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## FIZ1 is Expressed During Photoreceptor Maturation, and Synergizes with NRL and CRX at Rod-Specific Promoters *in vitro*

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### Abstract

FIZ1 (Flt-3 Interacting Zinc-finger) interacts and co-purifies with the rod-specific transcription factor NRL (Neural Retina Leucine zipper). We hypothesize that FIZ1 is part of an interface between cell-specific factors, like NRL, and more ubiquitous regulatory networks that vary the absolute expression levels of some rod-specific genes (i.e. *Rhodopsin*). As part of an ongoing exploration of FIZ1's role in neural retina, *in vivo*, we have taken the first look at FIZ1 expression in the developing mouse retina during the retinal maturation period. Using the normal C57/B6 mouse as a model, multiple approaches were used including: immunoblotting, immunohistochemistry, and quantitative real-time PCR. Functional implications of FIZ1/NRL interaction, on NRL- and CRX-mediated activation of the *Rhodopsin* (*Rho*) and cGMP-phosphodiesterase  $\beta$ -subunit gene (*PDE6B*) promoters, were examined by co-transfection assays. Immunoblot analysis revealed that FIZ1 protein levels were lowest in immature mouse neural retina (P0). FIZ1 concentration increased at least ten-fold as the neural retina matured to the adult state (P21 and later). Immunohistochemical comparison of immature post-natal and mature adult retina revealed increasing FIZ1 protein in photoreceptors, the inner plexiform layer, and the ganglion cell layer. Total retinal *Fiz1* mRNA content increased as the neural retina matured. The expected increase in *Rho* mRNA level was also monitored as a genetic marker of photoreceptor maturation. In transient co-transfection assays of CV1 cells, FIZ1 synergized with NRL to activate transcription from the *Rho* and *PDE6B* gene promoters with some differences. In the case of the *Rho* promoter, FIZ1 synergized when both NRL and CRX were present. With the *PDE6B* promoter, FIZ1 synergized with NRL alone, and the inclusion of CRX decreased this synergy.

**Conclusions**—These findings support previous evidence that FIZ1 is present in rod-photoreceptors. (Co-immunoprecipitation from nuclear-protein extracts with rod-specific NRL) FIZ1 expression increases in the neural retina during the retinal maturation period. Additionally, *in vitro* experiments demonstrate that FIZ1 has the potential to significantly increase the NRL-mediated activation of photoreceptor-specific promoters. While CRX is not a strong activator of the *PDE6B* promoter, alone or with NRL, CRX decreased the synergy of NRL with FIZ1.

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## Keywords

FIZ1; NRL; CRX; Rhodopsin; cGMP-phosphodiesterase  $\beta$ -subunit; transcription; retinal development; gene; retinal degeneration; mouse

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## 1. Introduction

Throughout retinal development, and in the mature retina, the precise control of photoreceptor gene expression is essential for photoreceptor integrity. Disruptions of these normal gene expression patterns cause photoreceptor degenerations. (Pacione, et al., 2003, Rattner, et al., 1999, Tan, et al., 2001) Transcription factors are integration points for signal transduction networks that orchestrate development. Homeobox transcription-factor genes are expressed in the earliest primordial eye tissue and are essential for vertebrate eye development (i.e. *Pax6*, *Rx*, *Six3*, *Six6*, and *Lhx2*). (Carl, et al., 2002, Mathers and Jamrich, 2000, Porter, et al., 1997) The bHLH (basic Helix Loop Helix) gene family of transcription factors also has pivotal roles in the unique patterning of transcription factors that guide retinal development (*Mash1*, *Math3*, *Ngn2*). (Akagi, et al., 2004, Brown, et al., 1998, Brown, et al., 2001, Hojo, et al., 2000, Kanekar, et al., 1997, Marquardt and Gruss, 2002, Moore, et al., 2002, Morrow, et al., 1999, Wang, et al., 2001, Yan and Wang, 1998)

Several transcription factors are clearly essential for photoreceptor development, and their mutations cause retinal degenerations: NRL, CRX, Otx2, Tr $\beta$ 2 (thyroid hormone receptor  $\beta$ 2), and NR2E3. (Bessant, et al., 1999, DeAngelis, et al., 2002, Freund, et al., 1997, Haider, et al., 2000, Haider, et al., 2001, Martinez-Gimeno, et al., 2001, Mears, et al., 2001, Ng, et al., 2001, Nishida, et al., 2003, Swain, et al., 1997, Swaroop, et al., 1999) However, our knowledge of the interactions of these cell-specific proteins with each other and upstream signal transduction proteins remains sparse. We do not know how this pool of cell-specific transcription factors interacts with the pool of ubiquitous proteins, which include the general transcription machinery and epigenetic regulators of chromatin/DNA structure. The challenge is to find links between these different functional protein pools: unique and ubiquitous. This requires far more data about, mostly unknown, protein interactions.

Our strategy is to use cell-specific proteins as starting points to discover new interaction networks. We have demonstrated the usefulness of this approach to uncover protein interactions that are functionally relevant to photoreceptor-specific gene expression, including: NRL + CRX, and NR2E3 + NR1D1 (RevErb- $\alpha$ ). (Cheng, et al., 2004, Mitton, et al., 2000) Peng et al., also using yeast two-hybrid traps, have demonstrated that NR2E3 can bind to CRX *in vitro*, supporting activation of the *Rhodopsin* (*Rho*) promoter, and contributing to repression of cone-opsin promoters. (Peng, et al., 2005) Recently, our use of protein interaction traps discovered FIZ1 binding to the NRL bZip domain. Indeed, native FIZ1 and NRL co-purify from retina nuclear protein extracts, and FIZ1 can alter NRL's activation of the *Rho* promoter. (Mitton, et al., 2003) FIZ1's amino acid sequence is highly conserved in mammals and the human and mouse genes are very similar. (Genbank Accession: **NM\_032836**) *FIZ1* mRNA is present in many human and mouse tissues, and FIZ1 protein is present in the photoreceptors and the ganglion cell layer of mature retina.

There is evidence that FIZ1 can exist in both the nuclear and cytoplasmic compartments, although its only documented cytoplasmic protein-interaction is with the tyrosine-kinase domain of a receptor essential for myeloid cell development. (Mitton, et al., 2003, Wolf and Rohrschneider, 1999) FIZ1 binds to FLT-3 (FMS-like receptor tyrosine kinase, FLK-2) in myeloid progenitors, and their association increases upon stimulation of cells with FLT-3-ligand. (Wolf and Rohrschneider, 1999) Mutations in the *FLT-3* gene constitutively activate Stat-5 and MAP-kinase pathways, promoting myeloid leukemia. (Hayakawa, et al., 2000, Nakao, et al., 1996)

Multiple-Zf proteins, like FIZ1, feature prominently in networks that regulate gene expression, especially those that involve nuclear-receptors. FIZ1 is not homologous to any known Zf-families and we know little of its basic functions in the cell. FIZ1 represents one potential interface between cell-specific transcription factors, like NRL, and the ubiquitous regulatory networks of the cell. Its ability to inhabit both the nuclear and cytoplasmic compartments makes FIZ1 an interesting candidate for this role. As part of our ongoing investigations into the function of this protein, we report that FIZ1's temporal and spatial expression would permit an interface role in the precise control of photoreceptor-specific gene expression. Retinal FIZ1 protein concentration increases substantially during post-natal maturation of the mouse retina, when maturation-related gene expression is on the rise (i.e. *Rho*). Furthermore, using co-transfection promoter-activation assays, combinations of specific quantities of expression vectors demonstrate that FIZ1 can promote activation of a minimal *Rho*, or *PDE6B* promoters in synergy with NRL and CRX.

## 2. Materials and methods

The study was designed under approval of Oakland University's animal care and use committee and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All eye tissues were obtained at the same time (mid-afternoon) to avoid circadian effects.

