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Krüppel-like Factor 15, a Zinc-Finger Transcriptional Regulator, Represses the Rhodopsin and Interphotoreceptor Retinoid-Binding Protein Promoters

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Abstract

Purpose—To identify novel transcriptional regulators of rhodopsin expression as a model for understanding photoreceptor-specific gene regulation.

Methods—A bovine retinal cDNA library was screened in a yeast one-hybrid assay, with a 29-bp bovine rhodopsin promoter fragment as bait. Expression studies used RT-PCR and β -galactosidase (LacZ) histochemistry of retinas from transgenic mice heterozygous for a targeted LacZ replacement of KLF15. Promoter transactivation assays measured luciferase expression in HEK293 cells transiently transfected with bovine rhodopsin or *IRBP* promoter-reporter constructs and expression constructs containing cDNAs for full or truncated KLF15, Crx (cone rod homeobox), and/or Nrl (neural retina leucine zipper). Data were analyzed with general linear models.

Results—The zinc-finger transcription factor KLF15 was identified as a rhodopsin-promoter—binding protein in a yeast one-hybrid screen. Expression was detected by RT-PCR in multiple tissues, including the retina, where KLF15-LacZ was observed in the inner nuclear layer, ganglion cell layer, and pigmented epithelial cells, but not in photoreceptors. KLF15 repressed transactivation of rhodopsin and *IRBP* promoters alone and in combination with the transcriptional activators Crx and/or Nrl. Repressor activity required both a 198-amino-acid element in the N-terminal domain and the C-terminal zinc finger DNA-binding domains.

Conclusions—The zinc finger containing transcription factor KLF15 is a transcriptional repressor of the rhodopsin and *IRBP* promoters in vitro and, in the retina, is a possible participant in repression of photoreceptor-specific gene expression in non-photoreceptor cells.

Normal visual function requires the establishment and maintenance of correct temporal and spatial expression of a large number of genes, including those encoding the components of the

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phototransduction machinery (e.g., rhodopsin, cone opsin, transducin, β -phosphodiesterase, arrestin) and those involved in retinoid recycling (e.g., interphotoreceptor retinoid binding protein [*IRBP*]). Many of these genes are expressed exclusively in photoreceptor cells and, based on their coordinated temporal and spatial patterns of expression during retinal development,^{1–3} are likely to share at least some of the same transcriptional regulatory mechanisms. The rod visual pigment rhodopsin, one of the most abundant and well-characterized retinal proteins, is expressed exclusively in rod photoreceptors in the retina^{4,5} and in a subset of pinealocytes in the light-sensitive pineal gland.^{6,7} Defects in rhodopsin structure, function, and cellular localization can result in both loss of visual function and photoreceptor degeneration. Mutations in rhodopsin account for approximately 30% of autosomal dominant retinitis pigmentosa.⁸ Rod photoreceptor survival also requires precise regulation of rhodopsin levels, as transgenic mice that either over- or underexpress wild-type rhodopsin demonstrate retinal degeneration.^{9–12}

Rhodopsin expression is regulated primarily at the transcriptional level.^{1,13} The rhodopsin proximal promoter region (RPPR), located -222 to +70 bp relative to the transcription start site, can drive photoreceptor-specific expression of a β -galactosidase (LacZ) reporter gene in transgenic mice¹⁴ and contains binding sites for both retina-specific and ubiquitous nuclear proteins.^{15–17} Among the transcription factors implicated in rhodopsin regulation are cone rod homeobox (*Crx*)^{18,19} and neural retina leucine zipper (*Nrl*).²⁰ Consistent with the proposal that photoreceptor-specific genes share transcriptional regulatory mechanisms, *Crx* transactivates multiple photoreceptor-specific promoters including rhodopsin, *IRBP*, β -phosphodiesterase, and arrestin,¹⁸ and regulatory targets of *Nrl* identified to date are rhodopsin^{20,21} and the rod-specific β -phosphodiesterase.²² Highlighting the importance of precise gene regulation in the retina, mutations in *Crx* and *Nrl* are associated with retinal degeneration. Homozygous *Crx*-knock-out mice fail to form photoreceptor outer segments (OS), lack detectable visual function, and have retinal degeneration.¹⁹ Mutations in *Crx* or *Nrl* have been identified in patients with various degenerative retinal diseases, including cone-rod dystrophy,^{23,24} Leber congenital amaurosis,²⁵ and both autosomal dominant, and autosomal recessive retinitis pigmentosa.²⁶ In addition, deletion of *Nrl* in mice leads to the loss of normal rods and an increase of conelike cells, suggesting an additional role for *Nrl* in photoreceptor cell fate determination.²⁷

Understanding the molecular mechanisms regulating photoreceptor-specific gene expression may provide new insights into the basis of retinal degenerative disease and aid in the development and targeting of gene-based therapies. To identify additional transcriptional regulators of photoreceptor-specific gene expression, we undertook a systematic analysis of the rhodopsin proximal promoter region by analyzing a series of promoter elements spanning the region from -176 to +52 (relative to the transcription start site) as baits in a yeast one-hybrid assay. We report the results of one of those analyses, which identified KLF15 as a possible transcriptional regulator of rhodopsin expression.

