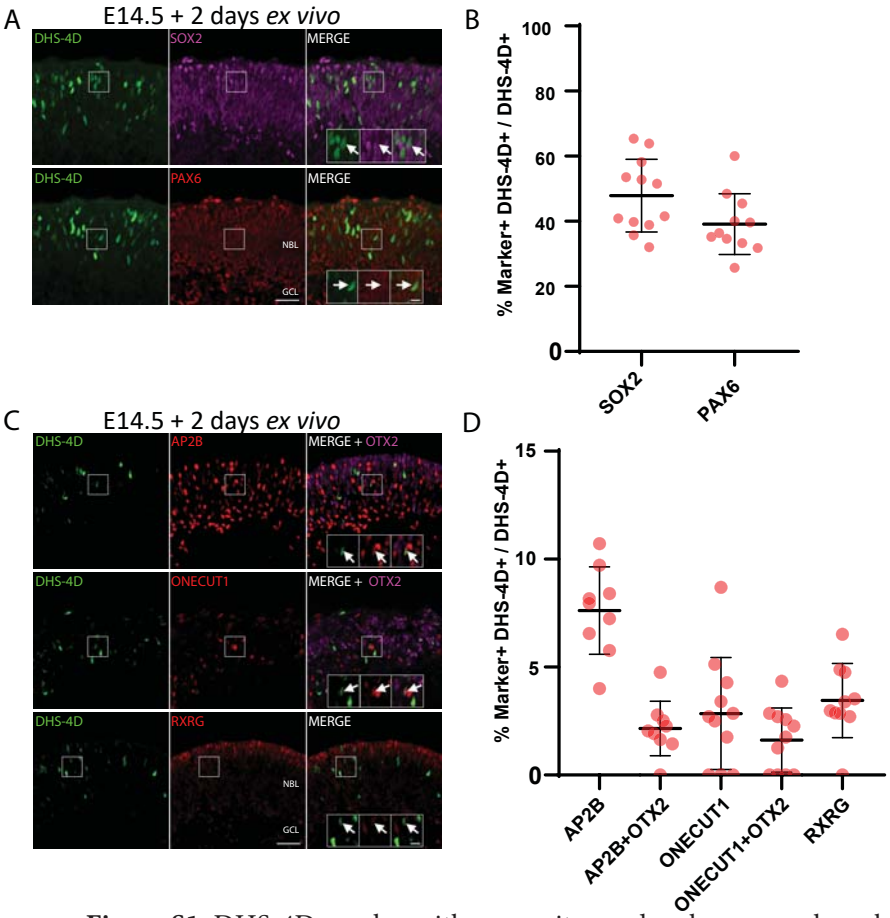

Figure S1: DHS-4D overlap with progenitor and early neuronal markers. (A) Representative histology of DHS-4D reporter electroporated into E14.5 retinal explants cultured for 2 days and stained for the progenitor markers SOX2 and PAX6. (B) Quantification of DHS- 4D overlap with SOX2 and PAX6. (C) Representative histology of DHS-4D reporter electroporated E14.5 explant retinas cultured for 2 days and stained for AP2B, ONECUT1, RXRG, and OTX2. (D) Quantification of DHS-4D overlap with AP2B, ONECUT1, RXRG, and triple overlap of AP2B and ONECUT1 with OTX2. Arrows mark double labeled cells in all panels. Dots represent quantified images from N=3 explants. NBL = neuroblastic layer, GCL = ganglion cell layer. Bars show the mean and s.d. Scale bars: 100μm; 25μm for insets.

