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The Atoh7 remote enhancer provides transcriptional robustness during retinal ganglion cell development

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The retinal ganglion cell (RGC) competence factor ATOH7 is dynamically expressed during retinal histogenesis. ATOH7 transcription is controlled by a promoter-adjacent primary enhancer and a remote shadow enhancer (SE). Deletion of the ATOH7 human SE causes nonsyndromic congenital retinal nonattachment (NCRNA) disease, characterized by optic nerve aplasia and total blindness. We used genome editing to model NCRNA in mice. Deletion of the murine SE reduces Atoh7 messenger RNA (mRNA) fivefold but does not recapitulate optic nerve loss; however, SE knockout (KO) trans heterozygotes have thin optic nerves. By analyzing Atoh7 mRNA and protein levels, RGC development, and survival, and chromatin landscape effects, we show that the SE ensures robust Atoh7 transcriptional output. Combining SE deletion and KO and wild-type alleles in a genotypic series, we determined the amount of Atoh7 needed to produce a normal complement of adult RGCs, and the secondary consequences of graded reductions in Atoh7 dosage. Together, these data reveal the workings of an evolutionary fail-safe, a duplicate enhancer mechanism that is hard-wired in the machinery of vertebrate retinal ganglion cell genesis.

Significance

Vertebrate retinal ganglion cells (RGCs) transmit all visual signals from the eye to the brain, are the pathogenic target in glaucoma, and require the Atoh7 competence factor to develop from multipotent progenitors. Atoh7 transcription is controlled by dual cis regulatory elements, including a remote shadow enhancer (SE). In humans, loss of the SE causes NCRNA disease, with congenital blindness due to optic nerve aplasia. We generated a mouse Atoh7 SE deletion model and analyzed its effects on transcription, retinal histology, and chromatin architecture. The mutant mice express 80% less Atoh7 mRNA but retain optic nerves, unlike NCRNA patients. By systematically varying dosage in a genotypic series, we show how the dual enhancers maintain robust plasticity during RGC genesis.

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The authors declare no competing interest.

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mice (16, 17, 30). NCRNA is caused by a 6.5-kb deletion, extending from 19.2 to 25.7 kb upstream of the TSS. The deletion spans three conserved noncoding elements (CNEs), which together recapitulate the expression pattern of endogenous Atoh7 in transgenic mice and zebrafish (26). These remote CNEs share no obvious DNA sequence homology with proximal and distal CNEs located close to the TSS, which constitute the “primary” enhancer (PE), but their expression patterns appear identical. The NCRNA deletion thus removes a remote “shadow” enhancer (SE) that is vital for full ATOH7 expression and RGC genesis but also, paradoxically, redundant with

Fig. 1. Targeted deletion of the murine Atoh7 SE. (A) Human and mouse Atoh7 locus maps. (Top) VISTA plots (114) show conserved noncoding elements within SEs (CNE1-3) and PEs (Di, Pr). Human NCRNA and mouse SE deletions (brackets) and transcription start sites (blue arrows) are indicated. Sequence identity (50 to 100%) is plotted for a 50-bp moving window. DNA segments are identified as intergenic (red), repetitive (green), coding (blue), or UTR (yellow). Primers used to amplify SE deletion (Δ) junction and WT endpoints are indicated below the mouse VISTA plot. (Bottom) Multiz species alignment, adapted from the University of California Santa Cruz (UCSC) browser (build mm10). (B) Agarose gel showing diagnostic PCRs from +/+, SE/+, and SE/SE genomic DNA. (C) Sanger chromatograms of PCR products spanning the SEΔ junction and WT endpoints. Deleted nucleotides (yellow highlight), Cas9 cut sites (arrowheads), and protospacer adjacent motifs (PAMs) are indicated. (D) Schema of Atoh7 alleles. (E) Adult eyes from Atoh7 genotypic series. The optic nerves are thin in SE/KO eyes (with 56.6 ± 10.6% of +/+ cross-sectional area, SI Appendix, Table S4) and absent in KO/KO eyes. (F) Histology of adult eyes (hematoxylin/eosin) with similar retinal morphology. X. tropicalis, Xenopus tropicalis; nt, nucleotides. (Scale bars: Top, 500 μm; and Middle and Bottom, 100 μm.)
the PE. Moreover, multiple genome-wide association studies (GWAS), in diverse human populations, suggest that ATOH7 is the major determinant of variation in optic disk size, a clinical indicator of RGC number—with peak log-odds (LOD) scores directly overlying the SE (31–35). How do these dual enhancers coordinately regulate Atoh7 transcription? How do Atoh7 coding and SE regulatory mutations differ in phenotypic severity and developmental progression?