### 2.1. Mouse retinal total RNA extraction

Mouse eyes (C57/B6 strain) were enucleated into PBS. Corneas were punctured with a 30-gauge needle and then removed completely, followed by removal of the lens. Eyecups were trimmed anteriorly to remove the iris and ciliary body, and the sclera/choroid/RPE layers were peeled off the neural retina. The still intact neural retinas were brushed free of isolated RPE-cell patches, and then separated from the optic nerve/disk. These neural retinal cups were fast-frozen to the inside of a 1.5 mL microfuge tube on dry ice, in groups of 4–8.

Total RNA was isolated using the Stratagene mini-Prep RNA kit (Stratagene, La Jolla, California, <http://www.stratagene.com>). Samples were processed using a 250  $\mu$ L homogenization volume and DNAase treatment. RNA samples were maintained in mild buffer (RNase free) with the addition of RNase inhibitor (Invitrogen Corporation, Carlsbad, California, <http://www.invitrogen.com>) and stored at  $-70^{\circ}\text{C}$ . Depending on the post-natal age of the retinas, 3–4  $\mu$ g of total RNA per retina were recovered as determined by optical density measurement (260 nm).

## 2.2. First Strand cDNA Preparation

For quantitative analysis of *Fiz1* mRNA using Amplitaq-Gold™ based RT-PCR, the reverse transcriptase reactions were performed using 2 µg of total RNA with Superscript-II™ enzyme as per the manufacturers protocol (Invitrogen). Single reactions resulted in over 40 µL of cDNA. First strand cDNA samples were split into aliquots and kept frozen.

For an additional quantitative analysis of *Fiz1* mRNA using a different RT-PCR system (Roche Applied Science, Indianapolis, Indiana, <http://www.roche-applied-science.com>), cDNA samples were prepared from 2 µg of total RNA using the Stratascript™ QPCR cDNA synthesis kit (Stratagene). The cDNA samples were stored at -70° C until further use.

## 2.3. Quantitative real time PCR

Mouse retinal *Fiz1* mRNA levels were measured using Amplitaq Gold™ enzyme (Applied Bio-Systems, Foster City, CA, <http://www.appliedbiosystems.com>), SYBR Green (Invitrogen) and plasmid cDNA standards in the pGemT-easy vector (Promega, Madison, WI, <http://www.promega.com>). Primers were first tested by touchdown PCR, using mouse retinal cDNA as template.

For Amplitaq Gold real-time PCR: PCR products were sub-cloned into the pGemT-easy vector as a source of cDNA standards. Reaction efficiencies were the same for templates in pGemTeasy as in retinal cDNA preparations. Expression of *Fiz1* mRNA was determined relative to *Gapdh* mRNA levels using equation-1 for Ct based analysis. Reaction efficiencies are used in this analysis, where  $E_g$  was the reaction efficiency for the target (*Fiz1* or *Rho*) and  $E_n$  the reaction efficiency for the normalizer gene (*Gapdh*). PCR products were confirmed by DNA sequencing.

$$\text{Relative template normalized} = \frac{(1 + E_g)^{-\Delta C_t_{\text{Gene}}}}{(1 + E_n)^{-\Delta C_t_{\text{GAPDH}}}} \quad \text{Equation-1}$$

Thermo cycling conditions and primers are summarized in Table 1. Samples were analyzed with an MX3000P real-time PCR system (Stratagene).

For an additional quantitative PCR analysis, retinal *Fiz1* mRNA levels were directly measured using standard curves prepared from QPCR Mouse Reference Total RNA™ (Stratagene). Another reference gene, *β-Actin*, was also analyzed directly using the same mouse universal reference RNA and a specific hydrolysis-probe (Universal library probe #106, Roche Applied science, Indianapolis, Indiana). This particular strategy with the hydrolysis probe and reference RNA, enabled confirmation of similar *β-Actin* reference mRNA levels (relative to total RNA) before and after the retinal maturation period. Realtime PCR reactions were performed using Faststart Taqman Probe Master (Rox) mix (Roche Applied Science) with SYBR green dye (for *Fiz1*) or with Universal Library probe for mouse *β-Actin*. Primers used, and thermocycling conditions are summarized in Table 2. Samples were analyzed with an MX3000P real-time PCR system (Stratagene).

#### 2.4. Retinal sections and immunohistochemistry

Eyes were obtained from mice (C57/B6) at mid-day. Globes were removed into PBS, and the corneas were punctured a few times with a 30-gauge 1/2 inch needle. Globes were fixed in 0.1 M phosphate buffer, pH 7.2, 4% paraformaldehyde, with gentle agitation for 15 minutes. Cornea and the lens were removed and the retinal cups fixed for 1 hour. Fixed tissues were infused with sucrose using 0.1 M phosphate buffer, pH 7.2, with 10, 15 and 20% sucrose. Tissues were infused with a 2:1 mixture of 0.1 M phosphate buffer, pH 7.2, 20% sucrose and OCT, for 1 hour at room temperature and then frozen in OCT.

Sections were cut (10  $\mu$ m) in a cryomicrotome and air dried onto positive-charged glass slides. Sections were washed (3  $\times$  5 minutes) in PBS 0.3% Triton X-100 (PBS-Triton), prior to blocking in PBS-Triton + 1.5% goat serum for 30 minutes. Sections were incubated at room temperature with 1/500 dilution of antibody to bovine FIZ1. Control sections were treated with pre-immune rabbit serum. Sections were then washed with PBS-Triton (2  $\times$  2.5 minutes) and incubated with 1/200 dilution of a biotinylated goat-anti-rabbit IgG in PBS-Triton for 30 minutes. Sections were washed (3  $\times$  5 minutes), and incubated with ABC-peroxidase complex (30 minutes) as per the manufacturer's instructions (Vector Laboratories, Burlingame CA). After a final washing (3  $\times$  5 minutes), the location of FIZ1 protein was visualized by reacting 8 minutes with DAB/hydrogen-peroxide/nickel substrate. Sections were photographed using phase contrast with care to use the same illumination and exposure times for controls and test samples.

#### 2.5. Retinal protein extraction and immunoblotting

Frozen retinas (10 – 14) were homogenized in a 1.5 mL Eppendorf tube with 250  $\mu$ L of ice-cold 50 mM Tris + 5 mM EDTA + 1 mM DTT, pH 7.4, using a hand held microtube homogenizer. PMSF (final concentration 1mM) was added just at homogenization. Homogenates were centrifuged (20,000g  $\times$  10 minutes, 4°C), and the supernatant was frozen before analysis. Protein concentrations were determined using the Bradford assay and aliquots were prepared for DISC-Page by heating (99°C, 5 minutes) with one-volume of 2x-Blue Juice denaturing/reducing buffer (Q-Biogene: Bromophenol blue, urea, SDS, 2-mercapto-ethanol). Denatured samples were centrifuged for 1 minute at 2,000g, prior to loading 25  $\mu$ g protein onto 12.5 % acrylamide gels in a Bio-Rad Mini-Protein-II apparatus. Proteins were electro-eluted in Towbin's buffer onto PVDF membranes.

Membranes were blocked for 1-hour in PBS + 5% fat-free milk powder + 0.1% Tween-20, and then washed with PBS-Tween (3  $\times$  5 minutes). Membranes were then incubated with a 1/1000 dilution of primary anti-body (anti-bFiz) in antibody diluent (PBS-Tween + 2.5% fat-free milk powder) for 1-hour, before washing and then incubating with peroxidase-labeled goat anti-rabbit IgG (1/10,000) for 1-hour. Membranes were subjected to chemiluminescent detection using the ECL-Plus Western Blotting reagents (GE-Health /Amersham). Film images were digitized and the relative amounts of FIZ1 protein were determined using Image-J, a Java-based version of the NIH-Image program. (Available at: <http://rsb.info.nih.gov/ij/index.html>)

In addition to loading equivalent total protein per gel lane, FIZ1 levels were normalized to the total protein actually transferred onto the PVDF membrane. Membranes were stripped of antibodies, washed in PBS-Tween, deionized water, and then stained with Electroblue colloidal protein stain (Q-Biogene) for 15 minutes. After washing in deionized water, the blots were scanned, and the relative amounts of total protein in each lane were determined using Image-J software.