Materials and Methods

Yeast One-Hybrid

Yeast one-hybrid screening of a bovine retinal cDNA-GAL4 fusion library (gift of Ching-Hwa Sung,²⁸ Weill Medical College of Cornell University, New York, NY) was performed as previously described.^{18,28} Bait plasmids pHISi1-bRho29 and pLacZ-bRho29 were constructed with synthetic DNA oligomers containing three tandem repeats of the bovine rhodopsin promoter sequence (bRho29: 5'-ATTAATAACGCCCCCAATCTCCGAGGTGC-3'). *EcoRI*/*MluI*-cut pHISi-1 and *EcoRI*/*Sall*-cut pLacZ bait plasmids were integrated into the genome of the yeast YM4271 by

homologous recombination. Screening was carried out on HIS⁺, LEU⁺, SD plates supplemented with 15 mM 3-amino-1,2,4 triazole.

Plasmids

The coding region of human KLF15 was PCR amplified from a human retinal cDNA library (gift of Jeremy Nathans, The Johns Hopkins University School of Medicine, Baltimore, MD) or from reverse transcribed human retinal RNA and subcloned into pCR-II-TOPO (Invitrogen, Carlsbad, CA). The expression construct hKLF15-pcDNA contains the full 1251-bp coding sequence of human KLF15, subcloned into *Bam*HI/*Eco*RI digested pcDNA3.1+HisC (Invitrogen) expression vector with the insert fully sequenced in both directions to confirm accuracy. KLF15 deletion constructs were generated as follows: for NΔ67, the 1056-bp *Stu*I/*Eco*RI fragment from hKLF15-pCR-II was ligated into *Bam*HI/*Eco*RI-digested pcDNA3.1+HisC with the *Bam*HI overhang filled in with Klenow; for NΔ265, the 5950-bp *Kpn*I fragment of hKLF15pcDNA was gel purified and religated. For NΔ291, a 605-bp fragment was PCR amplified from hKLF15pCR-II, digested with *Bam*HI and *Eco*RI, and ligated into *Bam*HI/*Eco*RI-digested pcDNA3.1+HisC. For CΔ126, a 961-bp fragment was amplified from hKLF15pCR-II, digested with *Bam*HI and *Xba*I and ligated into *Bam*HI/*Xba*I-digested pcDNA3.1+HisC. The expression constructs Crx-pcDNA3.1¹⁸ and Nrl-pED (gift of Anand Swaroop,²⁰ University of Michigan, Ann Arbor, MI) and the promoter-luciferase reporter constructs b-Rho225-luc²¹ and b-IRBP300-luc¹⁸ have been described.

Tissue Sources

Bovine tissues were obtained from a local abattoir. Human ocular tissues, obtained through the National Disease Research Interchange (Philadelphia, PA), came from postmortem donations with informed consent and in accordance with the tenets of the Declaration of Helsinki and with the approval of the Joint Committee on Clinical Investigation of The Johns Hopkins University School of Medicine. Rat and mouse tissues were obtained from animals that were handled and euthanatized according to the Animal Care and Use Policies of The Johns Hopkins University and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Radiation Hybrid Mapping

Human KLF15 was mapped with the Stanford G3 radiation hybrid panel (Invitrogen) using PCR with primers that amplified human, but not hamster, KLF15 (Table 1) in 50-μL reactions (2 mM MgCl₂, 200 μM dNTPs, 2 mM forward and reverse primers, and 50 ng DNA template), using *Taq* DNA polymerase (Invitrogen) according to the manufacturer's recommendations. Results were sent to the Stanford Human Genome Center (<http://www-shgc.stanford.edu>) for analysis.

RT-PCR

Total RNA was extracted from freshly isolated tissues homogenized in Trizol (Invitrogen) and purified according to the manufacturer's instructions. Bovine RNA samples were subsequently processed on separation columns (RNAqueous; Ambion, Austin, TX). For RT-PCR, total RNA was reverse transcribed (SuperScript; Clontech, Palo Alto, CA, for human, mouse, and rat, or ThermoScript; Invitrogen for bovine) with oligo-dT primers (Invitrogen) according to the manufacturers' instructions. First-strand cDNA was used as the template for PCR amplification, with gene-specific primers (Table 1). For KLF15, PCR conditions were 1.5 mM MgCl₂ with the addition of 10% glycerol (human, mouse, and rat) or 1× PCR enhancer (bovine) (Q Buffer; Qiagen, Valencia, CA). Amplification of acidic ribosomal phosphoprotein P0 (ARP) was performed according to published methods.²⁹

