Rbpj direct regulation of Atoh7 transcription in the embryonic mouse retina

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In vertebrate retinal progenitor cells, the proneural factor Atoh7 exhibits a dynamic tissue and cellular expression pattern. Although the resulting Atoh7 retinal lineage contains all seven major cell types, only retinal ganglion cells require Atoh7 for proper differentiation. Such specificity necessitates complex regulation of Atoh7 transcription during retina development. The Notch signaling pathway is an evolutionarily conserved suppressor of proneural bHLH factor expression. Previous in vivo mouse genetic studies established the cell autonomous suppression of Atoh7 transcription by Notch1, Rbpj and Hes1. Here we identify four CSL binding sites within the Atoh7 proximal regulatory region and demonstrate Rbpj protein interaction at these sequences by in vitro electromobility shift, calorimetry and luciferase assays and, in vivo via colocalization and chromatin immunoprecipitation. We found that Rbpj simultaneously represses Atoh7 transcription using both Notch-dependent and –independent pathways.

During vertebrate embryonic development, multipotent retinal progenitor cells (RPCs) undergo a prolonged period of differentiation during which six neuronal (retinal ganglion, cone and rod photoreceptor, amacrine, horizontal and bipolar) and one glial (Müller) cell type are produced and assembled into a highly laminated tissue. Multiple signaling pathways and transcription factors, including proneural basic helix-loop-helix (bHLH) transcription factors, regulate retinal cell fate, as reviewed in1. Among these factors, Atoh7 (Atonal homolog 7, Math5, Ath5) is dynamically expressed by subsets of embryonic RPCs and required for retinal ganglion cell (RGC) formation 2–6. Without Atoh7 function, essentially all RGCs fail to differentiate and adult animals lack optic nerves3,5,6. Although the Atoh7 retinal lineage includes all seven major cell types, gene activity is only required for RGC genesis. To understand how Atoh7 acts as an RGC competence factor requires deeper understanding of its mode of action as a DNA-binding protein, and the mechanisms tightly regulating its mRNA and protein. Here we focus on particular aspects of Atoh7 transcriptional regulation.

Previous studies of vertebrate Atoh7 genomic architecture defined two enhancer regions on the 5′ side of the lone Atoh7 coding exon, each containing multiple conserved noncoding elements (CNEs)7–10. In mice, the distant, shadow enhancer is 9.5 kb and the proximal, primary enhancer is 1.5Kb upstream of the Atoh7 ATG start codon. The primary enhancer is further subdivided into two CNEs, termed distal and proximal. The distal CNE contains validated Pax6 (paired domain) and Neurog2 (E box) binding sites, through which Pax6 activates transcription, and Neurog2 drives the initial wave of retinal neurogenesis2,10–12. However, the activities of these two factors cannot account for the rapid downregulation of Atoh7 in subsets of RPCs at differentiation.

