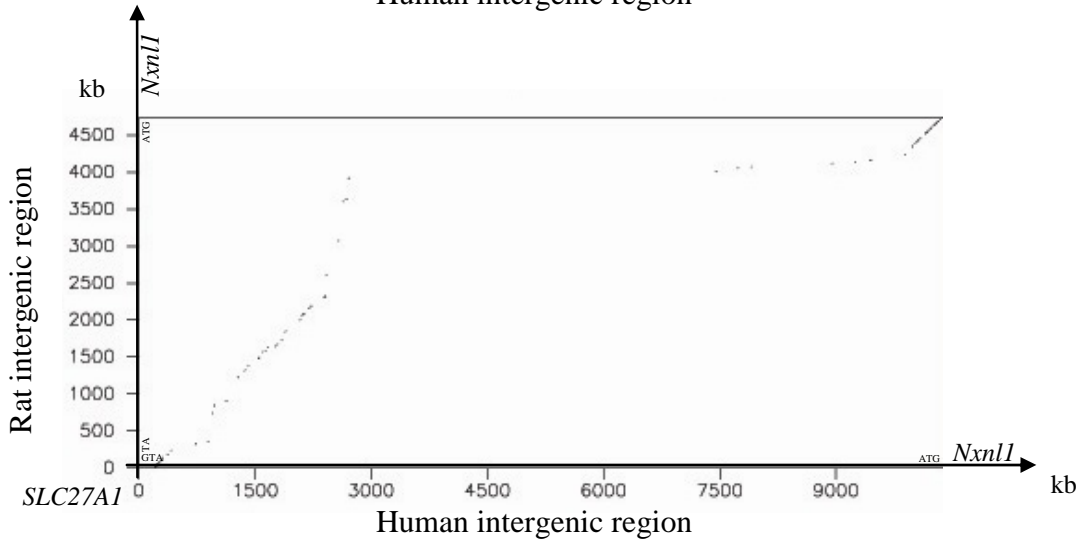
Supplementary Table 1. The 133 transcription factors predicted to bind an element within the 4.2 kb 5' upstream region of the *Nxn11* gene.

Supplementary Figure 1

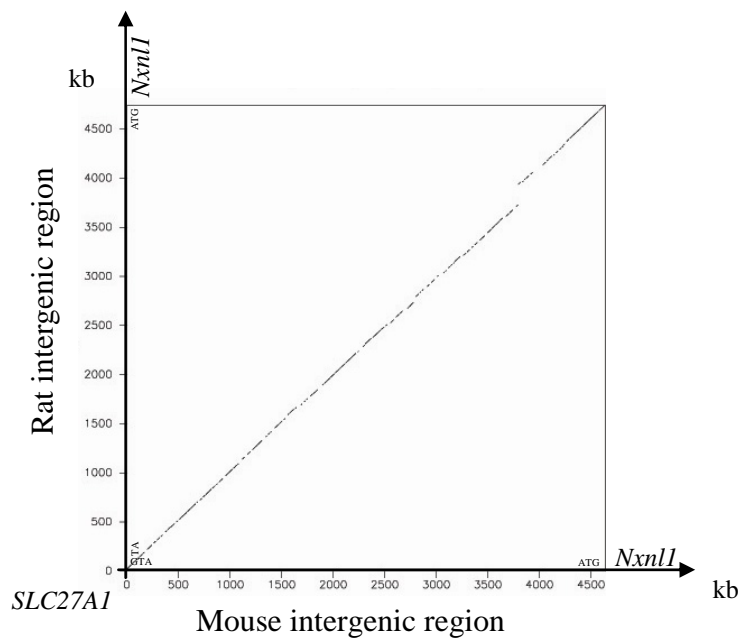
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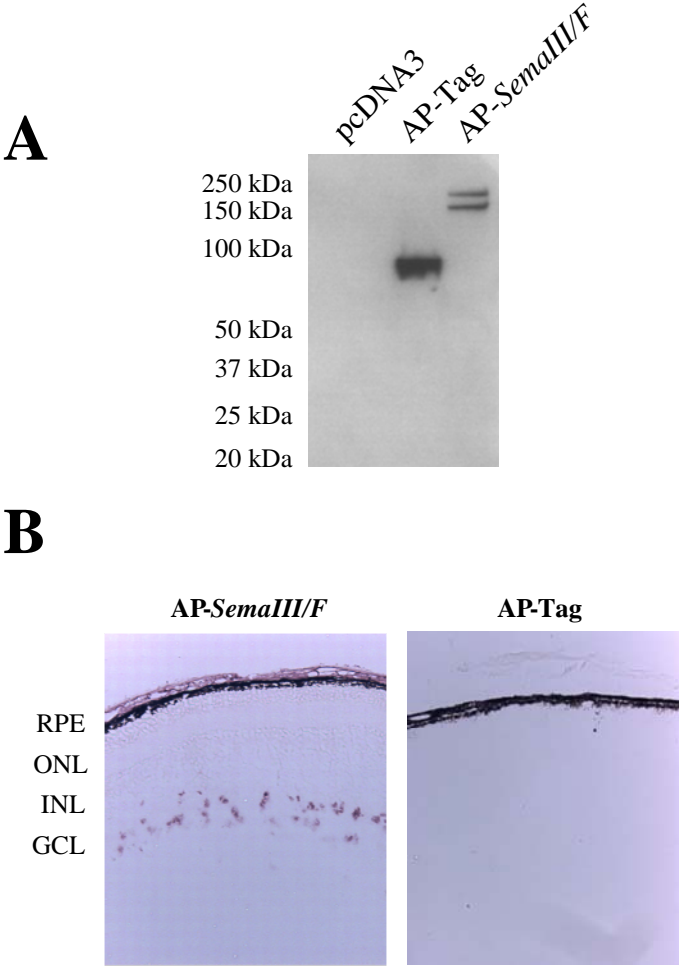
B



