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Expression of *Pax-6* mRNA in the Retinal Degeneration *(rd)* Mouse

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The homeobox gene *Pax-6* is expressed during eye development in both the retina and lens, and *Pax-6* mutations cause ocular abnormalities including retinal defects. We investigated the pattern of *Pax-6* gene expression in the *rd/rd* mouse model of inherited retinal degeneration in comparison with nondegenerative controls, using Northern blot, reverse-transcription (RT)-PCR and *in situ* hybridization analysis. We observed an increased level of *Pax-6* mRNA expression in the degenerative state, which appeared to affect equally the major *Pax-6* exon 5a transcriptional splice variants as detected by RT-PCR. By *in situ* hybridization, *Pax-6* mRNA was localized to the inner nuclear and ganglion cell layers of nondegenerative retina, but showed a more diffuse signal pattern in the *rd/rd* retina. This modulation of *Pax-6* mRNA levels and localization is suggestive of activation of expression in retinal glial cells and may reflect reorganization of cellular interactions in response to the degenerative processes.

The homeobox gene *Pax-6* encodes a transcription factor which has been highly conserved both structurally and functionally throughout evolution, and plays key roles in the development of the eye and other central nervous system structures in organisms as diverse as humans and squid (for reviews see (1, 2)). A closely homologous gene *eyeless* is essential for ocular development in *Drosophila* (3), and remarkably the murine *Pax-6* gene is capable of directing ectopic ommatidial eye formation in transgenic fruitflies (4). The Pax-6 protein has two DNA-binding motifs, a paired domain and a homeodomain, which control the targeting of the factor to regulatory sites in the genome. Mutations in *PAX6*/*Pax-6* are responsible for ocular abnormalities including human aniridia (5, 6) and the mouse and rat homologue *small eye* (*Sey*) (7, 8). In humans and mice, the heterozygous state for these mutations results in reduction of the iris, together with abnormalities of lens, cornea and retina. Homozygotes are anophthalmic and die shortly after birth, and the failure of eye development in these cases appears to be due to lack of formation of the lens placode, a structure which plays a key role in organizing the optic cup and is an early site of high levels of *Pax-6* expression in normal development (9, 10). *Pax-6* is able to regulate its own promoter (11), and moreover there appear to be numerous transcriptional and translational *Pax-6* variants, with alternative target promoter sequences and presumed functional differences (see (1, 2) for review; also (12)). These include a major splice variant with an additional exon of 42 bases which gives rise to a 14-amino-acid insertion between glutamine 44 and valine 45 of the paired domain; this exon, termed 5a in mouse and human, appears to be vertebrate-specific (1).

In addition to its roles in the anterior eye structures, *Pax-6* is expressed in the developing retina in the prospective amacrine and ganglion cells, suggesting a function in the differentiation of neural as well as ectodermal ocular tissues (13). In view of this and the evidence for retinal abnormalities associated with *Pax-6* mutations, we have investigated the pattern of *Pax-6* expression associated with a progressive inherited retinal degeneration, that displayed by the homozygous *rd* mouse. In these animals, recessive mutation of the gene for the β-subunit of the visual transduction enzyme, cGMP phosphodiesterase (PDE-β) leads to elevated retinal cGMP levels and rapid postnatal degeneration of the photoreceptor cells (reviewed in (14)). We here report that *Pax-6* mRNA is altered in the level and pattern of expression in the degenerating retina in comparison with nondegenerative controls, and discuss the possible significance of this in relation to the cellular and molecular changes accompanying photoreceptor loss.

TISSUES, MATERIALS AND METHODS

Tissues. Homozygous *rd/rd* mice and congenic nondegenerative C57BL/6 and *rd/+* control mice were the source of tissues for RNA extraction and sectioning for *in situ* hybridization. The animals were maintained in a 12 hours light, 12 hours dark cycle, and were killed by cervical dislocation and enucleated during the light phase. The

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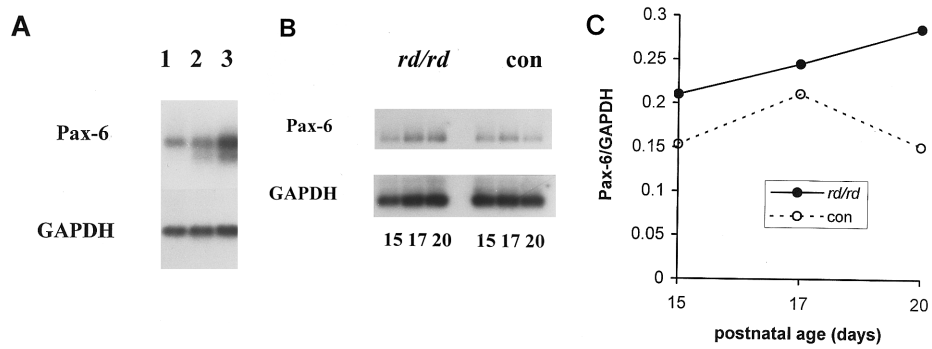