Given that Notch signaling regulation of bHLH factors occurs widely, including throughout the vertebrate nervous system, we wish to understand how this pathway controls Atoh7 expression at the molecular level, in the context of retinogenesis. Canonical Notch signaling initiates with a Delta-like or Jagged/Serrate ligand on one cell binding to a Notch receptor on an adjacent cell. This triggers proteolytic cleavage of the receptor to ultimately release its intracellular domain (termed NICD). The NICD complexes with a CSL protein (CBF-1, RBPJ-κ, 1Department of Cell Biology & Human Anatomy, University of California Davis School of Medicine, One Shields Avenue, Davis, CA, 95616, USA. 2Division of Developmental Biology, Cincinnati Children’s Hospital Research Foundation, 3333 Burnet Avenue, Cincinnati, OH, 45229, USA. 3Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati School of Medicine, Cincinnati, OH, 45267, USA. 4Present address: Department of Biology, University of Cincinnati Blue Ash College, Cincinnati, OH, 45236, USA. Joel B. Miesfeld and Myung-soon Moon contributed equally to this work. Correspondence and requests for materials should be addressed to N.L.B. (email: nlbrown@ucdavis.edu)
Recombination Signal Binding Protein for immunoglobulin kappa J region) in human/mouse, Su(H) (Suppressor of Hairless) in *Drosophila*, Lag-1 (lin12 and gfp-1 phenotype) in *C. elegans* and the Mami (Mastermind-like) co-activating factor. This protein complex activates downstream target gene transcription by binding to a variety of CSL sites within noncoding DNA. Well-characterized effector genes of the Notch pathway include the Hes (Hairy/Enhancer of Split (E(Spl)) and Hey gene families, which encode transcriptional repressor proteins. Intriguingly, during *Drosophila* retinal development, Notch signaling reiteratively regulates *Atonal* transcription. In undifferentiated cells anterior to the eye disc morphogenetic furrow (a moving differentiation boundary), Notch signaling activates *Atonal* in a continuous stripe of cells. But more posteriorly, the Notch complex, via E(Spl) activity, suppresses *Atonal* expression in cells adopting non-R8 photoreceptor fates. This latter activity embodies the classic neurogenic role of Notch signaling. Importantly, these phases of *Atonal* expression in cells adopting non-R8 photoreceptor fates. This latter activity embodies the classic neurogenic role of Notch signaling. Importantly, these phases of *Atonal* expression in cells adopting non-R8 photoreceptor fates. This latter activity embodies the classic neurogenic role of Notch signaling.
that were incubated with only one primary antibody (rabbit anti-Atoh7 or rat anti-Rbpj), prior to simultaneous application of both secondary antibodies (Fig. S1).

Rbpj binding to Atoh7 5′ regulatory DNA in vitro and in vivo. For initial assessment of Rbpj binding to the four putative CSL sites, we conducted in vitro electromobility shift assays (EMSA), using bacterially expressed and purified mouse Rbpj protein (residues 53–474), previously shown to bind DNA.42 Rbpj protein was incubated with biotin-labeled double stranded oligonucleotides, in which the putative binding site is centrally located (Fig. 4A, Supplemental Table S4). We noted that all four sites shifted upon incubation with Rbpj protein (Fig. 4A). When key nucleotides within each CSL site were mutated, based on previous CSL-DNA structural studies, binding was abolished. We conclude that Rbpj protein specifically binds to each CSL binding site in vitro.

We also performed isothermal titration calorimetry (ITC) to quantitate binding, using purified Rbpj with the oligomeric DNA duplexes that correspond to the four putative CSL binding sites (Fig. 4B–F and Supplemental Table S5). As positive and negative controls, we tested Rbpj with the CSL binding site from the Hes1 proximal promoter element (GTTAGTGGGGAAAGAAAG) and the non-specific sequence (GCTACTCATACCTAGAACG), respectively, and detected binding from the Hes1 site (~1 

**μM K d), but did not detect binding from the non-specific site (data not shown). The results showed Rbpj bound to three of the four putative CSL sites with comparable affinity to the well-characterized Hes1 site (Fig. 4B).43 However, we found...
that one consensus site, R3, displayed a lower binding affinity (Fig. 4E). While we were unable to separate any potential effects of nucleotide variation and flanking sequence, interestingly, R3 lies in between validated Pax6 and Neurog2 binding sites.

Next, we wished to determine which CSL consensus sites are occupied by Rbpj during in vivo retinal development. Previously we demonstrated that Pax6 occupies consensus site J in the human ATOH7 gene, using chromatin from Ad12Her10 retinal cell line. As a positive control, we performed Pax6 ChIP in parallel here, using mouse E14.5 retinal chromatin. This age was selected because it is the peak of Atoh7 expression. Dissected retinal cell line in which the human ATOH7 shadow enhancer drives BG-mCherry expression. For this we created a transgenic construct with 2.4Kb of mouse Atoh7 5’ DNA joined to a minimal human β-globin promoter (BG) and red fluorescent monomer Cherry reporter (mCherry). We then used antibody co-labeling to evaluate mCherry expression in retinal cryosections from E13.5 transient transgenic embryos. We found that endogenous Atoh7 significantly overlaps with Cherry+ cells (Fig. 6A) in the proliferative neuroblast layer, but not in the differentiated ganglion cell layer (GCL). This difference can be attributed to the greater stability of the fluorescent protein compared to Atoh7. Coexpression here was roughly equivalent to what was previously reported for another mouse transgenic line in which the human ATOH7 shadow enhancer drives BG-mCherry expression.