Duplicate regulatory elements have been described for several genes (36–39), including vertebrate retinal genes (40). Broadly speaking, PEs and SEs have distinct evolutionary origins but highly overlapping spatiotemporal domains, and they control transcription of the same gene. Their names do not indicate hierarchy or dominance; PEs are simply located closer to the TSS (41, 42). In some circumstances, one enhancer (PE or SE) can fully initiate and maintain gene expression by itself (43, 44)—yet both have been positively selected during evolution. In the case of ATOH7, the PEs and SEs may have complementary or synergistic roles that improve fitness. Because ATOH7 specifies the first retinal cell type, these enhancers control the onset of neurogenesis in the vertebrate eye.

To understand dual ATOH7 enhancer function and NCRNA pathogenesis, we generated SE deletion (Atoh7−SE) mice via CRISPR/Cas9 editing. By combining SE deletion and knockout (KO) alleles, we created a genotypic series with six graded levels of Atoh7 expression and quantitatively characterized effects on RGC genesis and retinal development. The SE deletion profoundly reduced Atoh7 transcription but did not alter its spatial or temporal pattern, or the architecture of surrounding chromatin. The number of adult RGCs was correlated with Atoh7 mRNA abundance following a saturation curve, and there were secondary defects in optic nerve head (ONH) morphology, axon fasciculation, and retinal vasculature. However, unlike humans with NCRNA disease, the optic nerves of homozygous Atoh7−SE mice were grossly normal. These dual modular enhancers can act additively to ensure robustness of expression, allowing developmental programs and regulatory networks to resist environmental or genetic perturbations.

Results
The SE Regulates Atoh7 Transcription. To model NCRNA, we deleted 5.6 kb of mouse chromosome 10 spanning the Atoh7 SE (Fig. L4). We generated three Atoh7 SE deletion alleles, with similar homozygous eye phenotypes, and chose one for further investigation (Fig. 1 B and C and SI Appendix, Fig. S1). Unlike NCRNA patients and Atoh7 KO animal models, the SE/SE mice had intact optic nerves (Fig. 1E). However, the optic nerves of trans heterozygous (SE/KO) eyes were noticeably thinner than wild-type (WT), with no change in retinal morphology (Fig. 1 E and F), suggesting that loss of the SE does impair Atoh7 expression and RGC genesis.

In principle, enhancers can regulate the level, timing, and/or spatial pattern of gene expression. To determine how SE deletion affects the level and timing of transcription in cis, we measured the relative abundance of Atoh7 messenger RNA (mRNA) transcripts in a triplex competitive reverse transcriptase (RT)-PCR experiment (Fig. 2). We compared the molar ratio of PCR products from littermate +/HA (hemagglutinin) and SE/HA retinas at the onset (E11.5), peak (E14.5), and offset (E16.5, P0.5) of Atoh7 expression (8) using the trans Atoh7H_A (HA epitope tag) knock-in allele (45) as an internal reference to calculate the ratio of mRNAs from untagged (UT) WT and SE alleles (Fig. 24). The HA epitope is coextensive with the endogenous Atoh7 protein in immunostained HA/+ retinas (ruling out allelic exclusion) (14) and has no detectable effect on RGC genesis (13, 45). If the SE deletion caused the temporal pattern to shift—delaying the onset of Atoh7 expression during development, for example—then the ratio of mRNAs originating from SE and HA alleles would be significantly reduced at early time points (E11.5) only. However, in these assays, the SE deletion caused a decrease in Atoh7 expression at every time point, by a similar magnitude (6.4–, 6.4–, 4.5–, and 5.2-fold at E11.5, E14.5, E16.5, and P0.5, respectively). The SE deletion thus uniformly reduces the level of Atoh7 expression four- to sixfold in cis, but does not alter the timing of transcription.

SE Mutants Have Fewer Atoh7+ and Isl1/2+ Cells. To evaluate how the SE deletion affects the level of Atoh7 protein and distribution of Atoh7+ cells, we immunostained midaxial retina sections at E14.5 (peak expression) (Fig. 3) with a validated antibody (14) and determined planimetric cell densities. This value broadly reflects the size of the RGC-competent progenitor pool. We observed significantly fewer Atoh7+ cells per 10,000-μm² retinal area for all five mutant genotypes in our series, compared to WT, but there were no differences in the overall retinal staining pattern (Fig. 3) or midaxial area (SI Appendix, Table S1). Across the genotypic series, the number of Atoh7+ cells was well correlated with the abundance of Atoh7 mRNA (Fig. 3H) (r² = 0.94, P < 0.01, df = 4), using 1.00, 0.16, and 0.00 as normalized expression values for WT, SE, and KO alleles, respectively, based on triplex RT-PCR data.