## 2.6. Tissue protein extraction (non-retinal)

Protein extracts were prepared as for retinal protein extractions; however, tissues were weighed frozen and homogenized with a hand-held polytron generator after addition of PMSF and five volumes of buffer. For electrophoresis, 25 µg of total protein (denatured, reduced) were loaded per lane. Immunoblotting for FIZ1 protein was accomplished as described above.

## 2.7. Mammalian cell expression of FIZ1 antigen

Monkey kidney cells (line CV1, American Type Culture Collection) were transiently transfected with the pDest26-bFIZ1 plasmid, expressing an N-terminal His-Tag fusion protein of FIZ1 (bovine) in the pDest26 vector (CMV promoter, Invitrogen). This was also the antigen for production and affinity purification of the FIZ1 antibody as previously described, and shares high homology to the human and mouse FIZ1 proteins. (Mitton, et al., 2003) Cells were transfected with 0, 0.6, or 1.2 µg of pDest26-bFIZ1 plasmid DNA. Plasmid DNA for transfections were prepared using the Qiagen maxi-prep kit (Qiagen, Valencia, CA, <http://www.qiagen.com>), and CV1 cells were transfected at 70% confluency in 24-well plates using Lipofectamine-plus™ (Invitrogen) as previously described. (Bessant, et al., 1999, Mitton, et al., 2003)

## 2.8 Transient co-transfection and promoter-activation assays

Monkey kidney cells (line CV1, American Type Culture Collection) were maintained in MEM-alpha (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 µg/ml, Invitrogen) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Cells were not used after more than eight passages. Cells were counted (Beckman Coulter Z1 Particle Counter), and plated in 24-well plates ( $1.7 \times 10^5$  cells/well) the day before transfection. Cells were transfected using Lipofectamine-2000 reagent as per the manufacturers standard recommendations for 24-well format: a DNA (µg) to reagent (µL) ratio of 1:3. (Invitrogen). Briefly, cells were adapted to antibiotic-free and serum-free medium for two hours, and then exposed to the transfection mixes for five hours (antibiotic- and serum-free).

The luciferase-reporter construct, pBR-130-luc, provided by Drs Donald Zack (Johns Hopkins) and Shiming Chen (Washington University), contained 130 bp of the bovine *Rhodopsin* proximal promoter region subcloned into the firefly Luciferase pGL2-basic vector (Kumar, et al., 1996). A similar construct, pGL2-*PDE6B* (-93/+53), provided by Dr. Debora Farber (UCLA), contained the human *PDE6B* minimal promoter region (-93/+53). (Lerner and Farber, 2000) The following expression constructs were used for transfection: pED-NRL for NRL (from Dr. Anand Swaroop, University of Michigan), pcDNA-bCRX for



CRX (Drs. Zack, Chen) and pDest26-bFIZ1 for FIZ1. (pDest26, CMV-promoter based, from Invitrogen.)

For the *Rho*-promoter, all transfection mixes contained 555 ng/mL (on cells) of pBR-130luc, and 19 ng/mL of the normalizer Renilla-luciferase construct pRL-CMV (Promega, Madison, WI, USA). Triplicate sample wells were co-transfected with expression vectors for NRL (55 ng/mL), CRX (110 ng/mL), and FIZ1 (165 ng/mL). Mixes contained either the desired expression constructs or corresponding empty plasmids.

For the *PDE6B*-promoter, all transfection mixes contained 220 ng/mL of pGL2-PDE6B-luc, and 19 ng/mL of the normalizer Renilla-luciferase construct pRL-CMV. (Final concentrations on cells.) Triplicate sample wells were co-transfected with expression DNAs for NRL (55 ng/mL), CRX (110 ng/mL), and FIZ1 (165 ng/mL)

After 48 hours, cells were rinsed in PBS and lysed using Passive Lysis Buffer (Promega). Firefly- and Renilla-luciferase levels were measured simultaneously in a single sample aliquot using Dual-Reporter Luminescence assay reagents and a Tuner Biosystems 20/20<sup>n</sup> dual-injector luminometer (both from Promega). An experiment included triplicate samples for each DNA combination, and each experiment was repeated at least three times. Individual experiments were analyzed independently. Individual sample activities (Firefly luciferase relative to Renilla luciferase) were normalized to the background activity of the reporter in the presence of empty expression vectors, to control for any unpredictable effects of vector DNA sequences. Data were analyzed for multiple comparisons using ANOVA and post-analysis with the conservative Tukey-Kramer procedure. (GB-Stat software, Dynamic Microsystems, Inc., Silver Spring, MD).

### 3. Results

#### 3.1. FIZ1 protein is present in several organs

Previous analysis (northern blots) indicated that many mouse and human tissues express *Fiz1* mRNA. (Mitton, et al., 2003, Wolf and Rohrschneider, 1999) However, data concerning FIZ1 protein for most non-retinal tissues was lacking. Our analysis shows that several of these tissues do contain FIZ1 protein. (Fig. 1A) Utilizing a polyclonal antibody, that was affinity purified to FIZ1 antigen, we detected a single major band in non-neural tissues and brain (approx. 65 KDa).

The specificity of the antibody to FIZ1 in immunoblotting was confirmed by specific expression of the FIZ1 antigen in transfected CV-1 cells. (Fig. 1B) This same antigen was used to produce the antibody (in rabbits), and for the antigen-specific affinity purification of the final IgG preparation (Invitrogen). Cells with increasing amounts of bovine FIZ1 were extracted for protein (denaturing) and examined by dot-blot. Under the conditions used, the antibody is specific to FIZ1 antigen.

#### 3.2. Neural retina FIZ1 protein expression increases during photoreceptor maturation

Previous investigators have shown that both *Rho* mRNA and protein levels begin to increase in the mouse retina around postnatal day P5-P6. This pattern holds for other rod visual

transduction proteins, such as arrestin and PDE6B. Photoreceptors begin to form inner segments at P5 and are fully functional by P21. Outer-segment length and Rhodopsin protein concentration continue to increase until P30. (Bowes, et al., 1988)

To examine the relative FIZ1 protein content of mouse neural retina throughout the retinal maturation period, samples were compared representing post-natal days P1, P9, P15, P21, and P32. (Fig. 2.) Measurement of the relative FIZ1 protein content revealed a constant increase throughout post-natal retinal development from the earliest time point, when FIZ1 content was relatively low. Image-based analysis of the relative FIZ1 content revealed at least a ten-fold increase from day P1 to day P32.

### 3.3. FIZ1 protein expression increases in photoreceptors and ganglion cell layer during retinal maturation

Next, we examined the expression of FIZ1 in fixed-frozen retinal sections at P0 (immature retina), P7 (early photoreceptor maturation) and P45 (fully mature retina). Results were consistent with the immunoblot data. At time P0, even with an extended peroxidase/DAB reaction time, there was no specific FIZ1 staining before the onset of background staining. (Fig. 3A.) FIZ1 protein was detectable at day P7, corresponded to the early stage of photoreceptor/neuronal maturation. Inner-segments were present at the P7 time point with faint immunolabeling for FIZ1. (Fig. 3B.) Low level staining for FIZ1 protein was also present at this time in ganglion cell layer.

In adult retina, the immunostaining agreed with our previous immunofluorescent data, detecting FIZ1 protein in photoreceptors and the ganglion cell layer. (Mitton, et al., 2003) (Fig. 3C.) Labeling for FIZ1 was strong in the photoreceptor inner and outer segments of mature neural retina. (Fig. 3D.) FIZ1 is present in photoreceptor nuclei as evident from co-isolation with NRL (rod-specific) from retinal nuclear protein extracts; however, this particular antibody does not intensely label FIZ1 in photoreceptor nuclei of mouse retinal sections. (Mitton, et al., 2003) This may be a result of lower concentration in nuclei (compared to cytoplasm), and some masking of the antigen in vivo.