Next, we performed luciferase assays in both HEK293T kidney-derived and AD12Her10 retinal-derived human cell lines, under identical conditions. This strategy was chosen because our previous study of Pax6 regulation of Atoh7 transcription suggested that retinal-specific context influences assay output. Yet we found no differences between these cell lines, although HEK293T cells endogenously express RBPJ, but not ATOH7, whereas AD12Her10 cells express both genes (Supplemental Fig. S2). Individual CSL site mutations (ΔR1, ΔR2, or ΔR4) did not affect transcriptional output, relative to wild type (Fig. 6A). By contrast, our mutation of CSL

Rbpj differentially regulates Atoh7 transcription. Although Rbpj binds to all four CSL sites in vitro and at least site R3 in vivo, it is unclear if it does so via the Notch complex (activation), or a corepressor complex (repression). To address these possibilities formally we used luciferase reporter assays to measure the activity and requirement for each site individually, versus simultaneous mutation of all 4 sites, within a previously identified primary gene Atoh7 enhancer. However, first we compared Atoh7 primary gene enhancer expression to that of the endogenous protein, which had not been previously reported. For this we created a transgenic construct with 2.4Kb of mouse Atoh7 5’ DNA joined to a minimal human β-globin promoter (BG) and red fluorescent monomer Cherry reporter (mCherry). We then used antibody co-labeling to evaluate mCherry expression in retinal cryosections from E13.5 transient transgenic embryos. We found that endogenous Atoh7 significantly overlaps with Cherry+ cells (Fig. 6A) in the proliferative neuroblast layer, but not in the differentiated ganglion cell layer (GCL). This difference can be attributed to the greater stability of the fluorescent protein compared to Atoh7. Coexpression here was roughly equivalent to what was previously reported for another mouse transgenic line in which the human ATOH7 shadow enhancer drives BG-mCherry expression.

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site R3 (ΔR3) derepressed luciferase activity over wild type levels, in both HEK293T and AD12Her10 cells, in the absence of exogenous Notch intracellular domains (Fig. 6A, Supplemental Fig. S2). We concluded that this particular binding site normally represses Atoh7 transcription. By contrast, when all four sites (ΔR1–4) were simultaneously mutated, there was a significant decrease in Atoh7 transcriptional levels (Fig. 6B, Supplemental Fig. 2). This is suggestive of coordinated, Rbpj-mediated transcriptional activation, among multiple (possibly all four) binding sites.

Rbpj occupancy of site R3 represses Atoh7, presumably reflecting corepressor complex activity. However, the coordinated enhancement via multiple sites might be attributable to direct regulation of Atoh7 transcription, via a Notch complex. Therefore, we tested for Notch-dependence by coexpressing the intracellular domains of Notch1 (NICD1) or Notch3 (NICD3) in our luciferase assays. Each receptor alone, as well as in combination, was previously shown to be required in vivo for particular aspects of Atoh7 expression. Plasmids containing the NICD1, NICD3, or a mixture of the two, were cotransfected with either the Atoh7 wild type or ΔR1–R4 mutant luciferase constructs. Increasing NICD1 levels stimulated Atoh7 transcription but had no impact on the ΔR1–R4 mutant (Fig. 6B). NICD3 or NICD1 + NICD3 coexpression did not affect wild type Atoh7 activity, but further suppressed transcription in the ΔR1–R4 mutant (Fig. 6B). We interpret these outcomes to mean that multiple CSL binding sites help maintain Atoh7 basal level transcription. The elevated luciferase activity seen after NICD1 overexpression might represent Notch-dependent regulation of sites R1, R2, and R4, or an ectopic effect of NICD1 acting as a Rbpj sink, de-repressing R3 similar to R3 mutagenesis. In addition, Notch-mediated regulation of Atoh7 must utilize other noncoding sequences based on the decrease of transcriptional activity with NICD3 overexpression, potentially through Hes consensus N-boxes.