We then performed a comparable analysis for LIM homeobox factors Isl1 and 2 using the same E14.5 retinas, which were costained with anti-Isl1/2 (Fig. 3). During normal development, retinal progenitors oscillate radically within the neuroepithelium via interkinetic nuclear migration (IKNM), coupled to the cell cycle (46). Following terminal mitosis at the retinal apex, nascent RGCs migrate basally. At day E14.5, Isl1 and Isl2 are expressed in differentiating RGCs and starburst amacrine cells (47) in the inner neuroblast layer and overlap with Atoh7 in a narrow postmitotic transition zone. As nascent RGCs traverse this zone, they down-regulate Atoh7 and up-regulate Isl1/2 (SI Appendix, Fig. S2). Isl1 expression is downstream of Atoh7 whereas Isl2 is immediately downstream of Isl1 (48–50); both are continuously expressed in mature RGCs. We observed a similar decrease in the planimetric density of Isl1/2+ cells across the Atoh7 genotypic series. The presence of Isl1/2+ cells in Atoh7 KO/KO retinas is consistent with previous results showing the existence of an alternate, independent mode of Isl1 regulation during RGC genesis (49). Nonetheless, the overall number of Isl1/2+ cells was correlated with Atoh7 mRNA levels (Fig. 3H) and the number of Atoh7+ cells (Fig. 3F), as expected. Similar decreases in Bm3+ (Pou4f) and Rbpms+ (multiply spliced RNA binding protein) cells were observed in Atoh7 SE/SE and SE/KO E14.5 retinas (SI Appendix, Fig. S3). The concordance between Atoh7 and Isl1/2 (ratio of double-positive to all positive cells) also decreased across genotypes but declined most abruptly between KO/+ (8.0 ± 1.7%) and SE/SE (3.0 ± 0.6%) retinas (SI Appendix, Fig. S2), reflecting a shorter temporal overlap between Atoh7 and its target, and potentially weaker transcriptional activation as Atoh7 protein levels are reduced. Loss of the SE thus causes a decrease in Atoh7 mRNA and Atoh7+ cells, and a corresponding decrease in Isl1/2+ Brn3+, and Rbpms+ cells, as indicators of RGC genesis.

Altered Timing of Atoh7 Expression within the Terminal Cell Cycle. At the onset of retinal neurogenesis (E11.5), a subset of progenitor cells experience a burst of Atoh7 transcription during terminal S-phase (13, 50, 51). As development proceeds (E13.5) and RPC cycles lengthen (52–54), this transient Atoh7 burst is progressively delayed, into terminal G2/M and G0 phases (8, 51). While perdurant SE and PE reporter transgenes have coextensive spatiotemporal and cellular patterns (26) and the overall timing of Atoh7 expression is broadly preserved at the tissue level in SE mutants, in stochastically phased RPCs (Fig. 2), these dual enhancers may act differently on a cellular scale, controlling distinct temporal features of Atoh7 transcription within the last
Atoh7 gene and cDNA are colinear with no introns. S5, 250-nt ROX size standard. (C) UT/HA cDNA peak area ratios are plotted at four ages, normalized to +/HA cDNA controls. The ratio of Atoh7 mRNAs transcribed from SE versus WT alleles is 0.16 ± 0.02 at E11.5, 0.16 ± 0.009 at E14.5, 0.22 ± 0.007 at E16.5, and 0.20 ± 0.015 at P0.5. (D) Temporal profile of Atoh7 mRNA extrapolated from qPCR (B) and triplex RT-PCR data. The SE deletion uniformly reduces Atoh7 expression at all time points. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

**Postnatal Atoh7 SE Mutants Have Fewer RGCs.** In a previous study, we showed that a 50% reduction in Atoh7 dosage impairs RGC genesis but does not alter the final number of RGCs, by comparing optic nerve axons in neonatal and adult Atoh7 +/+ and KO/+ mice (19). The developing nervous system thus compensates for defective RGC genesis by decreasing the extent of neonatal RGC culling (55, 56). However, in SE/KO mice, the deficiency in RGC genesis in the NCRNA animal model. In SE/SE mice, this defect is largely compensated by a reduction in postnatal RGC culling, since the range of these effects is similar, approximately twofold (Fig. 3) (56). However, in SE/KO mice, the deficiency in RGC genesis (fivefold) exceeds the buffering capacity of the transient RGC culling. To test this possibility, we determined the S-phase fraction of Atoh7+ cells by EdU (5-ethyl-2′-deoxyuridine) pulse labeling (Fig. 4). If Atoh7 activation were delayed in SE mutants, this value is expected to decrease. Instead, the S-phase fraction was significantly elevated in E14.5 SE/SE (34.1 ± 1.6%) and SE/KO (33.6 ± 7.4%) retinas compared to WT (23.1 ± 1.3%), suggesting that both enhancers (or the SE alone) may be needed to fully sustain the burst of Atoh7 transcription through the terminal cell cycle. At the same time, there was no change in the M-phase fraction, determined by PH3 (phosphohistone) staining (SI Appendix, Table S2). The accelerated decline in postmitotic Atoh7 expression in SE/SE and SE/KO retinas is supported by reduced Isl1/2 concordance (SI Appendix, Fig. S2).