FIG. 1. Northern blot analysis of *Pax-6* mRNA expression in murine retina. (A) Retinal RNA samples (3 μg each) from mice at postnatal age 16 days: Lane 1, C57BL/6 (+/+); 2, heterozygote (*rd*/+); 3, homozygote (*rd/rd*) probed with murine *Pax-6* cDNA (upper panel) or rat GAPDH (lower panel). (B) Retinal RNA samples (3 μg each) from *rd/rd* and control (C57BL/6: con) mice aged P15, 17 and 20 days, probed as in (A). (C). Expression of *Pax-6* mRNA standardized to GAPDH using laser densitometric analysis from Northern blots in (B).

rd/+ and *rd/rd* mice, in which the *rd* locus is closely linked to the light ear (*le*) character (15), were maintained in an inbred colony at St Thomas' Hospital. The C57BL/6 animals were obtained from Harlane UK, Bicester, UK.

RNA extraction, Northern blot analysis, reverse transcription PCR and Pax-6 cDNA cloning. Total RNA was extracted from pooled frozen tissues (from a minimum of 3 animals at each time point) using either the RNAXEL system (Eurobio Laboratoires, Les Ulis, France) or RNeasy kit (Qiagen, Crawley, UK) according to the manufacturers' instructions. For Northern analysis, 3 or 4 μg samples were denatured, electrophoresed in 1.3% agarose gels and blotted onto nylon membranes (Amersham Int., Slough, UK). Cloned inserts from plasmids were labelled with α-[^{32}-P]dCTP using the Rediprime kit (Amersham Int., Slough, UK) and hybridized to the blots in the presence of 50% formamide at 42°C overnight, followed by stringent washing and autoradiography. The probes were stripped off between separate hybridizations. A control probe for RNA loading was a cDNA for rat glyceraldehyde phosphate-3-dehydrogenase (GAPDH). Autoradiographic signal quantification was performed using a laser densitometer (LKB, Sweden).

Reverse transcription PCR (RT-PCR) was performed on single-stranded cDNA obtained according to the following conditions: 1 μg murine retinal RNA samples were incubated in 20 μl reactions with 1.25 units AMV reverse transcriptase (GibcoBRL, Paisley, UK) and random hexamer primers (Pharmacia Biotech, Uppsala, Sweden) for 60 min at 42°C, followed by heat inactivation of the enzyme. To generate cDNA clones for Northern and *in situ* analysis, amplifications were performed using primers MPAX-6.1: 5′-ATGCAGAACAGTCACAGCGGAGT-3′ and MPAX-6.2: 5′-TTACTGTAATCGAGGCCAGTACT-3′, in 30 μl reactions using 0.75 units *Taq* polymerase (GibcoBRL, Paisley UK) and 2 μl cDNA in a thermal cycler (Hybaid, Teddington, UK) with the following parameters: 94° 3 min, then 40 cycles of 94° 45 s, 56° 45 s, 72° 60 s, and final step of 72° 5 min. The product was cloned into the vector pCR2.1 (Invitrogen, Leek, The Netherlands) and confirmed by partial sequence analysis (using an ALF automated sequencer, Pharmacia Biotech, Uppsala, Sweden) to contain the canonical *Pax-6* coding sequence. Subcloned versions of this same insert and of a truncated (529 bp) fragment of the 3′-end of this cDNA in the vector pBluescript (Stratagene, La Jolla, CA, USA) were also constructed. For the analysis of alternative transcript expression, primers MPAX-6.3: 5′-CAGAAGATCGTAGAGCTAGC-3′ and MPAX-6.7: 5′-GAAGAACTCTGTTTATTGATGAC-3′ were used, with conditions as above except that an extension time of 35 s and 35 cycles of amplification were used. The products were resolved on ethidium bromide-stained 1.0% agarose (Pharmacia)/3.0% NuSieve GTG (FMC, Flowgen, Lichfield, UK) gels. Molecular size markers were obtained from GibcoBRL.