Multiple mammalian “co-repressor” genes encode proteins that interact with Rbpj via CSL binding sites. These genes are unrelated at the primary sequence level, but predicted to link Rbpj to HDAC machinery, and

Figure 3. Colocalization of Rbpj and Atoh7 proteins during retinal neurogenesis. (A–C) Double antibody labeling of retinal sections highlights essentially complete nuclear co-localization. The boxed area in each panel is shown at higher magnification to the right. (A–A”) At E11.5 Atoh7 protein is restricted to a subset of central RPCs (A’). (B–B”) By E13.5 the initial wave of neurogenesis has reached the periphery and Atoh7 is expressed more broadly throughout the apical neuroblast layer. (C–C”) Consistent with mRNA expression studies, Atoh7+ cells are localized to the peripheral retina (C’). Panel C is a composite stitched together from 4 overlapping 10× image fields. Colocalization of Rbpj (green) and Atoh7 (purple) is shown as white. Rostral is up in all panels. L = lens; Bar in A,C,E = 100 μm; in B,D,F = 50 μm. In (A’–C”) white arrows point to Atoh7+ cells that also contain high levels of Rbpj.
include Smrt/Ncor2, Spen/Mint/Sharp, Fhl1b/KyoT2, Rita, Skip, L3mbtl3 and Kdm1a/Lsd117–19. Interestingly, Kdm1a/Lsd1 is expressed in the retina from E17–P15, and its pharmacologic inhibition in retinal explants induced an upregulation of bHLH factor expression60. Because nothing further is known about the retinal expression of other Rbpj co-repressor genes, we used RT-PCR to test for transcription of four genes (Spen; Fhl1b; L3mbtl3; Kdm1a) in the E13.5 mouse retina (Fig. 6C). We found that all of these co-repressor mRNAs are expressed during embryonic retinal neurogenesis (Fig. 6C). Given that at least four of seven putative co-repressors are present in the mammalian retina, elucidation of their specific mechanisms of action will require in-depth, future studies.

Discussion

Previous studies of Notch signaling in the vertebrate retina genetically linked Rbpj activity to Atoh7 expression and retinal ganglion cell differentiation24–29. Notch activity normally suppresses both Atoh7 and RGC neurogenesis, but the molecular mechanisms for this remain unresolved. Here, we explored the possibility that Rbpj can directly regulate Atoh7 transcription.

Bioinformatic analysis of noncoding sequences surrounding the mouse Atoh7 gene identified four putative Rbpj-CSL consensus binding sites. While a CSL consensus sequence is useful for predicting Rbpj target genes13–15,
it is not the only nucleotide motif this protein can bind. One particular binding site arrangement, called SPS (Su(H)-paired site), consists of two CSL binding sites in a head to head configuration separated by ~16 nucleotides. These SPS elements are occupied by dimeric NICD/Rbpj complexes, to regulate transcription.

Although the Atoh7 upstream CSL binding sites lack a canonical SPS arrangement, there remains some possibility for such a mechanism, since a cryptic CSL element in the Hes5 promoter acts in a dimeric SPS complex.

Among the CSL sites analyzed here for the embryonic retina, R3 stands out as unique. Counterintuitively, CSL has the weakest affinity for R3 in vitro, but was the only site that ChIPped CSL in vivo with statistical significance.