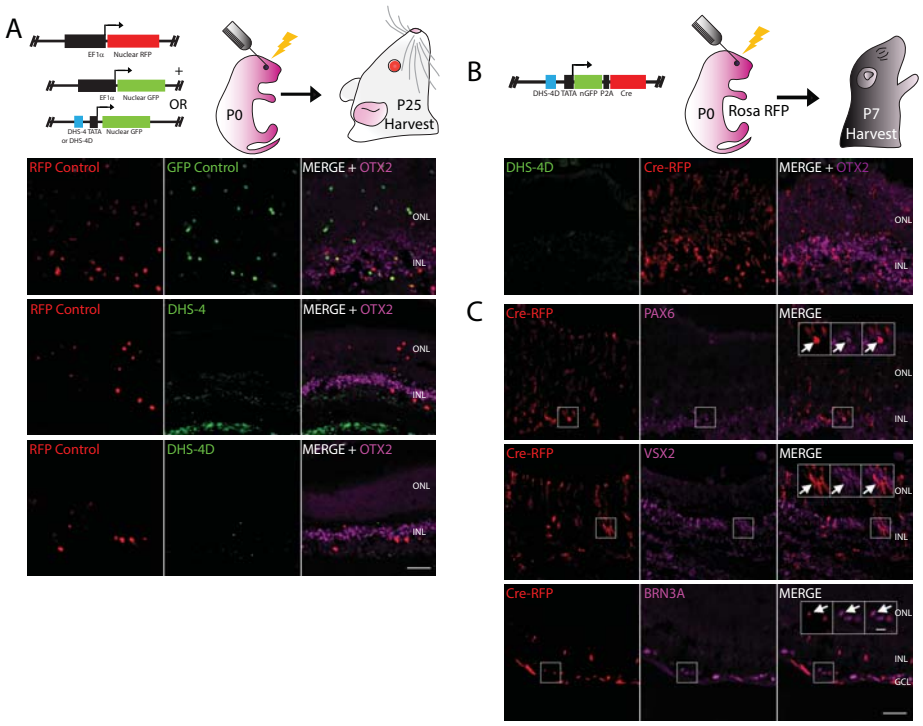

Figure S2: DHS-4 and DHS-4D long-term expression patterns. (**A**) Experimental design and histology of P0 CD1 mice electroporated in vivo with DHS-4, DHS-4D, or Control constructs harvested at P25. DHS-4 and DHS-4D lack activity in the mature retina. (**B**) Experimental design of lineage tracing utilizing DHS-4D driving GFP and P2A linked Cre. The plasmid was electroporated in vivo into P0 ROSA-RFP mice and the pups harvested at P7. (**C**) Histology of lineage traced mice stained for GFP, RFP, PAX6, VSX2, or BRN3A. DHS-4D lineage labeled cells (RFP+) contribute mostly to photoreceptor and bipolar cell fates. Arrows mark double labeled cells. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer. Scale bars: 100μm; 25μm for insets.

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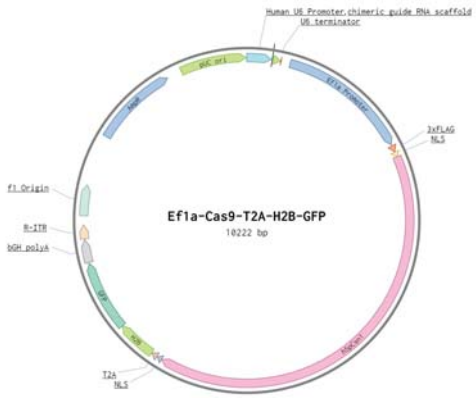

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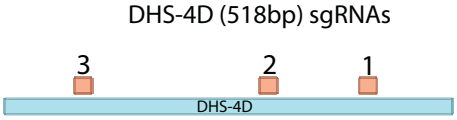

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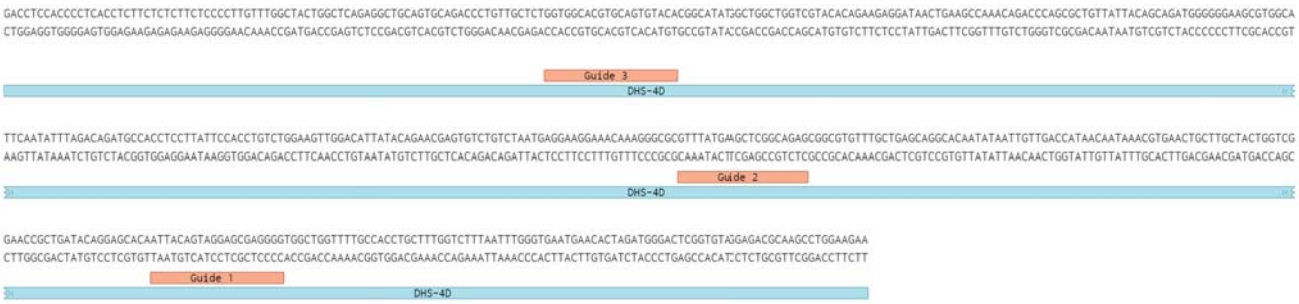

Figure S3: CRISPR/Cas9 DHS-4D knockout strategy. (A) Plasmid schematic of a modified PX458 CRISPR/Cas9 targeting construct. (B) Relative locations of CRISPR/Cas9 guides targeting DHS-4D. (C) Sequence base pair level location of CRISPR/Cas9 guides targeting DHS-4D.