LacZ Histochemistry and In Situ Hybridization

Eye cups from wild-type or heterozygous KLF15-LacZ mice (generated and genotyped as previously described³⁰) were fixed in 4% paraformaldehyde, cryoprotected, and frozen as previously described.³¹ Tissue was sectioned at 10 μ m and air-dried onto slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). β -Galactosidase (LacZ) histochemistry was performed by using standard methods.³² For in situ hybridization, digoxigenin-labeled, sense and antisense riboprobes were transcribed (Genius Kit; Roche, Indianapolis, IN) according to the manufacturer's instructions. Templates for probe synthesis were generated by nested PCR. In the first round PCR, oligo-dT primed, reverse-transcribed RNA from mouse retina was used as the template. In the second round, nested primers were used that incorporated either the T3 (sense) or T7 (antisense) promoters (Table 1). Before hybridization, slides were dried at 65°C for 1 hour, digested briefly with proteinase K and prehybridized at 58°C in hybridization buffer (2 $\times$ SSC, 10% dextran sulfate, 2.5 $\times$ Denhardt solution, 100 μ g/mL sheared salmon sperm DNA, 100 μ g/mL yeast tRNA, 40 U/mL RNase inhibitor, 50% formamide). Probes were added at 0.8 ng/ μ L for rhodopsin or at 2.0 ng/ μ L for *Crx* and hybridized overnight at 58°C. For detection alkaline phosphatase-conjugated, anti-digoxigenin antibodies were used (Roche).

Transient Transfections

In promoter transactivation assays, we used transient transfections of HEK293 cells (from human embryonic kidney), according to previously published procedures,^{18,21} with the exception that 2.5 μ g luciferase reporter and 0.025 μ g of a CMV-LacZ reporter (to assess transfection efficiency) were applied to each plate. Within each experiment, the total amount (in micrograms) of DNA used to transfect each plate was kept constant by addition of the corresponding empty expression vector DNA lacking a cDNA insert. At least two independent transfections were performed on different days. For analysis of full-length KLF15, *Crx*, and/or *Nrl*, at least four DNA precipitates were prepared for each condition and divided equally between two plates (≥ 8 plates per condition). For KLF15 deletion analysis, six DNA precipitates (12 plates per condition) were analyzed for each construct.

Statistical Analyses

Relative luciferase activity for each plate was calculated as the ratio of luciferase activity/LacZ activity to control for variations in transfection efficiency. Basal activity of each reporter construct in HEK293 cells was defined as the mean relative luciferase expression for control plates transfected with reporter constructs and empty expression vectors. Multiples of change (*n*-fold) from control were calculated for each plate as the ratio of relative luciferase (experimental) to the mean relative luciferase (control) and analyzed with a linear mixed model (SAS, Cary, NC).^{33,34} This method is analogous to analysis of variance (ANOVA), except that all data points are used in the analysis and the correlation observed between replica samples (two plates transfected with equal portions of the same DNA mix) is modeled directly, using the assumption that the replica samples were exchangeable experimental units. Differences were considered statistically significant if $P \leq 0.05$.

Results

Yeast One-Hybrid Screening: Cloning Bovine KLF15

A bovine retinal cDNA library (the generous gift of Ching-Hwa Sung²⁸) was screened in a yeast one-hybrid assay using a bait construct containing three tandem repeats of a 29-base-pair fragment of the bovine rhodopsin promoter located from -94 to -66 bp relative to the transcription start site. This element lies between consensus binding sites for the homeodomain protein *Crx*¹⁸ and the basic leucine zipper transcription factor *Nrl*.²⁰ Screening 2.14×10^6 transformants yielded 46 clones that grew on selective medium. Rescreening the positive

clones using a pLacZ-bRho29 bait construct was precluded by high background expression of LacZ; therefore, initial picks were retested using the pHISi-bRho29 bait. Of the 26 clones that regrew on HIS—, LEU— medium, five contained cDNAs with high sequence identity to the zinc-finger—containing transcription factor KLF15. Among the remaining clones, only three sequences were represented by more than a single clone (two clones each for opsin, transducin, and epsilon receptor-gamma chain). Because none of these genes are known to participate in transcriptional regulation, they were presumed to be false positives and were not analyzed further.

The largest of the five bovine KLF15 clones, none of which were full length, contained a 1783-bp insert that included an 894-bp open reading frame (ORF) at the 5' end followed by a TGA stop codon, an 889 bp presumptive 3'-untranslated region (UTR) and a polyA tail (accession number AY366084). Comparison to the National Center for Biotechnology Information (NCBI) database showed high sequence identity to cDNA and expressed sequence tag (EST) sequences for KLF15 from human, rat, and mouse. Within overlapping portions of the coding sequence, bovine KLF15 clones shared 83.1%, 77.8%, and 77.9% nucleotide identity with human, murine, and rat KLF15, respectively, as well as 60.7% nucleotide identity with *Xenopus* and 57.1% with zebrafish KLF15. The human, mouse, and rat KLF15 cDNAs extend 5' relative to the bovine KLF15 clones and include an additional 330 bp within the ORF and the 5' UTR sequence.

Comparison of the deduced amino acid sequences identified the longest, contiguous region of sequence identity as an 88-amino-acid region containing three Krüppel-type, Cys2-His2 zinc-finger domains located at the C-terminus (Fig. 1). Outside of the zinc-finger domains, there was somewhat greater divergence across species, although the N-terminal domain contained several conserved elements, including a 17-amino-acid sequence rich in acidic residues that was 100% identical in bovine, human, mouse, rat, and zebrafish KLF15 and differed at only three amino acid residues in *Xenopus* (Fig. 1). Despite the overall sequence conservation among the mammalian KLF15 orthologues, the N-terminal domain shares little homology with other known or predicted proteins and contains no protein motifs that can be identified by comparison to the BLOCKS+ database (available at <http://www.fhrc.org/blocks/> provided in the public domain by the Fred Hutchinson Cancer Research Center, Seattle, WA).