Similar phenomenon has been observed for Su(H), the fly CSL ortholog, binding of the sparkle (spa) enhancer in Drosophila, whereby the low affinity Su(H) sites are critical for proper gene expression and patterning driven by spa. The location of R3 within the distal CNE is flanked by a Neurog2-dependent Ebox (30 bp upstream) and a Pax6 binding site (20 bp downstream). Such spacing allows one or two helical turns to separate each of these sites. In the developing pancreas, this same distance is permissive for Rbpj physical interaction with the bHLH transcription factor Ptf1a. Interestingly, Ptf1a is also expressed in the developing retina and influences neurogenesis of several retinal cell types. In Drosophila and Xenopus there are other examples of CSL/Rbpj protein interactions with bHLH factors that activate transcription. Hence, we cannot discount the possibility that Neurog2 (or another bHLH factor) may physically interact with Rbpj. Alternatively, Rbpj occupancy of site R3 might affect local chromatin architecture, thereby displacing activating factors. Because we detected both Rbpj and Pax6 occupancy in E14.5 retinal chromatin (within the same preparation), a mutually exclusive binding mechanism would seem implausible. One caveat was the use of whole retina chromatin, which could obscure distinct configurations of transcription factor binding at the Atoh7 primary enhancer, among a heterogenic population of retinal cells. For example, in mitotically-active RPCs that do not express Atoh7, Rbpj could act as a repressor at site R3. When these cells enter their terminal mitosis, they may activate Atoh7 expression, via Pax6 or Neurog2 binding, which would also displace nearby Rbpj-corepressor complexes. Conversely, during terminal differentiation, this relationship could be reversed, with Rbpj-corepressor binding at site R3 dislodging either Pax6 or Neurog2. Only the generation of single-cell genomic datasets can map the occupancy of particular enhancer binding site to the developmental status of cells, at distinct stages of retinogenesis.

Here we also provided some insight into in vivo context for our biochemical data, via direct comparison of Rbpj and Atoh7 protein expression patterns. Rbpj is well established as ubiquitously expressed, so co-localization
with Atoh7 protein was already predicted. But, transcription factors that are coexpressed with their target genes typically activate transcription, not repress it. Indeed, Hes1, a known repressor of neurogenesis, displays mutually exclusive expression with βgal in Atoh7+/- eyes. Yet, our transcriptional activity data clearly indicate that Rbpj repression of Atoh7 is the major mode of regulation, which correlates with all previous genetic findings. However, we also note that Rbpj does not appear uniformly expressed, with brighter anti-Rbpj labeling coinciding with Atoh7 expression. The significance of this observation remains unclear. Better understanding will

Figure 6. Differential Notch-mediated regulation of Atoh7 transcription. (A) Colocalization of transient mCherry transgenic expression, driven by a 2.4 kb mouse Atoh7 enhancer, to endogenous Atoh7 protein in the E13.5 retina (White arrows point to colabeled cells). Both cytoplasmic and nuclear Cherry expression are seen, presumably due to the inefficiency of a synthetic nuclear localization sequence and reporter antibody sensitivity, which detected both nascent Cherry protein in the cytoplasm and its accumulation in the nucleus. Scale bar = 100 μm (B) Comparison of mouse Atoh7 transcriptional activity in HEK293T cells following single versus quadruple CSL site mutation. Transcriptional activity of individual CSL site mutants, and quadruple mutant within Atoh7–2.6Kb luciferase/pGL2 construct. Only site R3 in the distal CNE is required to suppress Atoh7 transcription. However, loss of sites R1–4 caused significant downregulation of Atoh7 transcription. n = 9 biological replicates (each performed in technical triplicate). (C) Cotransfection of activated NICD1, NICD3, or both constructs with Atoh7 wild type or RΔ1–R4 mutant luciferase constructs. n ≥ 3 biological replicates (each in technical triplicate). All luciferase experiments were normalized to a co-transfected Renilla control. A two-tailed, unpaired t-test with equal standard deviation and Gaussian distribution was used to determine p-value. (D) RT-PCR analysis of E13.5 retinal cDNA showing expression of all four co-repressor gene mRNAs. Distinct PCR primers for the Fhl1 (Kyo1) gene were used that amplified an exon common to all splice products (pan Fhl1), and the specific splice variants Fhl1b (KyoT3) and Fhl1c (KyoT2) that uniquely contain the Rbpj-interaction domain. All PCR reactions were run on a single gel, uncropped image provided in Supplemental Fig. S3. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p < 0.0001 and Figure S2 *p ≤ 0.05; **p ≤ 0.01; ***p < 0.001; ****p < 0.0001.
require the determination of which Rbpj regulation activities at work during distinct stages of retinal cell development. Moreover, we must clarify whether Notch1/3 signaling invokes canonical pathway regulation, namely Notch-Rbpj-Maml binding to Hes gene promoters, which may in turn directly repress Atoh7 transcription.