In situ hybridization. Linearized *Pax-6* cDNA plasmids were used to generate sense and antisense [^{35}S]UTP-radiolabelled (Amersham International plc, Slough, UK) cRNA probes (~10^4 cpm/ml) *in vitro* as described previously (16). Tissue sections (10 μm thick) were processed for *in situ* hybridization and dipped in Kodak (New Haven, CT, USA) NTB2 emulsion (1:1 in water). Three to four weeks later the slides were developed and stained with toluidine blue.

RESULTS AND DISCUSSION

We initially compared levels of *Pax-6* mRNA at postnatal day (P)16 in *rd/rd* and nondegenerative *rd*/+ and +/+ (C57BL/6) mouse retinas by Northern blot analysis (Fig. 1A). This time-point corresponds to the peak of photoreceptor cell death by apoptosis in the homozygous animal (17). In all genotypes, there was hybridization of a major transcript of approximately 2.5 kb in size (Fig. 1A, upper panel). Minor bands of smaller size, which may have been artefactual or alternative transcriptional forms, were present in *rd*/+ and *rd/rd* samples. The level of *Pax-6* expression was substantially increased at this time-point in the *rd/rd* retina (lane 3; approximately twofold higher than in *rd*/+ by laser densitometric analysis), and was not due to variable loading as the control probing with GAPDH showed, which detected a band of ~1.4 kb in size (Fig. 1A, lower panel). We extended the comparison to P15, 17 and 20 in a separate Northern analysis (Fig. 1B, C). Relative levels of expression standardized to GAPDH mRNA levels (Fig. 1C) showed that while there was some fluctuation in *Pax-6* mRNA in control C57BL/6 retinas, there was a higher and increasing level in the degenerative state, corresponding to an approximately twofold increase in *Pax-6* mRNA at P20 in the *rd/rd* retina.

In order to determine the pattern of *Pax-6* transcript variants in control and dystrophic retinas, selected RNA samples were subjected to reverse transcription PCR (RT-PCR) using primers located at sites spanning the region containing the differentially-spliced exon 5a

Exon 4 Exon 5
AGCATGCAGAACAGTCACAGCGGAGTGAATCAGCTTGGTGGTGTCTTTGTCAACGGGCGG

MPAX6.3 →
CCACTGCCGGACTCCACCCGGcagaagatcgtagagctagcTCACAGCGGGGCCCGGCCG

Exon 5a
TGCGACATTTCCCGAATTCTGCAGACCCATGCAGATGCAAAAGTCCAGGTGCTGGACAAT

Exon 6
GAAAACGTATCCAACGGTTGTGTGAGTAAAATTCTGGGCAGGTATTACGAGACTGGCTCC

ATCAGACCCAGGGCAATCGGAGGGAGTAAGCCAAGAGTGGCGACTCCAGAAGTTGTAAGC

AAAATAGCCCAGTATAAACGGGAGTGCCCTTCCATCTTTGCTTGGGAAATCCGAGACAGA

Exon 7
TTATTATCCGAGGGGGTCTGTACCAACGATAACATACCCAGTGTgtcatcaataaacaga
← MPAX6.7

gtTCTTCGCAACCTGGCTAGC

FIG. 2. Part of the murine *Pax-6* coding sequence. Exons 4, 5a and 7 are underlined and the target sequences of the PCR primers MPAX6.3 and MPAX6.7 are shown in lower case. PCR products using these primers are of predicted size 295 bp (exon 5a−) and 337 bp (5a+).

(1, 12) (Fig. 2). In addition, these primers were designed to be able to amplify further transcriptional variants reportedly expressed in bovine ocular tissue (18). Under the conditions of amplification used, two PCR products of the sizes predicted for the +5a (337 bp) and −5a (295 bp) exon transcripts were detected in all samples examined (Fig. 3). In each retinal sample, the intensity of the two bands was approximately equal (Fig. 3, lanes 1-6), whereas in the lens the canonical −5a form predominated (Fig. 3, lane 7), in agreement with that previously reported in bovine lens (18). Moreover, the bands in all samples from *rd/rd* retina stained more intensely than those from the C57BL/6 controls (compare lanes 1-3 with 4-6), although it should be noted that the RT-PCR was not designed as a strictly quantitative assay. No smaller bands corresponding to predicted alternative splicing products of exon 6 (18) were detected in any of the samples.