**Cone Genesis and Apoptosis in SE Mutant Retinas.** Beyond the absence of RGCs and optic nerves, adult Atoh7 knockout mice have thinner retinas, with a deficiency of later-born cell types that arises from decreased RPC proliferation (30). The cone-to-rod ratio is increased accordingly. In mutant embryos, retinal cell death is also broadly elevated (12, 16, 20). To evaluate cone genesis and apoptosis in SE mutants, we stained E14.5 retinas with thyroid hormone receptor beta (Thrβ2) and cleaved polyADP ribose polymerase (c-PARP) antibodies, respectively (SI Appendix, Fig. S4). Apoptotic cells were more abundant in KO/KO retinas, but no other genotypes, and there was no major change in cones.

**Fig. 2.** The SE regulates Atoh7 mRNA abundance. (A) Triplex RT-PCR strategy to quantify Atoh7 mRNAs in +/HA and SE/HA retinas. The FAM (+)-labeled 5′ primer (black arrowhead) recognizes both alleles; unlabeled 3′ primers are specific for UT (black/blue) or 3xHA knock-in (red) alleles. (B) Capillary electrophoresis profiles of PCR products from +/HA and SE/HA cDNA, and HA+/genomic DNA. The average ratio of UT (244 nt) and HA (273 nt) cDNA peak areas, at the right-hand corner of each trace, reflects the relative abundance of allelic transcripts. The gDNA trace (UT/HA product ratio = 1.8 ± 0.11, n = 3) was included as a PCR efficiency control, with a 1.0 fixed molar template ratio. Atoh7 gene and cDNA are colinear with no cycle.
culling mechanism so the number of surviving RGCs in adults is decreased (two- to threefold).

Since RGC myelination continues until postnatal week 16 in mice (57), axon counts may, in principle, underestimate the true number of ganglion cells in Atoh7 SE mutants. We therefore determined the total number of Rbpms-immunoreactive RGCs in P30 retinal flatmounts (58) using an automated cell counting algorithm (SI Appendix, Fig. S6). This allowed us to quantify RGCs with greater precision across the full Atoh7 genotypic series (Fig. 5 and SI Appendix, Table S5). Our WT C57BL/6 data match those obtained via other automated counting approaches (59, 60). Consistent with previous analyses (19), there was no significant difference in the number or density of RGCs in adult Atoh7 KO/+ and SE/+ retinas compared to WT, and KO/KO retinas had scant RGCs. However, both SE/KO and SE/SE retinas had significantly fewer RGCs. The distribution of RGCs was similar in all genotypes, with lowest density in the dorsal periphery and highest density in a central peripapillary stripe (SI Appendix, Fig. S7), as previously noted in mice (61) and rats (62). The number of adult RGCs varied with the size of the Atoh7+ progenitor pool, following a saturation curve (Fig. 5C).

Secondary Effects in SE Mutant Retinas. As RGC axons exit the eye and extend toward the diencephalon, they pass through the ONH, a discrete structure in the ventral posterior retina where the choroid fissure closes and hyaloid blood vessels enter (63). To investigate this structure and the glial deficiency in P1 mutant optic nerves (SI Appendix, Fig. S5), we counted astrocyte precursor cells (APCs) in the E14.5 ONH labeled with Pax2 antisera. These cells are packed in a wedge-shaped torus at the posterior pole of the optic cup, where it joins the optic stalk (Fig. 6A and B), and differ in their properties from Pax2+ cells located inside the stalk (63–66). There were fewer ONH Pax2+ cells in SE/KO and KO/KO mice compared to WT, with smaller reductions in SE/SE and KO/+ mice. Thus, like other phenotypes that depend on RGCs, ONH development is impaired in Atoh7 mutants, with increasing severity across the genotypic series.

Current models for retinal vasculogenesis highlight the importance of RGC axons, which haptotactically guide optic stalk astrocytes into the eye, where they direct endothelial cell migration and blood vessel development and SHH secreted by RGCs, which initiates this process (5, 6). These models are based on the major secondary vasculature defects associated with RGC...