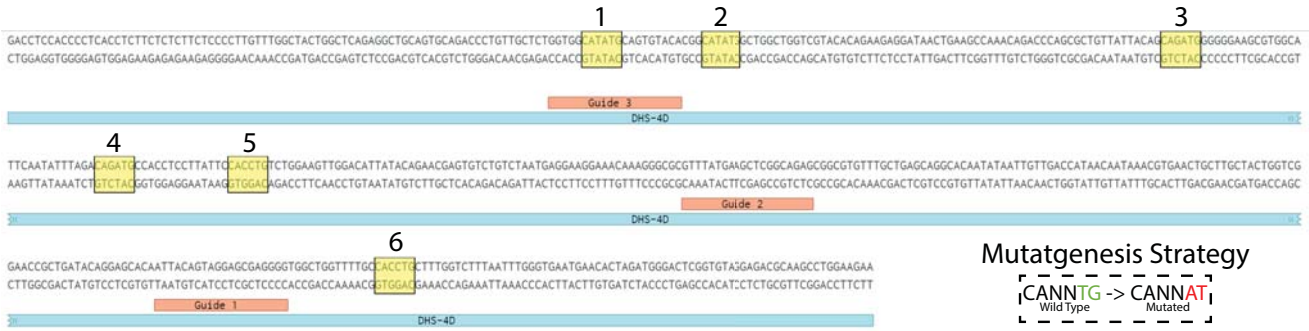

Figure S4: E-box sites within DHS-4D and the mutagenesis strategy. Locations and numbering of the six canonical CANNTG E-box sites identified relative to CRISPR/Cas9 guides. Mutagenesis strategy shown for reference.

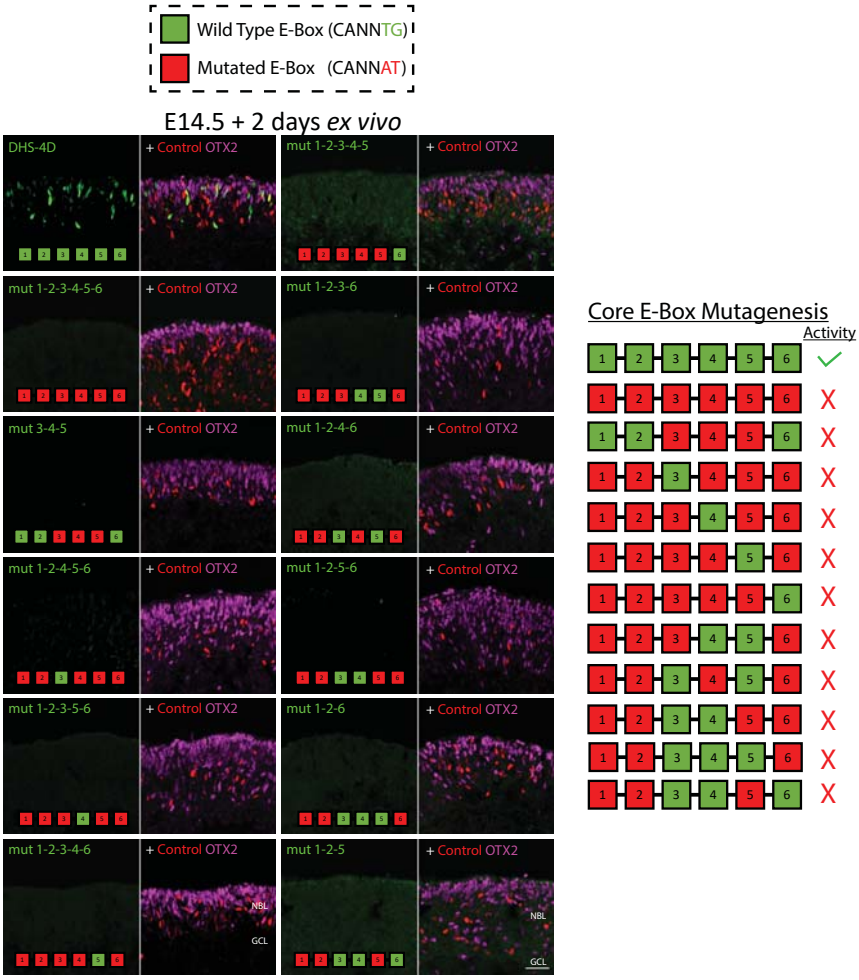

Figure S5: Core E-box mutagenesis series. Representative histology and summary of activity displayed by each mutant construct in the DHS-4D Core E-box mutagenesis strategy. E14.5 explants were electroporated and analyzed after two days of culture. Explants are stained for OTX2, GFP, and RFP. The X marks indicate constructs that lack activity or specificity. NBL = neuroblastic layer, GCL = ganglion cell layer. Scale bars = 100μm.