In our initial yeast one-hybrid screen, we used a bovine retinal cDNA library to take advantage of the large size and ready availability of bovine retina compared with mouse, rat, or human retina. However, because sequence information regarding bovine genes is limited and the bovine genome project has yet to be completed, we focused our genomic analysis on human, mouse, and rat *KLF15*. Mapping with the Stanford G3 Radiation hybrid library placed *KLF15* on the long arm of human chromosome 3 (3q21-22), between *D3S1269* and *D3S3606* with lod scores between 4.07 and 7.85, a region of synteny with rat chromosome 4 and mouse chromosome 6. This is consistent with the placement of *KLF15* to the 14.77-kb interval from 127,382,388 to 127,397,148 on the long arm of human chromosome 3 at 21.1 in the July 2003 freeze of the human genome database (available at <http://genome.ucsc.edu/> provided in the public domain by UCSC Genome Bioinformatics, University of California at Santa Cruz, Santa Cruz, CA) and confirms previous reports of the chromosomal location of *KLF15* genes in the human³⁵ and the mouse.³⁰

The genomic organization of *KLF15* was predicted by using in silico analysis of published cDNAs and spliced ESTs for human, mouse, and rat *KLF15* (see Supplemental Data at www.iovs.org/cgi/content/full/45/8/2522/DC1). All predicted transcripts encode identical proteins but differ within the 5' UTR (Fig. 2). Translation start sites are all located approximately 100 bp from the 5' end of exon 2, with the entire coding region contained in exons 2 and 3. The splice junction between exons 2 and 3 is located within the second zinc-

finger domain. For all species, the KLF15a transcript contains three exons and, for human and murine *KLF15*, represents the longest available cDNA. The human KLF15b transcript is supported by a single EST and uses an alternative splice site for exon 1. For rat, there are partial cDNAs that include either exons 1 and 2 or exons 2 and 3 (transcript r-KLF15a) but the published, full-length cDNAs all show alternative splicing and include an additional exon 1b (r-KLF15b). For mouse *KLF15*, all published cDNA and all the available EST data support the m-KLF15a transcript containing three exons. There is a 200-bp sequence within mouse intron 1 that shares 67% nucleotide identity with rat exon 1b; however, only the 5' splice junction is conserved, suggesting this is not an exon in the mouse.

KLF15 Expression in Retina and Visual System-Related Tissues

Because expression of *KLF15* in the retina or visual system-related tissues had not been reported, we tested it by using RT-PCR and detected *KLF15* expression in bovine (Fig. 3A), human, and mouse retina (Fig. 3C). *KLF15* expression in multiple nonretinal tissues has been demonstrated by Northern blot analysis in the human³⁵ and by RT-PCR in the mouse.³⁰ Consistent with these reports, by RT-PCR we detected *KLF15* transcripts in multiple bovine tissues, including the retinal pigmented epithelium (RPE)/choroid, iris/ciliary epithelium, cerebellum, lateral geniculate nucleus, visual cortex, kidney, and liver (Fig. 3A).

To determine the cellular pattern of KLF15 expression in the retina, we initially tried in situ hybridization. However, repeated efforts using multiple probes and hybridization conditions resulted in high background and inconsistent staining patterns that we attributed to the high GC content (overall 60% GC, with multiple regions >80% GC). Therefore, we examined retinas of heterozygous KLF15-LacZ mice in which the coding portion of exon 2 had been replaced by a nuclear LacZ gene, by using homologous recombination, and thus expressed LacZ as a reporter of the KLF15 expression patterns.³⁰ After LacZ histochemistry, most of the cells within the inner nuclear layer (INL) showed distinct nuclear staining and by their laminar position were most likely amacrine and bipolar cells, and possibly Müller glia (Fig. 4A). In the ganglion cell layer (GCL), there were scattered stained cells, presumably displaced amacrine cells or a subset of ganglion cells, as not all cells in the GCL were stained. LacZ-positive nuclei were also present in RPE cells and choroid (Fig. 4A), but no staining was observed in the retinal vasculature. Notably, there was no nuclear staining in the outer nuclear layer (ONL) that contained nuclei of rod and cone photoreceptors. In contrast, by in situ hybridization rhodopsin expression was detected specifically in the photoreceptor inner segments (IS) and the ONL (Fig. 4B), whereas *Crx* expression was detected in both the ONL and INL (Fig. 4C).

Effect of KLF15 on Transactivation of the Rhodopsin Promoter

To examine the effects of KLF15 on transcriptional activity of the bovine rhodopsin promoter, we used transient transfections of HEK293 cells with an expression vector containing the complete coding sequence of human KLF15 and the reporter construct, bRho225-luc, containing the bovine RPPR (-225 to +70 relative to the transcription start site) cloned upstream of the luciferase reporter gene.¹⁸ In this assay, KLF15 consistently reduced luciferase expression by 50% to 70% (Fig. 5A). Given the relatively low basal activity of this promoter in the 293 cells, these changes were numerically small; nevertheless, they were consistent and statistically significant ($P < 0.0001$) and suggested that KLF15 had repressor activity.