While highly speculative, we propose that lower levels of Rbpj protein expression are sufficient for binding to multiple CSL sites, which contributes to keeping the Atoh7 locus open and primed (yet transcriptionally silent) \(^{39}\), until its rapid, pulsatile expression is needed. Higher levels of Rbpj protein may subsequently be required to also engage in the activities of Notch and co-repressor complexes, which act at different regulatory sequences, and possibly at different rates to shut down Atoh7 transcription. Clearly additional transcription factors must simultaneously regulate Atoh7 (positively or negatively) since brighter-labeled Rbpj cells are capable of Atoh7 co-expression. The integration of these multiple modes of regulation allows for more precise modulation of target gene mRNA levels, particularly during highly dynamic developmental processes.

**Experimental Methods**

**Ethics Statement.** All mice were housed and cared for in accordance with the guidelines provided by the National Institutes of Health, Bethesda, Maryland, and the Association for Research in Vision and Ophthalmology, and conducted with approval and oversight from the Cincinnati Children’s Hospital Research Foundation and UC Davis Institutional Animal Care and Use Committees.

**Animals.** A Rbpj\(^{+/-}\) conditional allele (termed Rbpj\(^{CKO}\)) were maintained on a 129/SvJ background and genotyped as described\(^{41}\). α-Cre transgenic mice were maintained on a CD-1 background and genotyped as described\(^{42}\). Le-Cre mice were maintained on an FVB/N background and genotyped as described\(^{42}\). Z/EG lineage tracing mice (Tg(CAG-Bgeo/GFP)21Lb3/J) use the CMV enhancer/chicken actin promoter to constituatively express lacZ, which is replaced with eGFP expression upon Cre activation\(^{39}\). These mice were acquired from Jackson Labs (Stock Number 003920), maintained on a CD-1 background and genotyped as in\(^{39}\). Embryonic gestational age was determined by timed matings, with the date of the vaginal plug as E0.5.

The upstream, noncoding 2.4 Kb of mouse Atoh7 genomic DNA (nucleotides −3032 to −503 containing the primary enhancer but lacking the TATAAA box) was PCR amplified using primers with engineered XbaI and BglIII restriction sites, cloned into the Xba I-Bam HI sites of the pBGnCherry vector\(^{47}\) in the normal transcriptional orientation, and verified by Sanger sequencing. The Atoh7 fragment was PCR amplified (EXPAND Hi-Fidelity polymerase) from a previously subcloned 6.5 Kb mouse Atoh7 genomic DNA template\(^{47}\), digested with BglIII and XbaI and then purified. The pBGnCherry vector\(^{47}\) contains a minimal human β-globin promoter and monomeric Cherry red fluorescent protein reporter cassette (mCherry), as well as a synthetic amino terminal nuclear localization signal (MAPKKKKRQVEDY) downstream of the BamHI site. There is no intrinsic activity of this vector in transgenic mice\(^{47}\). Linearized DNA was microinjected into CD-1 mouse pronuclei by the CHRF Transgenic Core Facility. F\(_1\) embryos were collected at E13.5 and screened for live mCherry fluorescence with a Leica MZ12 dissecting scope equipped with a Texas-Red filter. We harvested 4 Cherry +/−/44 embryos and each Cherry-positive embryonic head was cryoembbed, sectioned and analyzed using immunohistochemistry and confocal imaging (see below).

**Bioinformatics of Rbpj binding sites.** Three kilobases of 5′ and three kilobases of 3′ noncoding genomic DNA from the mouse and human Atoh7 genes (Gene IDs 53404 and 220202) were aligned using the MacVector Clustal W algorithm (v. 12). CNEs were identified in multiple vertebrate genomes using the UCSC genome browser MultiZ alignment and conservation features and mm10 genome assembly (http://genome.ucsc.edu). Putative CSL/Rbpj binding sites were identified using the TRANSFAC MATCH program with matrices M01111 (VSRRBPJ_Q4) and M01112 (VSRRBPJ_01). Previously defined Pax6 paired domain and E-box binding sites within the mouse Atoh7 primary distal CNE are included for reference\(^{19,41,42}\).