Fig. 3. Abundance of Atoh7+ and Isl1/2+ cells decreases across the Atoh7 genotypic series. (A–F) At E14.5, Atoh7+ cells (magenta) reside within the neuroblast layer (nb) layer while Isl1/2+ cells (green) are located in the nb and ganglion cell layer (gcl). KO/KO retinas lack Atoh7 immunoreactivity. (G) Atoh7+ and Isl1/2+ cell counts per 10,000 μm². Each mutant genotype has fewer Atoh7+ cells than WT (+/+), and most have fewer Isl1/2+ cells. (H) In bivariate plots, the number of Atoh7+ and Isl1/2+ cells is directly related to Atoh7 mRNA abundance (for Atoh7+ cells, linear regression P < 0.01, r² = 0.94, df = 4). Atoh7 mRNA values were calculated from triplex RT-PCR data as 1.00 (+/+), 0.58 (SE/+), 0.50 (KO/+), 0.16 (SE/SE), 0.08 (SE/KO), and 0.00 (KO/KO). (I) The numbers of Atoh7+ and Isl1/2+ cells are strongly correlated across the genotypic series. *P < 0.05, **P < 0.01 and ****P < 0.0001. (Scale bar: 100 μm.)
agensis in Atoh7 mutant animals and humans with PHPV or NCRNA disease (5, 16, 18, 26). In each case, intravitreal fetal (hyaloid) vessels persist, and the adult retinal vasculature does not develop. Vascular endothelial cells (VECs) fail to enter the eye from the ONH, due to astrocyte migration and polarization defects in the absence of ganglion cells (6). In WT mice, the influx of VECs occurs postnatally, after RGC genesis is complete. Neither astrocytes nor VECs express Atoh7.

To determine how graded reductions in Atoh7 dosage and RGC density alter retinal vasculature and the formation of axon bundles, we examined flatmounts stained for Rbpm5 (RGC soma), Ib4-lectin (vessels), and Tubb3 (neural tubulin β3, axons). There were two noteworthy changes (Fig. 6C). First, the mean number of major vessels emanating from the ONH was significantly reduced in SE/SE and SE/KO retinas compared to WT, SE/+, and KO/+. However, despite having fewer primary vessels and 61% fewer RGCs (SE/KO), these mutant retinas were fully vascularized by smaller arterioles and veins. A potentially similar decrease in retinovascular branching points has been noted in children with optic nerve hypoplasia (67). Second, axon fasciculation was abnormal in SE/SE and SE/KO retinas. In both genotypes, we observed large bundles of Tubb3+ fascicles and a unique pattern of axon tracts converging on the ONH, unlike WT retinas (Fig. 6C). These anatomical findings were supported in vivo phase variance optical coherence tomography (pv-OCT) of the nerve fiber layer (NFL) and retinal vasculature in Atoh7 SE/SE and SE/KO mice (SI Appendix, Fig. S8).

Atoh7 Chromatin Accessibility in SE Mutant Retinas. To evaluate Atoh7 chromatin architecture, we compared E14.5 retinas from WT and SE/SE animals using assay for transposase-accessible chromatin with ATAC-seq (assay for transposase-accessible chromatin using sequencing) methods (68). As expected, Atoh7 SE and PE had signatures of open chromatin (Fig. 7A). The discrete open segments in these profiles correspond to known CNEs with two exceptions—a small open region 0.7 kb upstream from the distal (Di) PE element, which is not conserved, and SE element CNE1, which was not accessible above background. Based on read density, CNE2 appears to be most accessible, with 854 peak reads per nucleotide, followed by CNE3, Pr (proximal), and Di. Interestingly, CNE2 contains the lead GWAS single-nucleotide polymorphism for optic disk size in humans, rs3838145 (26, 31, 33), and shows greater accessibility than the distal CNE, which contains binding sites for established regulators of Atoh7 transcription (21–23, 69, 70). In SE mutant retinas, open signatures for CNE2 and CNE3 were missing, as expected, but accessibility of the surrounding chromatin was unchanged. These findings are reminiscent of ATAC-seq data from mice with α-globin enhancer deletions (71). In that study, individual Hbe enhancers were shown to influence transcription independently, in an additive manner, with single-element deletions causing only slight changes in the accessibility of surrounding chromatin.

Likewise, our findings suggest that the Atoh7 SE does not broadly regulate chromatin access across the locus. Instead, the SE and distal PE appear to work as separate cis regulatory modules to independently drive Atoh7 transcription (Fig. 7B). By itself, the PE can activate low levels of expression, which are sufficient for RGC genesis in mice. Although Isl1 and Pou4f2 are downstream of Atoh7, neither gene appears to be a direct transcriptional target (72), and their ATAC-seq profiles were unchanged in SE/SE retinas (SI Appendix, Fig. S9).