To investigate this further, we analyzed transcriptional activation of bRho225-luc in cotransfections of KLF15 with expression vectors containing *Crx* or *Nrl*, two transcription factors known to transactivate the rhodopsin promoter constructs in this assay.¹⁸ Cotransfection with KLF15 consistently repressed the positive transactivation of bRho225-luc by *Crx* (Fig. 5B) or *Nrl* (Fig. 5C), with statistically significant changes at all ratios of

KLF15:Crx ($P < 0.0001$ vs. Crx) and KLF15:Nrl ($P < 0.0001$ vs. Nrl; Fig. 5C). Previous studies have shown that cotransfection of Crx and Nrl results in a synergistic transactivation of this promoter.¹⁸ We found that in triple transfections, KLF15 reduced this synergy and decreased luciferase expression by more than 50% at all ratios tested ($P < 0.0001$ vs. Crx+Nrl; Fig. 5D).

Effect of KLF15 on Transactivation of the IRBP Promoter

Because photoreceptor-specific genes are known to share some regulatory elements, we also tested KLF15 with the luciferase-reporter construct (bIRBP300-luc) containing the proximal promoter region of bovine *IRBP* (-300 to +132).¹⁸ Again, KLF15 significantly reduced basal luciferase expression of bIRBP300-luc ($P < 0.0001$; Fig. 5E). With this promoter construct, transfection with Crx alone increased luciferase expression 4.6-fold as previously reported.¹⁸ Cotransfection with KLF15 resulted in a dose-dependent decrease in luciferase expression that was statistically significant ($P < 0.0001$ compared to Crx alone) at all ratios of KLF15:Crx tested (Fig. 5F). Luciferase expression was reduced to basal levels at 4:1 and 10:1 ratios of KLF15:Crx. In transfections with Nrl, there was extremely high variance in luciferase expression across all samples including controls containing Nrl alone, and no statistically significant differences were observed (Fig. 5G). This is consistent with the observation that the bovine *IRBP* promoter does not contain Nrl binding sites. In HEK293 cells cotransfected with Crx and Nrl (Fig. 5H), luciferase expression of bIRBP-luc was 12.8-fold greater than in the control, levels higher than observed after transfection with Crx or Nrl alone, but less than the synergistic 28.8-fold increase in luciferase expression observed using the bRho130-luc. Cotransfection with Crx and Nrl and increasing amounts of KLF15 (Fig. 5H) decreased luciferase expression of bIRBP300-luc at all concentrations tested ($P < 0.0001$).

Role of N-terminal and Zinc-Finger Domains in KLF15 Repressor Activity

To localize the repressor activity of KLF15, we analyzed a series of KLF15 deletion constructs using the same luciferase transactivation assay (Fig. 6). Deletion of the N-terminal 67 amino acids did not alter KLF15 repression of luciferase expression with the bRho225-luc reporter. Deletion of the N-terminal 265 amino acids abolished KLF15's repressor activity and converted KLF15 to a transcriptional activator. Transfection using either NΔ265 or NΔ291 resulted in a significant, dose-dependent increase in luciferase expression by bRho225 (Fig. 6B). In contrast, deletion of the three, C-terminal, zinc-finger domains (CΔ125) abolished KLF15 activity in this assay, and resulted in no changes in luciferase expression compared with the empty vector controls ($P = 0.6242$).

Discussion

The Sp/KLF family of zinc-finger transcription factors, of which KLF15 is a member, has been implicated in the regulation of multiple cellular processes including cellular proliferation, differentiation, normal development, and cancer. Sp/KLF proteins are known to function as transcriptional regulators of both tissue-specific and ubiquitously expressed genes,^{36,37} although there is functional heterogeneity, with some (e.g., KLF5/IKLF/BTEB2) functioning as transcriptional activators^{38,39} and others (e.g., KLF8/BKLF) as repressors.⁴⁰ We identified KLF15 as a potential transcriptional regulator of the bovine rhodopsin promoter in a yeast one-hybrid assay and have shown that KLF15 can repress transactivation of the photoreceptor-specific rhodopsin and *IRBP* promoters in vitro. Our findings are consistent with the previous report by Uchida et al.³⁵ who found that KLF15 (initially called KKLF/kidney-enriched Krüppel-like factor) was a repressor of the kidney-specific chloride channel-1 and -2 (*CLC-K1* and *CLC-K2*) promoters. In contrast, in a separate study, Gray et al.,³⁰ reported that overexpression of KLF15 using retroviral vectors increased glucose-4 transporter (*GLUT4*) expression in differentiated 3T3-L1 cells. Although differences in methodologies preclude precise comparisons, these studies suggest that KLF15 is a bifunctional transcriptional

regulator, perhaps dependent on which other factors are available. It is interesting to note that KLF15 and the transcriptional activator MAZ (myc-associated zinc finger gene) compete for the same consensus binding site in the *CLC-K1* promoter and that promoter activity in transiently transfected cells is dependent on the relative expression levels of these two factors.³⁵ MAZ is expressed in the retina with 5% (30/602) of human MAZ ESTs in the UniGene (NCBI) database isolated from eye libraries, with the majority from either fetal or adult retinas. However, there are currently no other published reports regarding MAZ in the retina.