**Immunohistochemistry.** Embryonic heads were fixed in 4% paraformaldehyde/PBS for 1 hour at 4 °C, processed through a sucrose/PBS series, cryoembedded and sectioned at 10 μm. Immunohistochemistry using our lab protocol\(^{2}\) to label with chick anti-GFP (Abcam, 1:1000, AB13970), rat anti-Rbpj (CosmoBio, 1:100, SIM-2ZRBP-1), rabbit anti-Atoh7 (Novus Biologicals, 1:500, NBP1-88639), or goat anti-mCherry polyclonal (SIM-2ZRBP-2) or mouse anti-β-actin (Sigma, 1:3000, A1978) primary antibodies, followed by HRP-conjugated (Invitrogen, LC2000). Standard western blotting was performed, using rat anti-Rbpj (Cosmo Bio Co, 1:500 SIM-2ZRBP-2) or mouse anti-β-actin (Sigma, 1:3000, A1978) primary antibodies, followed by HRP-conjugated anti-rat IgG or mouse IgG secondary antibodies (Jackson ImmunoResearch, Rat 112-035-175 1:5000, Mouse 315-035-003, 1:10,000). Blots were developed using a Supersignal West Pico Chemiluminescent substrate kit (Thermo Scientific, 34078), Kodak standard x-ray film and film developer.
Electrophoretic Mobility Shift Assay (EMSA). Single-stranded complementary oligonucleotides containing predicted CSL binding sites were labeled using a Biotin 3′ end DNA labeling kit (Thermo Scientific, 98918). Double-stranded DNA probes were made by annealing biotin-labeled complementary oligonucleotide pairs at room temperature for 1 hour. 0.5 μM purified mouse Rbpj protein (residues 53–474; Friedmann et al., 2008) and 1 nM of labeled oligonucleotide complexes, in the presence of 1.9 ng/μl poly(dI-C) (Sigma, 10108812001), were resolved on a 6% DNA retardation gels (Invitrogen, EC63652BOX) in 0.5 × TBE buffer and then transferred to nylon membranes. The LightShift Chemiluminescent EMSA kit assay (Thermo Scientific, 20148) was performed on the blots, which were developed using the Chemiluminescent Nucleic Acid Detection kit (Thermo Scientific, 89880), Kodak x-ray film and film developer.

Isothermal titration calorimetry of CSL-DNA complexes. The production and purification of bacterial Rbpj protein, residues 53–474, has been described. Oligonucleotides from Integrated DNA Technologies (IDT) (Fig. 4A) were hydrated, purified, quantified and annealed as in. All purified components were degassed, buffer matched and quantified as previously described. A typical experiment was performed at 5 °C using a MicroCal VP-ITC microcalorimeter with the oligomeric duplex (~100 μM) in the syringe and Rbpj (~10 μM) in the cell and consisted of 40 injections of 7 μl each. Data analysis used the ORIGIN software and was fitted to a one-site binding model, with binding data representing the average of n = 3 experiments.

Chromatin immunoprecipitation and Real-time PCR. ChIP was performed as described with several modifications. 30 E14.5 CD-1 pooled embryonic retinas were crosslinked with 1% formaldehyde and the reaction stopped by addition of 125 mM final concentration of glycine. Chromatin was sheared to 300–1000 bp size range with a Bioruptor UCD-200 sonicator + chiller (Diagenode), for 20 minutes at high power with 15 sec ON/30 sec OFF cycles. Either 3 μg rat anti-Rbpj antibody (Cosmo Bio Co, SM-2ZRB1P) or rat IgG (Jackson ImmunoResearch, 012-000-003) were incubated with 40 μg sonicated chromatin overnight at 4 °C. Immune complexes were collected with Protein G agarose beads (Sigma, P7700), washed several times and eluted using 0.5 M NaHCO3, 1% SDS elution buffer. Pax6 ChIP was run in parallel from each retinal chromatin prep, by incubating 20 μg of sheared chromatin with 1 μg anti-Pax6 (Covance, PRB-278P), or rabbit IgG (Jackson ImmunoResearch, 011-000-003), coupled to Protein A sepharose beads (GE Healthcare, 17-0780-01). Input and immunoprecipitated chromatin samples were initially analyzed by performing 30 cycles of PCR amplification and agarose gel electrophoresis, then quantified by real time PCR, using Table S4 primers, fast SYBR Green master mixes and a StepOnePlus PCR system (Applied Biosystems, 4385612 and 4376600). A standard curve using serial dilutions of 1% input chromatin was used to calculate the percent input of each sample. The p-values were determined by ANOVA and a Bonferroni posthoc test (Rbpj) or a student’s unpaired, 2-tailed t-test (Pax6) with GraphPad Prism software (v6).