Discussion

Atoh7 SE Deletion Mice Do Not Model NCRNA Disease. Murine Atoh7 mutations differ from NCRNA disease in two important ways. First, as shown here, the SE deletion reduces but does not block RGC genesis, so mutant mice retain optic nerves, whereas, in NCRNA disease, there is no radiological evidence for optic nerve remnants, chiasm, or tracts (26). While NCRNA deletion effects on human ATOH7 transcription are unknown, the relative contributions of SE and PE may differ between species. Alternatively, the fraction of Atoh7+ progenitors that adopt...
ganglion cell fates may differ radically between primates and rodents. In mice, only 11% of Atoh7+ cells develop into RGCs, but each is competent to do so (8, 20), providing a buffer for the embryo from environmental or genetic perturbations. Indeed, as we reduced Atoh7 activity and the size of the RGC-competent progenitor pool (Fig. 3H), the number of adult RGCs did not decline dramatically until these values fell below 20% of WT (Fig. 5C). Presumably, the fraction of Atoh7+ RPCs acquiring RGC fates was increased. However, if most primate Atoh7+ cells were intrinsically biased toward RGC fates, a fivefold decrease in Atoh7+ cell densities would severely limit ganglion cell development. The developmental plasticity of Atoh7+ cells may thus be greater in mice than humans. Likewise, nonautonomous Atoh7 effects on RGC genesis (8) may differ between species. Finally, RGC culling provides a basis to compensate for RGC deficiency and was diminished in SE homozygotes (19) to explain the huge difference in phenotypic severity between human and mouse SE deletions.

Second, in Atoh7-null mice, the hyaloid vasculature persists in the vitreous and invades the retina but does not proliferate excessively (5, 6, 77); however, in NCRNA disease, there is a dense profusion of retrolental fetal vessels, which fill the vitreous and retract, causing bilateral retinal detachments at birth. Indeed, while most anatomical features of the eye scale with size, cell dimensions and the physics of O2 diffusion do not. Since eye globe volumes are >400 times larger in humans than mice, and RGCs are required for adult retinal blood vessels to develop, the magnitude of retinal hypoxia and vigor of the secondary neovascular response are expected to be much greater in NCRNA disease. Likewise, RGC axons provide anatomical support to stabilize the posterior globe. In the larger human eye, exposed to greater biomechanical force (78, 79), the retina may detach more readily than it does in mice when this structure is missing, severing any remaining RGC axons. While these secondary effects may contribute to NCRNA disease pathology, our data suggest impaired RGC fate specification is the major primary defect.

RGC Genesis Depends Strictly on Atoh7 Activity. Using the hypomorphic SE allele, we extended previous analysis (19) to define Atoh7 dosage thresholds for RGC phenotypes. Two findings are striking. First, the abundance of Atoh7+ and Isl1/2+ cells depends directly on Atoh7 mRNA (Fig. 3H), which is uniformly decreased by the SE deletion at all ages (Fig. 2). The transient burst of Atoh7 appears necessary to kindle transcription of Isl1 and Pou4f2, which cooperatively instruct progenitors to differentiate as RGCs and positively regulate their own expression (48–50, 80, 81). Second, the number of mature RGCs (P30) is relatively stable until the Atoh7+ progenitor pool is diminished by >70% (Fig. 5C). Adult +/+ and KO mice have a similar number of RGCs, and SE/SE mice have only 16% fewer RGCs despite having 84% less Atoh7 mRNA. Indeed, widespread transgenic expression of Atoh7 in >80% of retinal progenitors during terminal mitosis, and continuously thereafter in CxRk lineal descendants (photoreceptor and bipolar cells), does not increase the number of RGCs (82).

Timing of Atoh7 expression is also critical. Rescue of RGC genesis in Tg(Crx>Atoh7-ires-Cre); Atoh7 KO/KO mice is partial and restricted to progenitors that exit mitosis during the normal temporal envelope for RGC births (82). In this respect, the narrow time window for Atoh7 transcription, mediated by dual enhancers, is reminiscent of Drosophila atonal (83) and Sry, the HMG-box testis-determining factor. Sry is transiently expressed in bipotential gonads where it coactivates Sox9 transcription and initiates male sexual differentiation (84). When timing of the Sry pulse is shifted, even by 6 h, testis development is severely disrupted (85–87). Accordingly, dual Atoh7 enhancers may be needed as a fail-safe to ensure robust mRNA transcription during a short time interval. Despite these constraints,
the period of RGC competence and Atoh7 expression can be prolonged under certain circumstances, leading to a marked increase in ganglion cells (88–90).

Decreased Atoh7 Activity Causes Secondary Malformations. Loss of RGCs in Atoh7 mutants triggers a chain of downstream effects involving the ONH, retinal astrocytes, and vasculature. Fewer Pax2+ glia are present in ONH domains of Atoh7 SE/SE, SE/KO, and KO/KO mice (Fig. 6 A and B). These Pax2+ APCs enter the optic cup and give rise to A1 astrocytes, which are needed to establish the retinal vasculature (64, 65). Their migration depends on SHH signaling from pioneer RGC axons (3, 4, 66). Reduced ONH dimensions may also physically restrict migration of astrocytes and VECs into the retina.