We have mapped the KLF15 repressor domain to a 198 amino acid fragment (amino acids 68-265) within the N-terminal domain, a region that is rich in proline residues and contains an highly conserved acidic element. The *Drosophila* Krüppel gene contains a proline-rich repressor element that requires an adjacent acidic domain for transcriptional repression.⁴¹ However, acidic and proline rich domains have also been implicated in transcriptional activation,⁴² and the KLF15 repressor domain overlaps the portion of the protein required for transactivation of the Glut4 promoter (amino acids 58-200).³⁰ Various mechanisms can regulate the switch between transcriptional repression and activation by other Sp/KLF family members. The coactivator E2F and the corepressor histone-deacetylase competitively bind to a single C-terminal domain of Sp1 and regulate transcriptional activation or repression by Sp1.^{43,44} Sp3 has distinct activation and repression domains that confer opposing functions, depending on the number and location of Sp3-binding sites within a promoter.⁴⁵ KLF1/EKLF typically acts as a transcriptional activator of the erythrocyte-specific β -globin promoter⁴⁶ but can function as a repressor when recruited to the promoter by interaction of its zinc finger domains with other proteins.⁴⁷ Future studies to characterize the influence of promoter structure and to identify KLF15 interacting proteins will be designed to determine the mechanisms that modulate transcriptional regulation by KLF15.

Deletion of the N-terminal domain converted KLF15 from a transcriptional repressor to an activator in our transactivation assay, whereas a similar deletion converted KLF15 from an activator to a repressor of the Glut4 promoter in 3T3-L1 cells.³⁰ This reversal of KLF15 activity may reflect a dominant negative effect of the truncated proteins. By RT-PCR, we found that KLF15 is expressed in 293 cells (data not shown), suggesting that it may contribute to the low levels of basal expression of rhodopsin promoter constructs in this cell line. Truncated KLF15 could compete with the endogenous full-length protein for promoter-binding sites and de-repress the rhodopsin promoter. Consistent with this hypothesis, we found that in co-transfections we could titrate repression or activation of the rhodopsin promoter construct by varying the ratios of full length and Δ 291 KLF15 (Otteson DC, Zack DJ, unpublished results, 2001). Although we favor a DNA-dependent model of KLF15 transcriptional regulation, zinc-finger domains can also mediate protein—protein interactions; therefore, it is also possible that the truncated KLF15 could sequester corepressors or coactivators necessary for promoter repression/transactivation by the full-length protein.

Previous analysis of DNA binding by KLF15 using electrophoretic mobility shift assays identified the CACCC³⁰ and GGGGnGGnG (reverse complement: CnCCnCCCC)³⁵ as minimal and extended KLF15-binding sites, respectively. The rhodopsin promoter element used as bait in the initial yeast one-hybrid assay contains a CGCCCC sequence that differs from the minimal KLF15 binding site at a single base and shows considerable overlap with a previously proposed extended site. This element is highly conserved between bovine and human rhodopsin promoters, but surprisingly, it is not present at the corresponding location in the mouse and rat rhodopsin promoters (Fig. 7). An examination of the rhodopsin proximal promoters of mouse and rat identified several minimal CnCCC (or the reverse complement GGGnG) binding sites and an additional extended G/C rich domain that could serve as alternative KLF15 binding sites. Differences in the position and extent of other promoter elements (e.g., Eopsin-1 and Ret-1)^{15–17,48} within the bovine and rat rhodopsin promoters

have been identified, although the biological significance of these variations on rhodopsin expression remains to be determined. Changes in transcription factor binding sites could reflect differences in the binding specificity of orthologous transcription factors in different species. For KLF15, this seems somewhat unlikely, given the high sequence identity within the zinc-finger domains. However, there are sequence differences outside of KLF15's zinc-finger domains, and a recent study showed that that sequence specificity of identical zinc-finger domains could be influenced by differences in the flanking, nonfinger residues.⁴⁹ Thus, subtle differences in binding specificity of KLF15 could be reflected in species-specific changes in the DNA binding sites. Changes in transcription factor binding sites may also indicate differences in the regulation of photoreceptor-specific gene expression in different species, although thus far this does not appear to be the case, at least among mammals. Perhaps a more attractive hypothesis is that the relative locations of some transcription factor binding sites can change as a result of mutation or chromosomal rearrangement during evolution. In a recent study comparing well-characterized promoters of 51 genes, the investigators found that transcription factor bindings sites evolved over time and estimated that 32% to 40% of human functional transcription factor binding sites were not functional in rodents.⁵⁰ It seems logical that new sites would evolve at a similar rate; therefore, positive selection for compensatory mutations could serve to maintain binding sites for critical transcriptional regulators, albeit with possible alterations in location or orientation, whereas nonessential binding sites could be lost.