C



Supplementary Figure 2



Supplementary Table 1.

Gene ID NCBI	Gene Name	Gene Symbol
11622	aryl-hydrocarbon receptor	Ahr
11634	autoimmune regulator (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy)	Aire
23983	poly(rC) binding protein 1	alpha-Cp1 / Pcbp1
11695	aristaless-like homeobox 4	Alx-4
4205	myocyte enhancer factor 2A	Mef2a
21418	transcription factor AP-2, alpha	Ap2 / Tcfap2a
16597	Kruppel-like factor 12	Ap2rep / Klf12
11774	adaptor-related protein complex 3, beta 1 subunit	Ap3b1 / Ap3
83383	transcription factor AP4	Tcfap4 / Ap4
11835	androgen receptor	Ar
11819	nuclear receptor subfamily 2, group F, member 2	Arp1 / Nr2f2
11911	activating transcription factor 4	Atf4
12014	BTB and CNC homology 2	Bach2
20997	brachyury	Brachyury / T
12611	CCAAT/enhancer binding protein (C/EBP), gamma	C/Ebpgamma / Cebpg
13052	coxsackie virus and adenovirus receptor	Cxadr / Car
12358	cartilage homeo protein 1	Cart1
71702	cell division cycle 5-like (S. pombe)	Cdc5
13047	cut-like homeobox 1	Cux1 / Cutl1 / Cdp
12590	caudal type homeo box 1	Cdx1
211151	churchill domain containing 1	Churchill / Churc1
12677	visual system homeobox 2	Chx10 / Vsx2
252877	nuclear matrix protein 4	Ciz / Nmp4
17863	myeloblastosis oncogene	c-Myb / Myb
17187	Max protein	c-Myc:Max / Max
21422	transcription factor CP2	Tcfcp2 / Cp2
12951	cone-rod homeobox containing gene	Crx
54006	deformed epidermal autoregulatory factor 1 (Drosophila)	Deaf1
21417	zinc finger E-box binding homeobox 1	Zeb1 / deltaEf1
1403641	regulatory protein E2	E2
13555	E2F transcription factor 1	E2f1
13560	E4F transcription factor 1	E4f1
13653	early growth response 1	Egr1 / Krox
13712	ELK1, member of ETS oncogene family	Elk1
13798	engrailed 1	En1
104035	repeated epilation	Er
21677	TEA domain family member 2	Tead2 / Etf