One unique phenotype in the Atoh7 series is an alteration in axon fasciculation, with coarse bundling of axons converging on the ONH in SE/SE and SE/KO retinas (Fig. 6 C). Hyperfasciculation may be mediated by reduced expression of downstream cell adhesion molecules, such as L1CAM (91), but has not been reported in Pou4f1-3, Isil, Isil2, Sox4, or Sox11 mutants, which have comparable reductions in RGC abundance (48–50, 92). However, Pou4f2 and Isil1 mutants do exhibit axon pathfinding defects at the chiasm (49). Intra-retinal axon misrouting has been noted in Bmp7 mutants, which lack an ONH (63), and in Tg(Crx>Atoh7-ires-Cre); Atoh7 KO/KO mice, with delayed RGC genesis (82).

The Atoh7 SE Does Not Control Access to Surrounding Chromatin. CNEs often indicate the position of enhancers and, when active, exhibit specific histone acetylation (H3K27ac) and methylation (H3K4me1) marks, p300 enrichment, and features of open chromatin (42, 93, 94). The Atoh7 SE is structurally and functionally conserved; as such, the human SE drives reporter expression appropriately in transgenic mice and zebrafish (26). However, CNEs 1 to 3 are not equally accessible. In ATAC-seq profiles, CNEs 2 and 3 are open, consistent with data from developing mouse (95–97) and human (98) retinas, but CNE1 has a closed configuration. It may thus function as a repressive element to inhibit Atoh7 transcription in proliferating RPCs (99), mature retinal neurons, or nonretinal tissues, or it may have an architectural role (94); however, CNE1 is unlikely to regulate neighboring genes as these are separated from the Atoh7 syntenic block in teleosts (26). Indeed, CNE1 has sequence similarity with a repressive element upstream of Xenopus homolog Ath5, which prevents precocious retinal expression (70).

If this element augments activity of the mouse Atoh7 PE, it may help explain the species difference in SE deletion phenotypes. One function of dual Atoh7 enhancers may be to modulate chromatin looping and accessibility over time as progenitors progress through a series of competence states (101). Remarkably, the SE deletion did not alter the E14.5 retinal ATAC-seq profiles of chromatin surrounding Atoh7 or other biologically relevant genes. The SE thus does not control or gate access of transcription factors to the PE or promoter. Indeed, if these two enhancers acted cooperatively and looped to form a single holo complex with transcription factors at the Atoh7 promoter, the SE deletion would be expected to reduce accessibility of PE chromatin, as has been observed for hypersensitive sites in the β-globin locus control region (LCR) (102). Instead, the Atoh7 SEs and PEs
Methods

Atoh7 tandem arrangement helps establish a robust RGC progenitor. E16.5, and P0.5) was used to direct complementary DNA (cDNA) synthesis (Appendix). Directing cDNA synthesis with ATG, UTR, and WT activity (45).


does not depend on SE. (b) Proposed mechanism for Atoh7 regulation by SE and PE during retinal histogenesis. SE (CNE2/3 elements) and PE (distal element) loop independently with the Atoh7 promoter to activate transcription and are functionally redundant. Each modular enhancer is sufficient to control spatiotemporal expression, but they act additively to increase activity.

appear to act redundantly to rapidly boost expression, similar to mouse rod opsin enhancers (40), β-globin and cone opsin LCRs (103–105), and dual Pax6 lens enhancers (44, 104). In this respect, the Atoh7 SE functionally resembles SEs originally defined in Drosophila for knuppel, knirps, and svb genes (36, 37, 106). This tandem arrangement helps establish a robust RGC progenitor pool for the developing eye.

Methods

Animals. Atoh7<sup>SE</sup> mutant mice were generated by injecting fertilized C57BL/6J oocytes with CRISPR/Cas9 ribonucleoprotein (RNP) complexes that cleave genomic sites flanking the Atoh7 SE (SI Appendix, Supplemental Methods and Table S6). Two further alleles were combined with Atoh7<sup>SE</sup> on an isogenic C57 background in different experiments. Atoh7<sup>Atoh7<sup>SE</sup></sup> (Atoh7<sup>y<sup>y</sup>y</sup>) is a null (KO) allele with in-frame lacZ reporter (16). Atoh7<sup>HA</sup> has three tandem HA epitope tags at the protein C terminus, intact 3′ untranslated region (UTR), and WT activity (45).

Triplex RT-PCR. Total RNA from HA/+ and SE/+ littermate retinas (E11.5, E14.5, E16.5, and P0.5) was used to direct complementary DNA (cDNA) synthesis (SI Appendix, Supplemental Methods), and PCR was performed using three primers—one shared forward primer end-labeled with 6-carboxyfluorescein (FAM) and two reverse primers specific for WT (WT, SE) or HA alleles (SI Appendix, Table S6). Denatured products were resolved by capillary electrophoresis. Peak areas reflect the molar abundance of WT and HA cDNAs. Since Atoh7 has no introns, we included an HA+ genomic DNA (gDNA) control to assess differential amplification of WT and HA alleles.