It is well accepted that both transcriptional activation and repression are critical to the establishment and maintenance of cell-type—specific patterns of gene expression.^{51–54} To date, much of the research on regulation of gene expression in the retina has focused on the transcriptional activators, although some repressors of photoreceptor-specific gene expression have been identified. The majority of the repressors appear to repress by protein—protein rather than protein—DNA interactions. Polyglutamine-expanded Ataxin-7,^{55,56} barrier to auto-integration factor (Baf),⁵⁷ and phosducin⁵⁸ all physically interact with Crx and can repress its transcriptional activity in vitro. The zinc-finger—containing protein Flt3-interacting zinc finger protein (Fiz1) can interact with Nrl and reduce Nrl-mediated transactivation of a bovine rhodopsin promoter construct.⁵⁹ However, the N-terminal fragment of Fiz1 containing the Nrl-interaction domain lacks independent repressor activity, and the ability of Fiz1 to bind DNA has not been determined. MOK2, an unrelated zinc-finger transcriptional repressor, binds to specific elements in the IRBP promoter, but thus far appears to repress only the *IRBP* promoter.⁶⁰

Crx, one of the known transcriptional activators of rhodopsin and other photoreceptor-specific genes, is expressed in differentiated photoreceptor cells. Paradoxically, Crx is also expressed in nonphotoreceptor cells in the INL⁶¹ and yet these cells do not express rhodopsin (see Figs. 4B, 4C). This raises questions regarding the mechanisms that prevent Crx from activating expression of rhodopsin or other photoreceptor-specific genes in the INL. Our finding that KLF15 can repress promoter transactivation by Crx and is coexpressed with Crx in a subset of INL cells makes it an attractive candidate to participate in repression of photoreceptor-specific gene expression in the INL. If this hypothesis is correct, mutation of KLF15 binding site(s) in the rhodopsin promoter or the loss of KLF15 expression could result in misexpression of rhodopsin and other photoreceptor-specific genes in the inner retina or the RPE. Experiments are currently under way to test this possibility.

Supplementary Material

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Figure 1.

Alignment of KLF15 deduced amino acid sequences. *Shaded areas*: identical amino acids; *bold and underscored sequence*: zinc-finger binding domains (ZF1, ZF2 and ZF3); *filled bar*: conserved acidic domain; *dotted line*: portion of KLF15 not included in bovine cDNA clone; *dashes*: spaces introduced for alignment. Numbering of amino acids is based on human KLF15. B, *Bos taurus* (AY366084); H, *Homo sapiens* (NM_014079); M, *Mus musculus* (NM_023184); R, *Rattus norvegicus* (NM_053536); X, *Xenopus laevis* (AY116304); Z, *Danio rerio* (AY116305).

Figure 2.

Genomic organization of KLF15. Schematic diagram of predicted KLF15 genomic organization based on comparison of cDNA and EST clones to genomic database sequences. (□) Untranslated regions; (■) coding regions; length of introns (in kilobases) are indicated below each schematic; length of exons and supporting EST clones are provided in online Supplemental Data. These analyses used Human Freeze July 2003; Mouse Freeze October 2003, Rat Freeze June 2003.

Figure 3.

KLF15 is expressed in the visual system. (A, C) RT-PCR analysis of KLF15 in bovine tissues. *Left to right*: each group of three lanes contain PCR amplification for 30, 35, or 40 cycles, using oligo-dT primed, reverse transcribed RNA from (lane 1) retina; (lane 2) RPE/choroid; (lane 3) iris/ciliary epithelium; (lane 4) cerebellum; (lane 5) visual cortex; (lane 6) lateral geniculate nucleus; (lane 7) pineal; (lane 8) kidney; (lane 9) liver; (lane G) genomic DNA (negative control); (lane H) water (negative control). (B, D) Amplification of equivalent aliquots of the same RT reactions as (A) and (C) using primers for the ubiquitous housekeeping gene ARP²⁹ as a control for loading and quality of RT reactions. (E) Amplification of KLF15 from oligo-dT—primed reverse transcribed mRNA: (lane 10) human retina; (lane 11) mouse retina; (lane 12) mouse brain; (lane 13) rat liver; (lane H) water negative control. (F) Amplification of equivalent aliquots of RT reactions from (E) using primers for ARP. The discontinuities resulted from removal of irrelevant lanes.

Figure 4.

KLF15 is expressed in nonphotoreceptor cells in the retina. Photomicrographs of 10- μ m cryosections through the retina of (A) adult, albino KLF15-LacZ transgenic³⁰ and (B–D) wild-type (C57/B16) mice. (A) Blue-stained nuclei are positive for LacZ histochemistry and identify KLF15 expressing cells in the INL, GCL (*filled arrow*), RPE (*filled arrowheads*), and choroid (*open arrowheads*); LacZ-negative nucleus in GCL (*open arrow*). (B) In situ hybridization for rhodopsin antisense-probe showing rhodopsin-positive cells in the ONL and strong staining of photoreceptor inner segments (IS). (C) In situ hybridization of *crx* antisense probe, showing *crx*-positive cells in the ONL and INL. (D) In situ hybridization of the *crx* sense probe showing no staining. Scale bar, 100 μ m.