### 3.4. Neural retina *Fiz1* mRNA content increases during photoreceptor maturation

Several key rod-photoreceptor genes, such as *Rho* and *Arrestin*, are expressed during the period of photoreceptor maturation. We examined the expression of the *Fiz1* gene compared to the *Rho* gene, since FIZ1 protein levels were increased during this same period. The relative mRNA levels for *Rho* and *Fiz1* were examined at several time points, spanning the maturation period, using quantitative RT-PCR (real time). *Rho* gene expression increased throughout this period, as expected. (Fig. 4A.) This pattern agreed with previously published data (northern blot), and provided a genetic marker for photoreceptor-specific gene expression during maturation. (Bowes, et al., 1988) Relative *Fiz1* mRNA levels also increased in concentration throughout the maturation period when examined relative to *Gapdh* mRNA levels using the Amplitaq Gold real-time PCR method. (Fig. 4B.)

As a confirmation, *Fiz1* mRNA levels were also examined using a different tissue sample set with normalization to a different reference gene:  $\beta$ -*Actin*. Both *Fiz1* and  $\beta$ -*Actin* mRNA levels were determined using absolute calibration with a Universal Mouse Reference RNA



standard (Stratagene). This analysis method also indicated that neural retina *Fiz1* mRNA levels increased 4-fold from the immature (P6) to the mature (P22) state. (Fig. 4C). This analysis was repeated twice. The levels of  $\beta$ -*Actin* mRNA per total retinal RNA from early (P6) and mature (P22) neural retina were not different. (Data not shown) While the data indicate *Fiz1* mRNA levels do not increase as much as the very highly expressed *Rho* mRNA, a 3–4 fold increase was apparent.

### 3.5. Synergistic activation of *Rho* and *PDE6B* gene promoters by *NRL*, *CRX* and *FIZ1*

To explore the functional implications of *NRL*-*FIZ1* interaction, we co-transfected CV1 cells with *Rho*- or *PDE6B*-promoter reporter plasmids and various combinations of *NRL*, *CRX* and *FIZ1* expression constructs. *FIZ1* synergistically activated *NRL*-mediated transcription of the *Rho*, and *PDE6B* gene promoters with some differences. Representative experiments are shown in Figure 5.

In *Rho* promoter assays (Figure 5A), *FIZ1* alone had no effect on promoter activation. *NRL*, or *CRX*, could activate the promoter about 5-fold compared to empty vectors ( $p < 0.01$ , ANOVA, Tukey-Kramer post analysis). *NRL* combined with *CRX* resulted in synergistic activation of the *Rho* promoter: 33-fold relative to empty vectors ( $p < 0.01$ , Tukey-Kramer). These experiments utilized relatively more *NRL* and less *Fiz1* DNA (more similar amounts) than previously found to inhibit *NRL*-mediated activation of the *Rho* promoter. Here, *FIZ1* had little effect on *NRL*- or *CRX*-mediated activation of *Rho* promoter. However, with both *NRL* and *CRX*, *FIZ1* increased the total activation synergy by over 30% relative to *NRL* plus *CRX* ( $p < 0.01$ , Tukey-Kramer).

Using the same relative expression DNA concentrations for the *PDE6B* promoter (Figure 5B), *CRX* alone had no significant effect compared to empty vectors (N.D., Tukey-Kramer). The same was true for *FIZ1* alone. *CRX* combined with *FIZ1* did result in a 10-fold activation of the promoter relative to empty vectors ( $p < 0.01$ , Tukey-Kramer). *NRL* alone was a strong activator of the *PDE6B* reporter: 12-fold relative to empty vectors ( $p < 0.01$ , Tukey-Kramer). While *CRX* had no effect when added to *NRL*, *FIZ1* was synergistic with *NRL* and doubled promoter activation to 26-fold (*NRL* versus *NRL*/*FIZ1*,  $p < 0.01$ , Tukey-Kramer). Furthermore, when *CRX* was combined with *NRL* and *FIZ1*, there was a 40-percent decrease in the total promoter activity compared to *NRL*/*FIZ1* ( $p < 0.01$ , Tukey-Kramer). These results indicate that *FIZ1* can promote *NRL*-mediated activation of this minimal *PDE6B* gene promoter.

## 4. Discussion

The *Rhodopsin* (*Rho*) gene is an excellent model to explore the pathways that precisely regulate rod-specific gene expression. *Rho* expression is rod-specific and the amount of Rhodopsin protein is regulated at the level of transcription. (Treisman, et al., 1988) Transcription factors that regulate the *Rho* gene also regulate other rod-specific genes that share a similar expression pattern. (Kumar, et al., 1996, Lerner, et al., 2001, Mears, et al., 2001, Rehemtulla, et al., 1996) *Rho* expression varies during development, dramatically increasing during maturation of rod outer-segments. (Bowes, et al., 1988) In mature retina, *Rho* expression continues to be regulated to follow the circadian patterns of rod outer-

segment formation. (Bowes, et al., 1988, Matsumoto and Bok, 1984, von Schantz, et al., 1999)

The *Rho* proximal promoter region is sufficient for rod-specific expression and includes several DNA sequence elements that bind to proteins *in vitro*. These include the NRL response element (NRE). NRL binds to NRE elements in several rod-specific genes: *Rho*, *Pde6b*, *Pde6a* and *Gnat-1*. (Ahmad, 1995, Chen, et al., 1997, Chen and Zack, 1996, DesJardin and Hauswirth, 1996, Kumar, et al., 1996, Lerner, et al., 2001, Mears, et al., 2001, Morabito, et al., 1991, Nie, et al., 1996, Rehemtulla, et al., 1996) NRL can bind directly with CRX to synergistically co-activate the *Rho* promoter. (Mitton, et al., 2000) FIZ1 represents a model to study an interface between these crucial transcription factors and the more ubiquitous regulatory machinery of the cell.

#### 4.1. FIZ1 protein expression in various tissues

Western blots of protein extracts from several tissues, previously shown to contain *Fiz1* mRNA by northern blot, confirmed the presence of FIZ1 protein. Previous analysis of FIZ1 protein content in several hematopoietic progenitor and fibroblast cell-lines seemed to suggest that endogenous FIZ1 protein is easier to detect in more differentiated cells (i.e. NIH-3T3). (Wolf and Rohrschneider, 1999) We previously found that proliferating COS and CV-1 cells contain very low levels of FIZ1. (Mitton, et al., 2003) One could speculate that the function of FIZ1 is associated with processes during and after maturation of cell-type.

#### 4.2. FIZ1 protein expression in retinal development

Examination of the mouse neural retina at several points of postnatal development revealed that retinal FIZ1 expression is developmentally regulated. Previous work had only examined mature retina. FIZ1 protein levels were minimal in neonatal mouse retina, when about half of the retinal precursor cells have not completed a final mitosis (P0 in the mouse.) A clear and dramatic increase, in FIZ1 protein content was seen through the period of neuronal maturation (approximately P5 to P21).

While 50% of the cells destined to the rod-photoreceptor fate are born by P0, expression of the *Rho* gene does not occur until P4-P5. This expression pattern is similar for other rod-specific visual transduction genes, including *Pde6b*. (Bowes, et al., 1988) If FIZ1 has influence on the expression level of photoreceptor specific genes, such as *Rho*, it is expressed during the correct time period.