Immunostaining, Embryonic Cell Counts, EdU Labeling, and Optic Nerve Histology.

Tissue cryosections (10 μm) were prepared and stained, following standard procedures (SI Appendix, Supplemental Methods and Table S7), and were imaged using a Leica SPE confocal microscope (Wetzlar, Germany). Optical sections were collected every 2 μm, producing a layered 2-stack. Composite Atoh7, Isl1/2, Brn3, Rbpms, c-PARP, and Thuj2 images were stitched from nasal and temporal retinal fields using Leica LASX software. Cells were counted in maximum intensity projections of two midlevel optical planes, with n ≥ 3 biological replicates and equal exposures for all genotypes. Planimetric densities were determined using neuroretinal areas, calculated by Fiji (107). To evaluate the onset of Atoh7 expression during terminal S-phase, E14.5 gravid females were injected 90 min before harvest with 12 μg/g EDU, which was detected in Brn3 nuclei using Alexa 488 or 594 azide.

Ultrathin optic nerve sections from P30 and P1 mice (n = 3 +/+, SE/SE, SE/KO) were stained with toluidine blue or processed for electron microscopy to determine cross-section areas and myelinated axon counts (SI Appendix, Supplemental Methods) (19). P30 and P1 optic nerve areas were calculated by perimeter tracing (Fiji). P30 myelinated axons were counted (19, 108) for each nerve at 14 locations (10 peripheral, four central). Counts per field were averaged for each sample, and axons per nerve were calculated as mean axon density × area.

Retinal Flatmounts. Adult retinas were dissected, fixed and flattened (photoreceptors downward), and immunostained (SI Appendix, Supplemental Methods and Table S7). After staining, retinas were mounted (RGCs facing up) and imaged at 200× using an ApoTome, collecting six individual planes in 2-μm steps. Images were tiled to form whole-retina composites and Z-stacks compressed as maximum intensity projections (ZenPro, Zeiss). To count and map RGCs, retinal flatmounts were stained with anti-Rbpm5. Composite images were traced to generate area maps (Fiji) and processed to enumerate positive cells and plot RGC density using custom Python codes (SI Appendix, Supplemental Methods). Automated and manual counts were highly correlated (SI Appendix, Fig. S6). In flatmount images, orientation is indicated with D (dorsal), V (ventral), N (nasal), and T (temporal) axes.

Live OCT. Using custom OCT system hardware and methods (109, 110), we imaged fundi of anesthetized mice in phase variance mode (pv-OCT), capturing multiple consecutive B-scans per retina position to visualize retinal blood flow and map vasculature. Scans were processed extensively to generate sectional intensity and phase variance images and depth-coded en face views (SI Appendix, Supplemental Methods). Images were processed using Matlab (Mathworks, Natick, MA) and Fiji software. Retinal B-scans were processed, to generate intensity and phase variance images, and flattened using a custom Matlab script. Intensity images were cropped to include theNFL and resliced to generate en face views. pv-OCT images were cropped to encompass the inner retina and coded for depth.

ATAC-Seq. Dissected pairs of E14.5 +/- and SE/SE retinas (n = 3 each) were processed into single cell suspensions (SI Appendix, Supplemental Methods). For each sample, 100,000 cells were exposed to Tns transposase and extracted following a scaled ATAC-seq protocol (68) with the Nextera DNA Library Prep Kit (Illumina, San Diego, CA). ATAC-seq libraries were amplified from transposed genomic DNA using barcoded primers (SI Appendix, Table S6) and established guidelines (SI Appendix, Supplemental Methods), with quality assessment by Qubit assay, high sensitivity DNA bioanalyzer (Agilent, Santa Clara, CA), and qPCR (KAPA Library Quantification; Roche, Indianapolis, IN). The six sample libraries were pooled (300 nM each) and size-selected (1.8x ratio SPRI beads; Beckman Coulter, Brea, CA), giving a final library with 1.9 ng/μl (17.5 nM) DNA and 300-base pair (bp) mean fragment size. This was sequenced using an Illumina HiSeq4000 instrument with paired-end 100 format (Novogene, Santa Clara, CA) and analyzed to generate bigWig alignment files (SI Appendix, Supplemental Methods).

Statistics. Genotype groups were compared using Student’s t test and Tukey multiple comparison tests, assuming cell count and mRNA data are normally distributed, with nonparametric bootstrap confirmation (pooled resampling × 1,000 iterations) implemented in R (111–113). Error is reported as SD. Parametric significance is indicated in display figures as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Data Availability. The automated cell counting (cellcounter9.py) and density heatmap (cellheatmap_v38B.py) computer codes are available at https://github.com/jbmiesfeld/Atoh7-remote-enhancer. ATAC-seq data were deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (accession no. GSE146897).

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