By *in situ* hybridization, the normal developmental pattern of *Pax-6* mRNA expression from P15 to P20 was shown to be localized particularly to the inner part of the inner nuclear layer (INL), where amacrine and horizontal neuron nuclei are situated, and to the ganglion cell layer (GCL) (Fig. 4B, D and F). No expression was detected in the outer nuclear layer (ONL) or the retinal pigment epithelium when silver grain distribution was carefully examined. These results accord with the pattern observed in chick and quail retina during later embryonic and postnatal development (13, 19); a similar localization of Pax-6 protein to these nuclear layers is also seen in mouse retina (20). In the degenerative state (Fig. 4A, C, E), changes in tissue organization, with the progressive loss of photoreceptors and thinning of the outer nuclear layer to a single row of mainly cone nuclei by P20, were as previously described (14). *Pax-6* mRNA displayed a pattern of inner retinal expression at P15 in *rd/rd* retina similar to that of the control (Fig. 4A, B). At P17 (Fig. 4C), however, there was a more diffuse pattern of labelling throughout the degenerative retina. By P20 (Fig. 4E), the signal in the *rd/rd* retina was diffuse, with a loss of the stratified appearance. The labelling was more evenly distributed within the inner retina, particularly in the region extending from the inner plexiform layer to the ganglion cell layer. This diffuse pattern of signal was consistent with that seen for genes expressed primarily in glial cells (e.g. Müller glia), such as that encoding glial fibrillary acidic protein (GFAP) (16). No specific signal was seen using a sense cRNA control probe (not shown).

Abnormal ocular expression of *Pax-6* can have serious consequences for eye development as the aniridic, corneal and retinal dysplasia mutants demonstrate (1, 5-8). Additionally, overexpression of ocular *Pax-6* in one transgenic mouse strain led to complete absence of the photoreceptor layer (21). These findings suggest that ocular cells are highly sensitive to the precise local levels of *Pax-6* expression during development. The normal function of Pax-6 in the anterior eye appears to be primarily in driving the formation of the lens placode from surface ectoderm (9), and the lens may generate crucial signals controlling retinal development. However, in the retina itself, *Pax-6* expression has been implicated in the control of neuronal differentiation such that, together with other transcription factors such as Prox-1 and Chx-10 (13), specific retinal cell lineages may be defined by the subsets of factors expressed within them. If this is the case, then the elevation and relocalization of *Pax-6* mRNA expression we have observed associated with photoreceptor degeneration could suggest reactivation of functions normally occurring during early retinal development. These could include the promotion of cell-cell interactions *via* the regulation of extracellular matrix proteins and neural cell adhesion molecules (22, 23), and could reflect the reorganization of tissue architecture and synaptic connectivity accompanying photoreceptor cell loss. Part of the altered expression may derive from

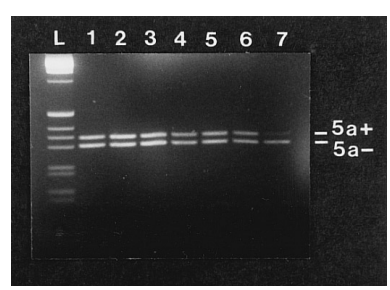

FIG. 3. Reverse-transcription PCR amplification from murine retinal RNAs using primers MPAX6.3 and MPAX6.7 to detect differentially-spliced *Pax-6* transcripts. Lane L, Molecular size markers; then *rd/rd* retinal cDNA at 1, P15; 2, P16; 3, P18; and C57BL/6 retinal cDNA at 4, P15; 5, P16; 6, P18; and C57BL/6 lens cDNA (P20). Bands of the predicted sizes of 295 bp (exon 5a-) and 337 bp (5a+) were detected in all samples.