In frozen sections of neural retina, immunolabeling for FIZ1 protein was quite intense in the inner segment region. The antibody used can detect FIZ1 protein in retinal nuclear protein extracts, but does not label FIZ1 well in nuclei on sections. (Mitton, et al., 2003) This could reflect sensitivity limitations on sections, or a masking of antigen. Several nuclear proteins have been difficult to detect in retinal sections, possibly due to masking of antigen within the large protein complexes involved in gene regulation. Different antibodies to NRL, for example, have displayed varied utility on retinal sections and blots. (Rehemtulla, et al., 1996, Swain, et al., 2001) The location of FIZ1 in photoreceptor nuclei is supported by co-immunoprecipitation with NRL from retinal nuclear extracts. (Mitton, et al., 2003) Intense

labeling of FIZ1 in photoreceptor inner segments, suggests that this protein is also present in the photoreceptor cytoplasmic compartment.

The presence and appearance of FIZ1 in other regions of the retina, such as ganglion cells, suggests that FIZ1 may have a maturation-related function in these cells as well. During this time of development, retinal neurons begin to express genes that are functionally cell-specific (i.e. *Rho*), and synaptogenesis progresses in bipolar, photoreceptor, and ganglion cells. (Bachman and Balkema, 1993, Rich, et al., 1997) Could FIZ1 have different interactions with other transcription factors involved in maturation-related gene expression in these other retinal cell-types? At this time, we can only speculate.

#### 4.3. FIZ1 can modulate NRL and CRX-mediated activation of rod specific gene promoters, Rho and PDE6B

Previously we demonstrated that FIZ1 interacts with NRL using yeast two-hybrid screening, GST-pulldown and co-immunoprecipitation from retinal nuclear-protein extracts. To test the functional implications of FIZ1/NRL interaction, we analyzed its role in NRL/CRX-mediated activation of the *Rho*, and *PDE6B* gene promoters *in vitro*. Previous tests had demonstrated an inhibition of NRL-mediated activation of the *Rho* promoter with greater quantities of FIZ1 expression DNA and lower quantity of NRL expression DNA. Continuing our explorations with this assay system, we included a second rod-specific promoter (*PDE6B*). We reduced the differences in quantities among the different expression plasmids and decreased the total DNA concentration, to minimize the over expression required for data collection. Under these parameters it is apparent that FIZ1 can contribute to the activation synergy at the minimal *Rho*, and *PDE6B* gene promoters. Lerner et al., (2005), reported a similar experience with the Sp4 transcription factor (Zn-finger protein). Excessive amounts of Sp4 expression vector had an inhibitory effect on promoter reporters, while a reduced amount synergized with CRX on the *Rhodopsin* promoter.

FIZ1 alone had little effect on the *Rho* proximal-promoter, and a small additive effect with NRL or CRX. When combined with NRL and CRX, FIZ1 synergized to give the maximum activation of all the combinations tested. With the *PDE6B* minimal-promoter, maximum activation resulted from FIZ1 in synergy with NRL alone. Addition of CRX to NRL and FIZ1 resulted in a decrease of NRL-FIZ1 synergy. We also observed slight activation of *PDE6B* promoter by CRX and FIZ1.

Different effects of FIZ1 at these promoters could be attributed, in part, to the different effects of NRL and CRX at these same promoters. Our results with NRL and CRX in co-transfection assays are consistent with previous reports. NRL and CRX clearly synergize on the *Rho* proximal-promoter, but CRX does not produce this strong effect on the *PDE6B* minimal-promoter, in spite of the presence of a CRX binding element (Chau, et al., 2000, Lerner, et al., 2002, Mitton, et al., 2000). Our results here, comparing the two promoters, show that CRX lacks synergy with NRL on the *PDE6B* promoter. However, recent Chromatin immunoprecipitation studies by Peng et al., (2005), indicate that CRX co-occupies the mouse *Pde6b* gene promoter with NRL and NR2E3 *in vivo*. Lerner et al., have recently shown that CRX can synergize with Sp4 (a zinc-finger transcription factor) to activate the *Rho* promoter, and may slightly inhibit Sp4-mediated activation of the *PDE6B*

promoter. (Lerner, et al., 2005) Our results here indicate that CRX can decrease the activation synergy of FIZ1 with NRL at the *PDE6B* promoter.

At the molecular level, the different effects of NRL, CRX, and FIZ1 on these two promoters could result from the different combinations and spacing of DNA-binding elements in the promoters. It is also possible that an unknown factor may associate at one promoter and not the other; it is wise to keep in mind that we do not possess a complete inventory of all proteins present on these promoters in vivo. (Peng, et al., 2005) Furthermore, NRL and CRX interaction does not require interaction with DNA. (Mitton et al., 2000) Therefore, the same protein combinations could be present on different promoters, yet use a different balance of protein and DNA associations. These alternative structures could propagate different effects on the transcriptional apparatus.

Same-site reorientation is another mechanism, acting on bZIP proteins, that may have implications for the NRL-mediated promoters used in this study. Erklasnon, et al. (1996) have shown that the orientation of the AP-1 (Fos/Jun) DNA binding surface can be reversed, on the same DNA element, due to interaction of its leucine zipper with NFAT. This ability of bZIP proteins to reverse orientation, on the same DNA-binding element, would reverse the orientation of their N-terminal activation-domains. It is possible to envision similar effects on NRL.

Interaction of a bZIP and Homeodomain (HD) were discovered through studies of NRL/CRX association. (Mitton, et al., 2000) Likewise, CRX and NR2E3, interact via their DNA-binding domains. (Peng et al., 2005) Studies of Sp4 indicate that the Sp4 DNA-binding domain is also sufficient and necessary for interaction with the CRX HD. (Lerner, et al., 2005) Currently we do not know if FIZ1 has DNA-binding activity; however, the FIZ1-binding domain of NRL is the bZIP domain, including the leucine zipper. (Mitton et al., 2003) We would note that FIZ1 binding to NRL's bZIP domain is compatible with a synergy effect. While the basic portion of a bZIP dimer interfaces with dsDNA, the leucine-zipper portion of the complex extends away from the DNA normal axis. This leucine-zipper dimer is free to interact with other proteins along its entire length. X-ray crystallographic models of NFAT and AP1, bound to DNA, have illustrated this clearly. (Chen et al., 1998)

The human *FIZ1* gene is located near several other genes important to retinal development, such as the *CRX* gene, on Chromosome-19. This chromosome is particularly rich in Zf-proteins. Analysis of the human and mouse genome sequences does not reveal any significant homology for *FIZ1* with other known or theoretical genes. This includes other Zf-proteins, some of which fall into closely related families based on overall amino-acid sequence. FIZ1 represents a unique Zf-protein in its overall structure. FIZ1 has eleven Zn-fingers organized into groups of four, two, two and three Zn-fingers. The N-terminal (four fingers) and C-terminal (three fingers) groups are capable of interacting with the kinase domain of the RTK, Flt-3/Flk-2. (Wolf and Rohrschneider, 1999)

Zn-finger domains typically provide for DNA-protein and protein-protein interactions. Zf-proteins feature prominently as regulators of gene repression, activation and chromatin reorganization (supporting both repression and activation). Several Zf-proteins (EKLF, SP1,

GATA-1) utilize motifs of two or three Zn-fingers to recruit the chromatin remodeling complex SWI/SNF to the  $\beta$ -Globin gene promoter. (Armstrong, et al., 1998, Emerson, 2002, Kadam, et al., 2000) At this time we cannot rule out the possibility that FIZ1 may have the ability to interact directly with DNA, in light of the fact that FIZ1 has multiple Zn-finger domains. Promoter activation studies here cannot answer this particular question; however, it is possible for a protein to alter the activation context with or without DNA association. For example, current evidence suggests that NR2E3 exerts its effects through interaction with CRX without binding to DNA directly. (Peng et al., 2005)

NR2E3 (a nuclear receptor) is required for normal photoreceptor development, and recent studies have demonstrated NR2E3 can interact with CRX and regulate transcription at *Opsin* promoters. (Cheng, et al., 2004, Haider, et al., 2000, Peng, et al., 2005) Nuclear receptors, as a family, are noted for both repression and activation effects that are mediated through Zn-finger proteins. (Belandia, et al., 2002) Recent studies also indicate that the interaction of NR2E3 with NRL or CRX may not be direct and additional transcriptional regulatory proteins might be necessary for the formation of a stable complex *in vivo*. Appearance of FIZ1 during retinal maturation is consistent with our hypothesis that FIZ1 is required for the precise regulation of genes imparting cell-specific functionality. FIZ1 may be one additional regulatory protein, which forms a complex with NRL, CRX, Sp4 and NR2E3, and is necessary for regulating the precise control of photoreceptor-specific genes such as *Rho* and *PDE6B*.