Figure 5.

KLF15 represses transactivation of rhodopsin and *IRBP* promoters. (A) HEK293 cells were cotransfected with 2.5 μ g of bovine rhodopsin-luciferase reporter construct (bRho225-luc) together with the indicated amount (in micrograms) of KLF15 expression vector. Luciferase activity (in relative light units) was corrected for transfection efficiency using β -Gal as the internal control. Histograms show a mean increase (n -fold) in relative luciferase expression compared with control transfections, using an empty expression vector. (B) Same as (A), except 0.25 μ g Crx expression vector was added to all samples. (C) Same as (A) except 0.25 μ g Nrl expression vector was added to all samples. (D) Same as (A) except 0.25 μ g Crx and 0.25 μ g Nrl were added to all samples. (E–H) Same as (A–D) except that the reporter construct was bovine IRBP (-300 to +132)-luciferase (bIRBP-luc). Error bars, 95% confidence interval of the mean; * $P < 0.004$, ** $P \leq 0.008$, *** $P < 0.0001$ versus control transfections (KLF15 0 μ g, first histogram in each graph).

Figure 6.

Deletion of N-terminal repressor domain converts KLF15 to a transcriptional activator. **(A)** Schematic of KLF15 showing location of acidic domain (*stippled area*) and zinc-finger domains (*arrowheads*); *filled bars*: portions of KLF15 included in deletion constructs. **(B)** Results of luciferase reporter assays using KLF15 deletion constructs and bovine rhodopsin-luciferase reporter (bRho225-luc). Histograms show mean change (*n*-fold) in relative luciferase expression compared with control transfections using an empty expression vector. Error bars, 95% confidence interval of the mean; **P* < 0.05; ***P* = 0.001; ****P* < 0.0001 versus control.

KLF15 minimal		CACCC	
bovine	-94	ATTAATAAC <u>CGCCCC</u> CAATCTCCGAGGTGC	-66
human	-92	ATTATGAAC <u>CACCCC</u> CAATCTCCCAGATGC	-64
mouse	-82	AGTATGAT-----ATCTCGCGGATGC	-62
rat	-82	AGTGTGAT-----ATCT <u>CCC</u> GGATGC	-62

Figure 7.

Variation of predicted KLF15 binding site in bovine, human, mouse, and rat rhodopsin promoters. Alignment of bovine rhodopsin promoter element (used as bait in yeast one-hybrid assay) with equivalent regions of rhodopsin proximal promoter region from human, mouse, and rat KLF15. C-rich sequences with similarity to KLF15 minimal binding site (CACCC)³⁰ are in *bold* and *underscored*. Positions are relative to transcription start site for each species. *Shaded area*: conserved bases; *dashes*: spaces added for alignment.

Table 1

PCR Primers

Method	Gene (species)	5' Primer (Forward)	3' Primer (Reverse)	Annealing Temp (no. cycles)	Size (bp)
PCR for NΔ291	<i>KLF15</i> (human)	5'-CAAGCCTGTTGGATCCGGACC-3'	5'-AGCTCACTCATTAGGCACCCCAAGGC-3'	60°C (25)	605
PCR for CΔ126	<i>KLF15</i> (human)	5'-TGCAGGATCCGCCCTTGCCAG-3'	5'-TGGGTTCTAGAGGAACCTTCTGGC-3'	60°C (25)	961
RT-PCR	<i>KLF15</i> (bovine)	5'-CAAGAGCAGCACCTGAAG-3'	5'-AAACCTGCCGTCTGTCGGTCTCTCC-3'	60°C (30–40)	989
RT-PCR	<i>KLF15</i> (human, murine, rat)	5'-ATGCACAAATGTACTTTCCCTGG-3'	5'-AACTTCTTCTCGCACACAGGACAC-3'	60°C (30–40)	212
G3-genomic mapping	<i>KLF15</i> (human)	5'-CTCCTGTGTCTCTTTCCTGCAG-3'	5'-TCAGGATCACCCAAAGGAACCTC-3'	55°C (35)	438
In situ (RT-PCR)	Rhodopsin (murine)	5'-GAAGGGATTAAATGGAAGGGAAC-3'	5'-GTCTCGATGCTTTTCTCAGTCCT-3'	55°C (40)	558
In situ (nested)	Rhodopsin (murine)	5'-AATTAAACCCTCACATAAGGGAGATATG-GCTGCTTCAAAGACAGAG-3'	5'-TAATACGACTCACTATAGGGAGACCAAGCA-GTCACATAACAAGCAG-3'	50°C (40)	267
In situ (RT-PCR)	<i>crx</i> (murine)	5'-GATTCTTACAGCCCCATCTCTGC-3'	5'-CTATAGCTCTGGCCTGACAAGG-3'	56°C (40)	359
In situ (nested)	<i>crx</i> (murine)	5'-AATTAAACCCTCACATAAGGGAGAGGTT-CACCTACCACAGC-3'	5'-TAATACGACTCACTATAGGGAGACTGAAATA-GGAACTTGG-3'	42°C (10) + 62°C (30)	205