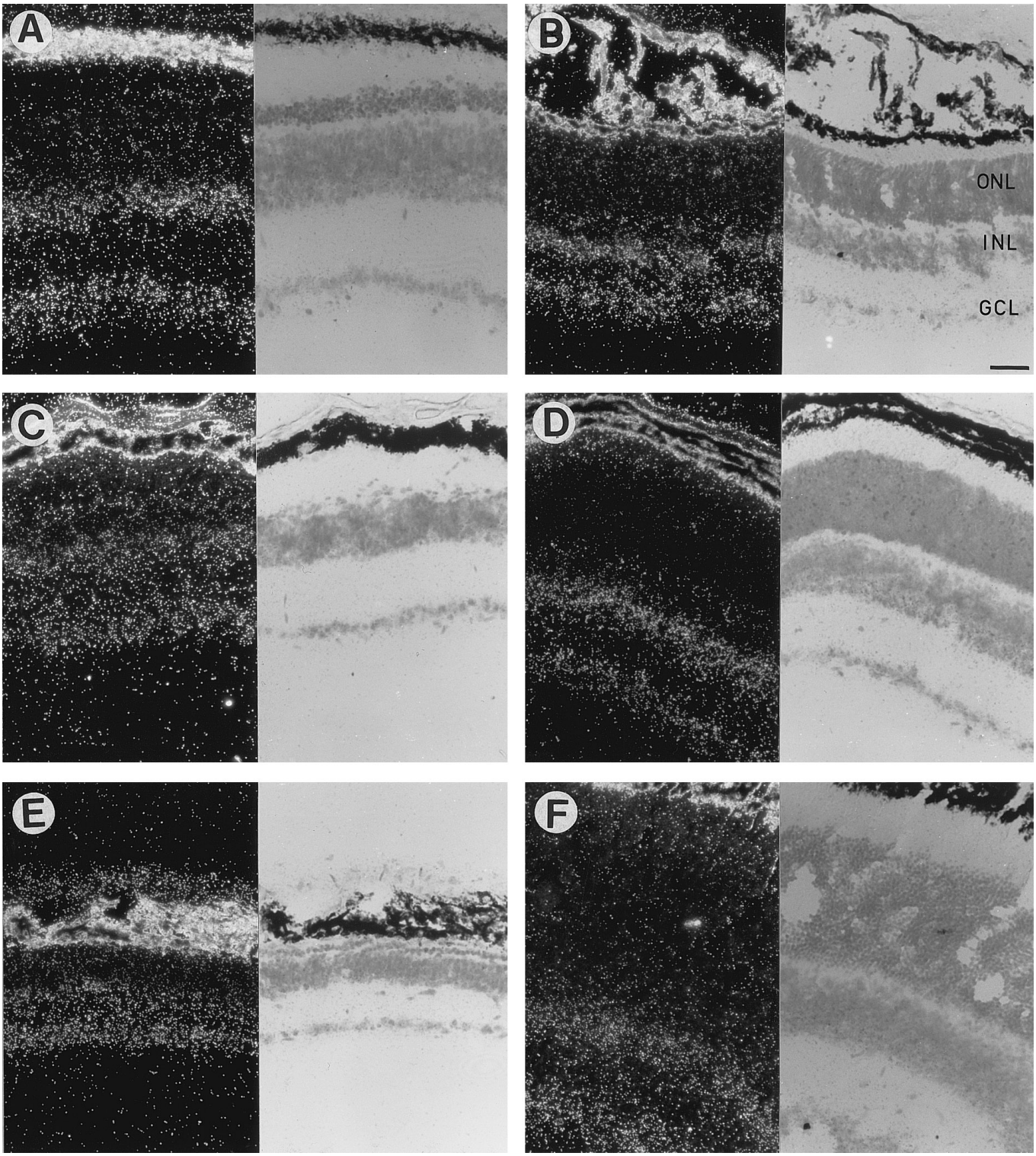

FIG. 4. *In situ* hybridization analysis of *Pax-6* mRNA expression in postnatal murine retina. Autoradiographs of retinal sections following hybridization with ^{35}S-labelled murine *Pax-6* antisense cRNA probe. Darkfield and brightfield views are shown for each section. Panels A, C, E: *rd/rd* retina at P15, 17 and 20 respectively. Panels B, D, F: C57BL/6 retina at P15, 17 and 20 respectively. Abbreviations (panel B): ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Bar = 100 μm. Bright regions near the top of each darkfield section correspond to light-scattering from pigmented layers.

activation of *Pax-6* expression in reactive glial cells, as the *in situ* hybridization pattern at later time-points in *rd/rd* suggests. The gliotic response occurs rapidly to neuronal damage caused by genetic, toxic or physical factors, and it will be of interest to assess *Pax-6* expression in other neurodegenerative states, including models of induced neuronal death such as the light-damaged retina, as well as in conditions such as retinitis pigmentosa and Alzheimer's disease.

Prominent amongst the target genes regulated by Pax-6 are members of the crystallin family of genes (2), which encode proteins abundantly expressed in the lens, and of which some (notably αB-crystallin) are known to have stress-inducible chaperone functions in other tissues. Our recent studies have indicated strikingly altered patterns of crystallin mRNA expression in the *rd* mouse retina (manuscripts submitted). In the light of this, modulation of *Pax-6* expression in the degenerating retina may arise from a subset of cellular signals activated in response to the stresses induced during photoreceptor apoptosis. These signals may correspond particularly to those orchestrating tissue-protective mechanisms, potentially including the up-regulation of the crystallins themselves. Further analysis of the changing patterns of transcription factor expression and downstream regulated genes in retinal degenerative states should provide additional insights into the complex phenomenon of photoreceptor cell death.

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