In summary, FIZ1 protein content is developmentally regulated in post-natal retinal development. Total FIZ1 protein and mRNA content increase during the photoreceptor maturation period in mouse neural retina. The timing of this increase in *Fiz1* expression corresponded to the increasing expression of photoreceptor-specific genes, such as *Rho* and *PDE6B*. Transient co-transfection studies indicate that FIZ1 can work with NRL and CRX, to alter the activation of rod-specific gene promoters. Further studies will examine the interaction of FIZ1 with CRX and NR2E3.

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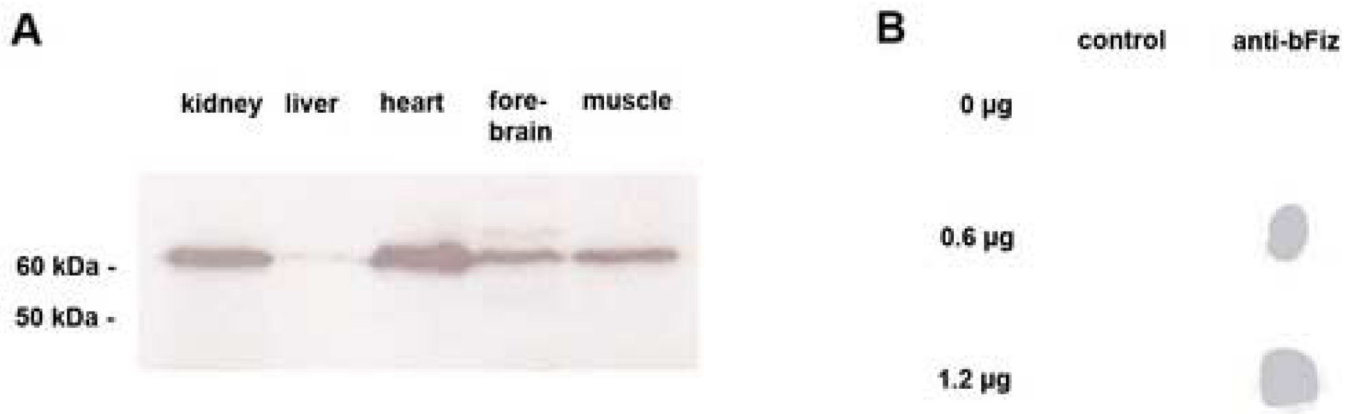
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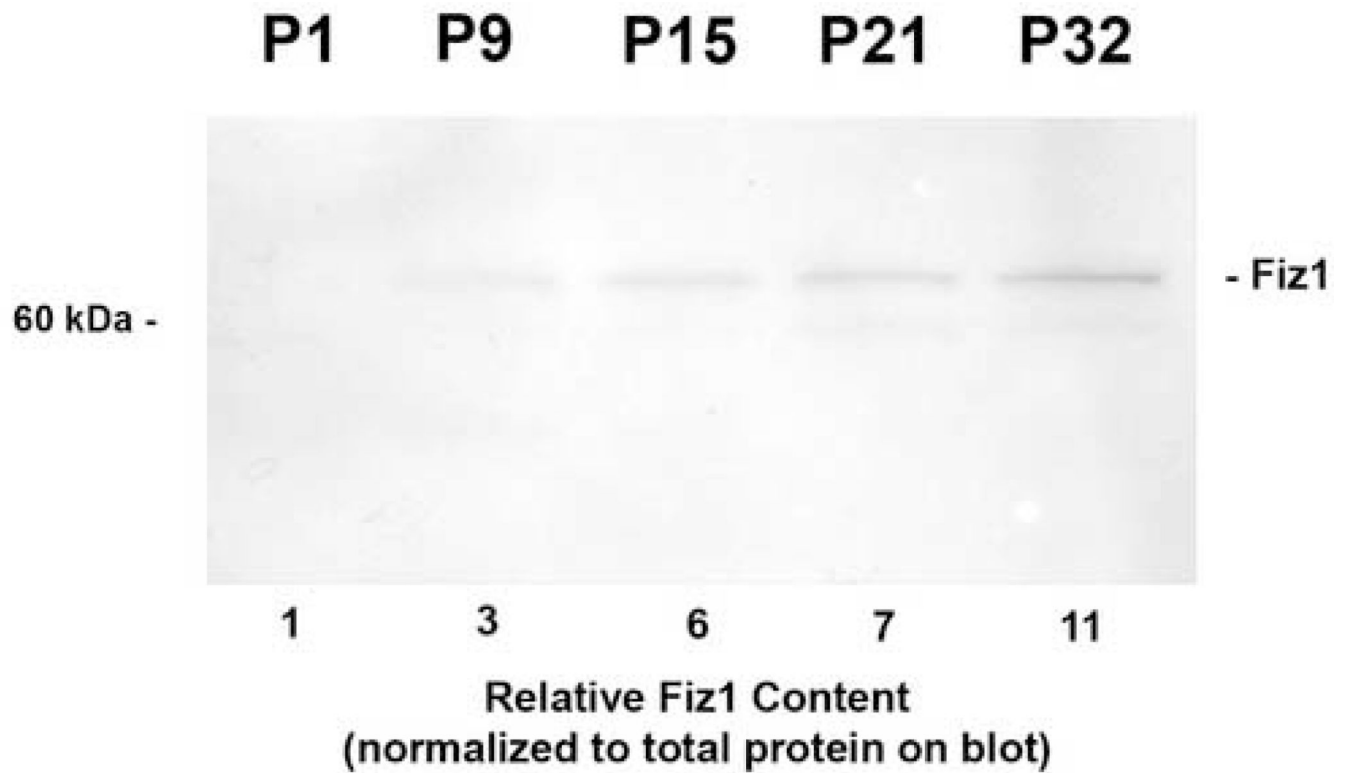
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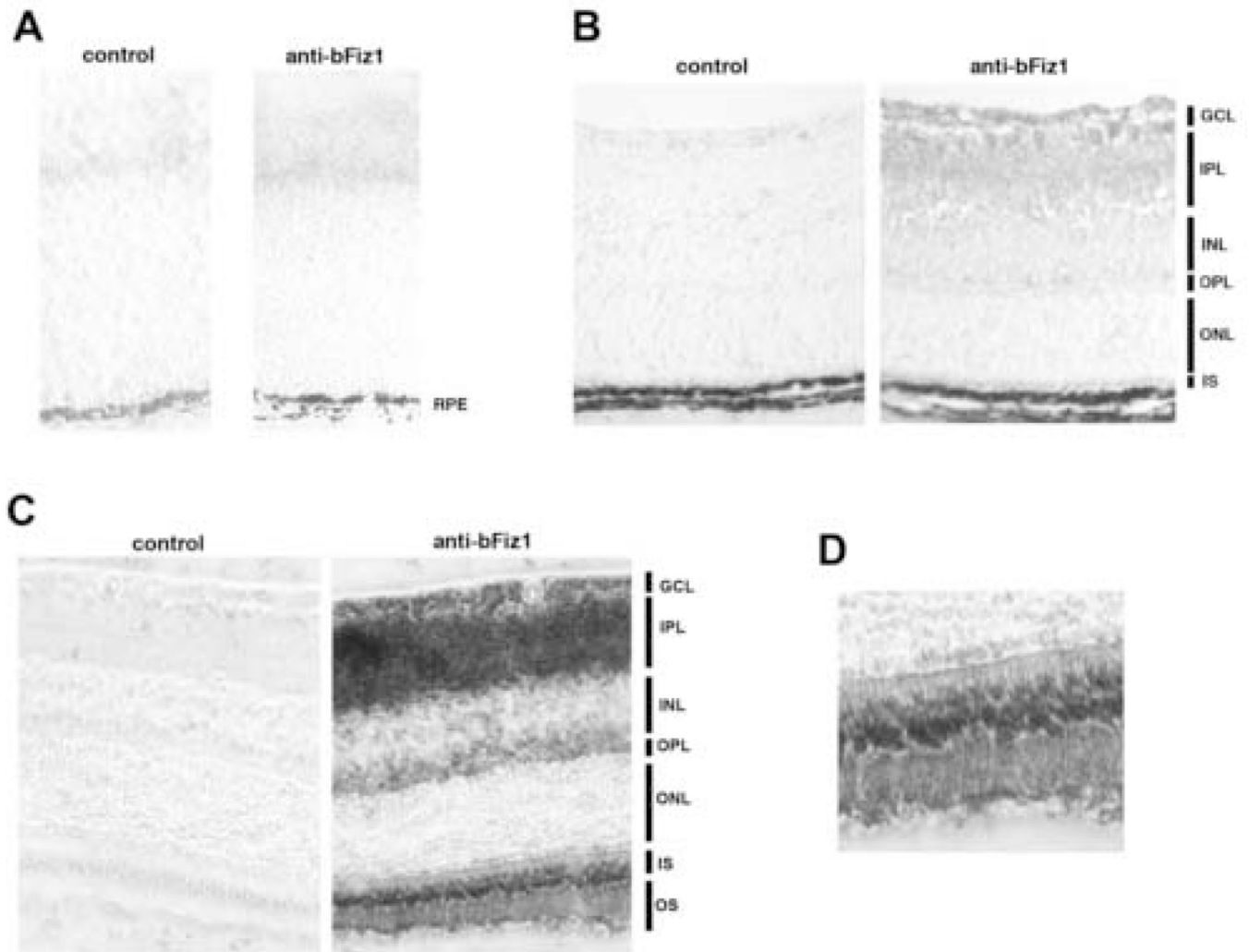