14013	ecotropic viral integration site 1	Evi1
2186	bromodomain PHD finger transcription factor	Fac1 / Bptf
20371	forkhead box P3	Foxp3
14463	GATA binding protein 4	Gata4
14531	glial cells missing homolog 1	Gcm
14536	nuclear receptor subfamily 6, group A, member 1	Gcnf / Nr6a1
209334	Gen homolog 1, endonuclease (Drosophila)	Gen
14581	growth factor independent 1	Gfi1
14781	grizzled	Gr
15110	heart and neural crest derivatives expressed transcript 1	Hand1
18071	nescient helix loop helix 1	Nhlh1 / Hen1
217082	hepatic leukemia factor	Hlf
15361	high mobility group AT-hook 1	Hmga1 / Hmg ly
21405	HNF1 homeobox A	Hnf1a / Hnf1
2305	forkhead box M1	Hnf-3 / Foxm1
171302	forkhead box E3	Hnf-3 / Foxe3
15378	hepatic nuclear factor 4, alpha	Hnf4a
15379	one cut domain, family member 1	Hnf-6 / Onecut1
12419	chromobox homolog 5 (Drosophila HP1a)	Cbx5 / Hp1
15499	heat shock factor 1	Hsf / Hsf1
18609	pancreatic and duodenal homeobox 1	Pdx1 / Ipf1
54123	interferon regulatory factor 7	Irf7
16476	Jun oncogene	Jun / Ap1
16842	lymphoid enhancer binding factor 1	Lef1
85437	ZCRB1 zinc finger CCHC-type and RNA binding motif 1	Lf-a1 / Zcrb1
16871	LIM homeobox protein 3	Lhx3
14238	forkhead box F2	Foxf2 / Lun1
22260	nuclear receptor subfamily 1, group H, member 2	Lxr / Nr1h2
22778	IKAROS family zinc finger 1	Lyf1
17132	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	Maf
56218	POZ (BTB) and AT hook containing zinc finger 1	Mazr
17260	myocyte enhancer factor 2C	Mef2c / Mef2
15405	homeo box A9	Hoxa9
17268	Meis homeobox 1	Meis1a
237412	predicted gene, EG237412	Mif1
71371	AT rich interactive domain 5B (MRF1-like)	Arid5b / Mrf2
17764	metal response element binding transcription factor 1	Mtf1
17928	Myogenin	Myog
18015	neurofibromatosis 1	Nf1
21909	T-cell leukemia, homeobox 2	Ncx / Tlx2
307820	nuclear factor of activated T-cells 5	Nfat5 / Nf-at

18030	nuclear factor, interleukin 3, regulated	Nfil3 / E4bp4
18033	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	NF-kappaB / Nfkb1
13591	early B-cell factor 1	Ebf1 / Olf1
18519	K(lysine) acetyltransferase 2B	p300 / Pcaf / Kat2b
22059	transformation related protein 53	p53 / Trp53
18506	paired box gene 4	Pax4
5037	phosphatidylethanolamine binding protein 1	Pebp
18736	POU domain, class 1, transcription factor 1 (Pit1, growth hormone factor 1)	Pit1
18771	Pbx/knotted 1 homeobox	Pknox1 / Pbx
18986	POU domain, class 2, transcription factor 1	Pou2f1 / Oct1
19013	peroxisome proliferator activated receptor alpha	Ppar
18667	progesterone receptor	Pr
18171	nuclear receptor subfamily 1, group I, member 2	Pxr
217371	Rab40b, member RAS oncogene family	Rar
19724	regulatory factor X, 1	Rfx1
104336	zinc finger protein 104	Roaz
19401	retinoic acid receptor, alpha	Rara / Roralpha1 / Rora
30928	zinc finger protein 238	Zfp238 / Rp58
68750	ras responsive element binding protein 1	Rreb1 / Rreb1
20181	retinoid X receptor alpha	Rxra
20186	nuclear receptor subfamily 1, group H, member 4	Fxr / Nr1h4
20204	paired related homeobox 2	Prrx2 / S8
26423	nuclear receptor subfamily 5, group A, member 1	Sf1
17128	MAD homolog 4	Smad4
20682	SRY-box containing gene 9	Sox9
20683	trans-acting transcription factor 1	Sp1
20687	trans-acting transcription factor 3	Sp3
79401	spermatogenic leucine zipper 1	Spz1
20787	sterol regulatory element binding transcription factor 1	Srebp1 / Sreb1
20807	serum response factor	Srf
21674	sex determining region of Chr Y	Sry
20841	zinc finger protein 143	Staf / Zfp143
20850	signal transducer and activator of transcription 5A	Stat5a
20892	stimulated by retinoic acid 13	Stra13
21423	transcription factor E2a	Tcfe2a / E47
21406	transcription factor 12	Tcf12 / Heb
21685	thyrotroph embryonic factor	Tef
21676	TEA domain family member 1	Tead1 / Tef1
83602	general transcription factor II A, 1	Gtf2a / Tfla
14886	general transcription factor II I	Gtf2i / TflI-I

21815	TGFB-induced factor homeobox 1	Tgif
499766	transcription termination factor, RNA polymerase I	Ttf1
22278	upstream transcription factor 1	Usf1
22282	upstream transcription factor 2	Usf2
22337	vitamin D receptor	Vdr
217166	nuclear receptor subfamily 1, group D, member 1	Nr1d1 / vErbA
15218	forkhead box N1	Foxn1 / Whn
50505	excision repair cross-complementing rodent repair deficiency, complementation group 4	Ercc4 / Xpf1
394455	VENT homeobox 1, gene 2	Xvent1
22632	YY1 transcription factor	Yy1
21417	zinc finger E-box binding homeobox 1	Zeb1 / Areb6
22666	zinc finger protein 161	Zfp161 / Zf5
22772	zinc finger protein of the cerebellum 2	Zic2
241322	zinc finger and BTB domain containing 6	Zbtb6 / Zid
3783744	bZIP transcription factor	Zta / Bzlf1