Luciferase Assay. The upstream 2.6 Kb noncoding DNA from the mouse Atoh7 locus was previously cloned into the pGL2 luciferase vector. Rbpj binding site mutations were generated using PCR-based site directed mutagenesis and verified by Sanger Sequencing. Either 3.5 × 105 HEK293T or 5 × 105 AD12HER10 cells were plated per well of a 6-well tissue culture plate. After 48 hours (~60% confluency) cultures were transfected according to the FuGene6 (Promega, E2692) protocol with a 5:1 FuGene6 to DNA ratio, with the DNA constituting 500 ng of luciferase plasmid and 50 ng of Renilla control plasmid (pRL). In Notch ICD overexpression experiments, 100 ng of NICD1/pBK-CMV, 100 ng of NICD3/p3XFLAG-CMV-7TM, or a mixture of 50 ng of each plasmid with 100 ng of NICD1/pBK-CMV, 100 ng of NICD3/p3XFLAG-CMV-7TM, or a mixture of 50 ng of each plasmid were cotransfected with the luciferase and Renilla plasmids. Cells were washed in PBS, harvested 48 hours after transfection in 500 μL of PLB (Promega) and cell pellets stored at −80 °C. Cell extracts were assayed in technical triplicate using the Luciferase Assay System (Promega, E1980) on a Perkin Elmer Victor X5 workstation. Luciferase activity levels were normalized to the control Renilla activity, and p-values determined with GraphPad Prism (v6) software, using a two-tailed, unpaired t-test with equal standard deviation and assuming a Gaussian distribution.

RT-PCR. Total RNA was extracted using the RNeasy micro kit (Qiagen, Cat No 74004) from 1 pair of dissected E13.5 retinas, or using Trizol (Invitrogen Cat No 15596026) for HER-10 cells. For embryonic retinal RNA 100 ng was reverse transcribed into cDNA using the iScript Synthesis kit and product protocol (BioRad, Cat No. 178891). For HER-10 cells, 6.5 μg of total RNA was first treated with 1 U of 10 U/μL of Dnase (Roche Cat No. 0471672801) by incubating at 37 °C × 35 min, 80 °C × 5 min, 90 °C × 3 min. Then 2 μg of treated RNA was used for cDNA synthesis, with Superscript III and manufacturer protocol (Invitrogen/ThermoFisher, Cat No. 18080093). Both experiments included a mock synthesis (lacking total RNA) performed in parallel. For embryonic retinas, 1 μL of cDNA was combined with individual primer sets (Supplemental Table S4) and Go-Taq polymerase (Promega, cat # M7122) for 35 cycles of PCR at 95 °C × 30 sec, 55 °C × 30 sec, 72 °C × 30 sec. PCR products were electrophoresed on a 2% TAE agarose gel. Alternatively, 1 μL HER-10 cDNA was combined with individual primer sets (Supplemental Table S4), 1 × PCR Buffer and dNTPs, 1 × Masteramp (Epicentre/Illumina, ME81210) and 1 U of Taq polymerase (5U/μL Roche/Sigma, Cat No. 11146173001) for 35 cycles of PCR at 95 °C × 30 sec, 60 °C × 30 sec, 72 °C × 30 sec. PCR products were electrophoresed on a 1% TBE agarose gel.

Data availability. All data generated or analyzed during this study are included within this published article and its Supplementary Information files.
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Author Contributions
J.B.M., M.-S.M., R.A.K. and N.L.B. conceived and designed the experiments; J.B.M., M.-S.M., A.N.R. and A.N.C. performed the experiments; J.B.M., M.-S.M., R.A.K. and N.L.B. analyzed the data and wrote the paper.

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