**Fig. 1.**

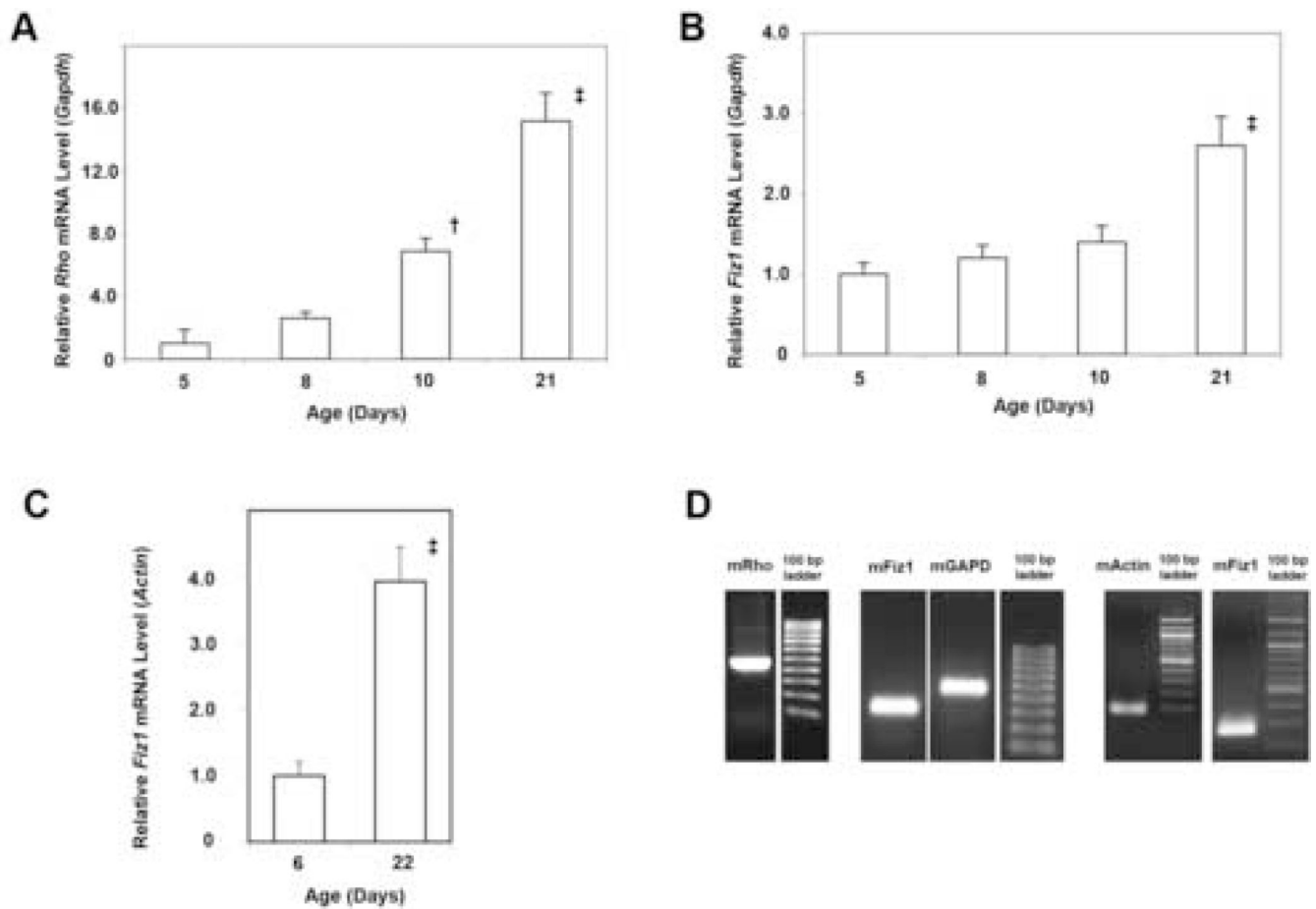
FIZ1 protein is present in several non-ocular tissues that express *Fiz1* mRNA. Expression of FIZ1 protein in mouse tissues was examined by Disc-PAGE and immunoblotting with affinity-purified antibody to the FIZ1 protein. **A.** FIZ1 protein was detected in all tissues examined. (25  $\mu$ g total protein per lane) Equivalent loadings were confirmed by direct staining of protein on the blot itself as described in methods. **B.** Dot blots of protein extracts from CV-1 cells transiently expressing the bovine FIZ1 protein used for production of the FIZ1 antibody. Lysates of cells transfected with 0  $\mu$ g, 0.6  $\mu$ g and 1.2  $\mu$ g of the pDest26-bFiz mammalian expression plasmid were dotted onto nitrocellulose, and then incubated with or without (control) affinity-purified antibody to FIZ1.



**Fig. 2.** FIZ1 protein concentration increases during photoreceptor maturation. Relative levels of FIZ1 protein, in total neural retina (mouse), were examined by Disc-PAGE and immunoblotting with affinity-purified antibody to the FIZ1 protein. Relative FIZ1 protein content was determined using the total protein actually transferred to the blot, as described in materials and methods.

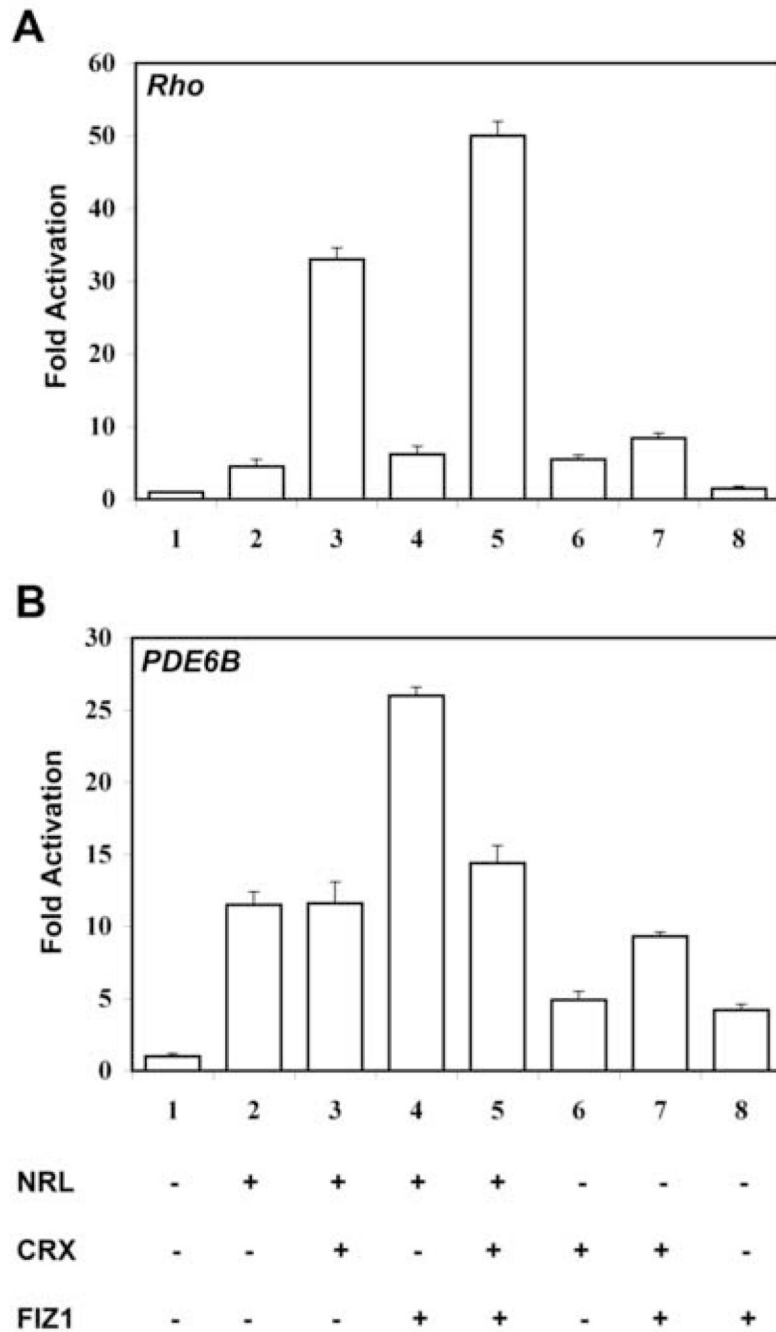


**Fig. 3.** FIZ1 protein content increases in photoreceptors and the ganglion cell layer during maturation of the mouse retina. **A to C.** Affinity purified antibody to bFIZ1 (rabbit). Control using pre-immune rabbit serum. Secondary antibody: biotinylated goat anti-rabbit. Detection: avidin-biotin-complex peroxidase, DAB/H<sub>2</sub>O<sub>2</sub> substrate (Vector Labs). Visualized with phase contrast using equivalent exposures. **A.** Immature retina, day P0; the FIZ1 protein levels are below detection. **B.** Before outer-segment formation, day P7. The timing of increased FIZ1 protein content is similar in photoreceptors and the ganglion cell layer. Inner segments are just starting to form at P7. **C.** Mature adult retina. FIZ1 protein was detected in the photoreceptor inner (IS) and outer segments (OS), foot pedicle region in outer plexiform layer (OPL), ganglion cell layer (GCL) and inner plexiform layer (IPL). **D.** Enlargement of the inner and outer segment region as shown in **C.**



**Fig. 4.** *Fiz1* Gene Expression Increases During Photoreceptor Maturation and the Onset of *Rhodopsin* Gene Expression. **A.** Total *Rho* mRNA, normalized to *Gapdh*, in the mouse neural-retina, measured with the Amplitaq-gold real-time PCR method. (Relative to age P5: †  $p < 0.05$ ; ‡  $p < 0.01$ , ANOVA Tukey-Kramer.) **B.** Total *Fiz1* mRNA, normalized to *Gapdh*, in the mouse neural retina, measured with the Amplitaq-gold real-time PCR method. (Relative to age P5: ‡  $p < 0.01$ .) **C.** Additional confirmation of total *Fiz1* mRNA levels comparing immature and mature mouse neural retina using direct calibration to universal mouse reference RNA, and normalization to  $\beta$ -*Actin* mRNA. (‡  $p < 0.01$ ) **D.** Examples of real-time PCR products for mouse *Rho* (mRho), *Fiz1* (mFiz1), *Gapdh* (mGAPD) and  $\beta$ -*Actin* (mActin) using the primers in Tables 1 and 2. PCR products were confirmed by direct DNA sequencing.



**Fig. 5.**

FIZ1 activation synergy with NRL and CRX at the *Rho* and *PDE6B* promoters.

**A.** Fold activation of the *Rho* proximal promoter in co-transfected CV-1 cells. FIZ1 synergy required the presence of both NRL and CRX. All transfection mixes contained the *Rho*-promoter luciferase-reporter construct, pBR-130luc, and the normalizer Renilla-luciferase construct pRL-CMV. Triplicate sample wells were co-transfected with expression vectors for NRL, CRX, and FIZ1 in the combinations as indicated (expression vector +, corresponding empty vector -). Firefly luciferase activity was normalized to Renilla-luciferase activity for

each sample. Fold Activation is shown relative to reporter DNA in the presence of all empty vectors. Bars indicate SD (n = 3).

Fold activation of the *PDE6B* minimal-promoter in co-transfected CV-1 cells. FIZ1 synergy required the presence of NRL, and was inhibited by CRX. All transfection mixes contained the *PDE6B*-promoter firefly-luciferase reporter, pGL2-PDE6B-luc, and the normalizer Renilla-luciferase construct pRL-CMV. Triplicate sample wells were co-transfected with expression DNAs for NRL, CRX, and FIZ1 in the combinations as indicated (expression vector +, corresponding empty vector –). Firefly luciferase activity was normalized to Renilla-luciferase activity for each sample. Fold Activation is shown relative to reporter DNA in the presence of all empty vectors. Bars indicate SD (n = 3).

**Table 1**

Primers for quantitative PCR analysis of mouse retinal gene expression using Amplitaq Gold Method.

<i>Fiz1</i>	TGTAGCGAATGTGGCAAGAGTTTC	AGGGCTTTTCACCAGTGTGGAC	247bp
<i>Gapdh</i>	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452bp
<i>Rho</i>	TCAGAAGGCAGAGAAGGAAGTCAC	TGGTCTTGGTGGATGGATGC	528bp
<b>Thermo cycling:</b>	95°C/8min., 40 × (95°C/30sec., 60°C/45sec., 72°C/60sec.), melting curve (90°C – 50°C)		

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**Table 2**

Primers for quantitative PCR analysis of mouse retinal gene expression using Roche SYBR green and  $\beta$ -actin comparitor.

<b><i><math>\beta</math>-Actin</i></b>	TGACAGGATGCAGAAGGAGA	CGCTCAGGAGGAGCAATG	75bp
<b><i>Fiz1</i></b>	TGTAGCGAATGTGGCAAGAGTTTC	AGGGCTTTTCACCAGTGTGGAC	247bp
<b>Thermo Cycling</b>	95°C/10 min., 40 × (95°C/30sec., 62°C/45sec., 72°C/30sec.), melting curve (90°C – 50°C)		

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