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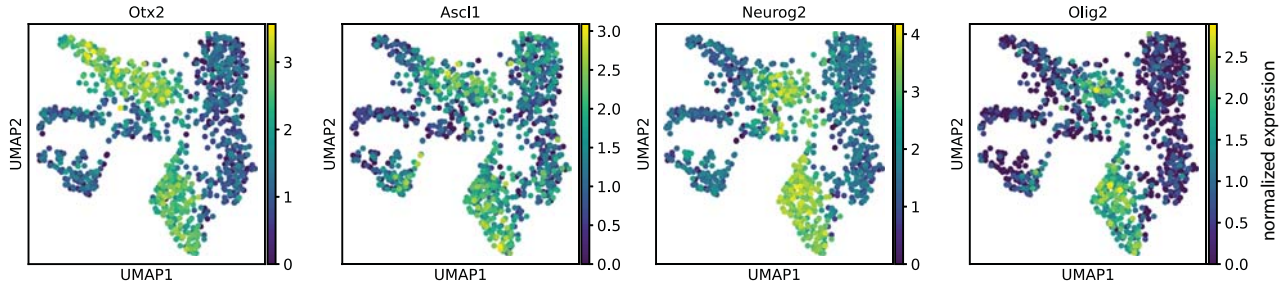

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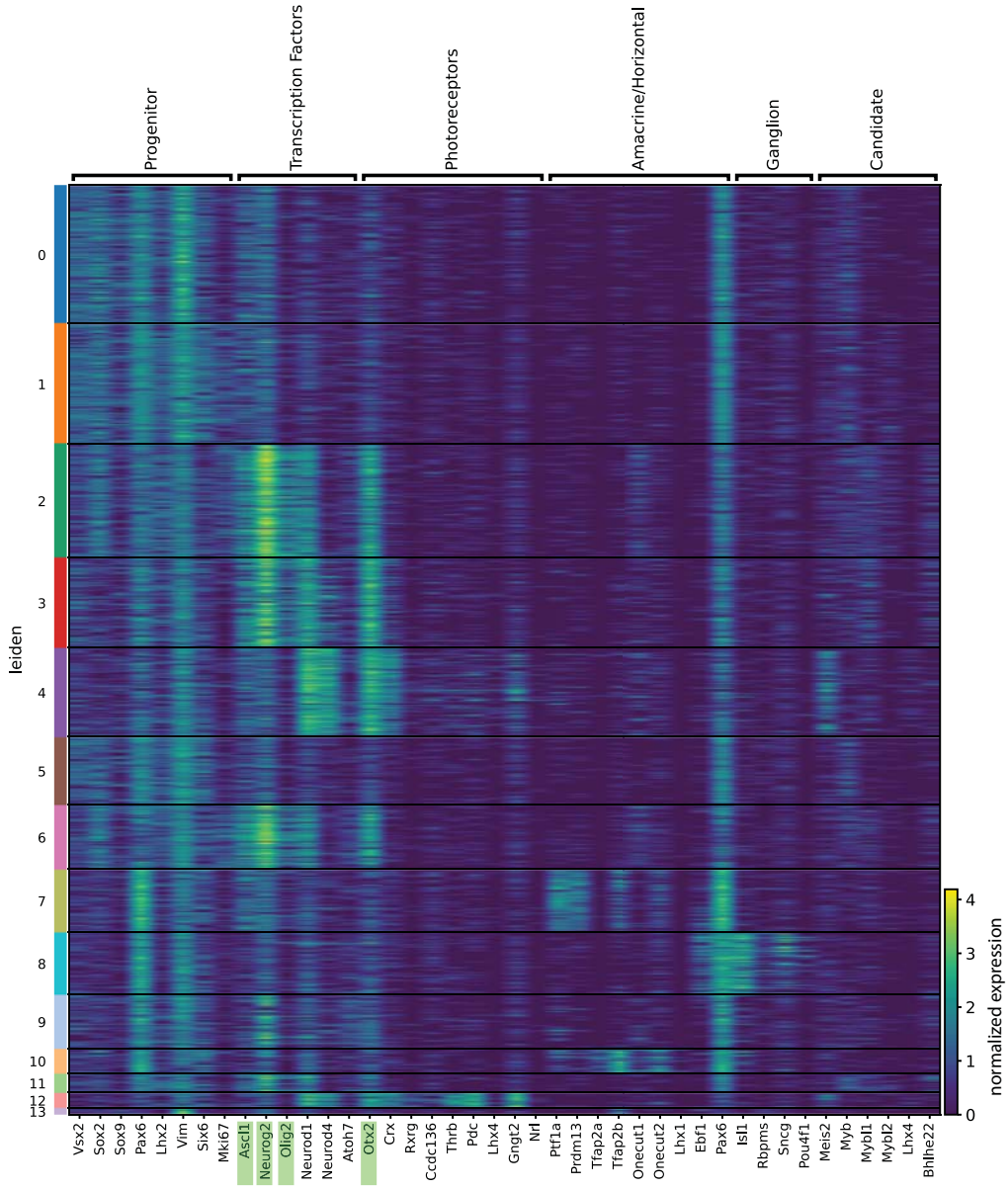

Figure S6: Additional single cell RNA sequencing findings. (A) UMAP plots with normalized expression values of *Otx2*, *Ascl1*, *Neurog2*, and *Olig2*. Note the high degree of overlap between these four transcription factors in UMAP space. (B) Heatmap representation of normalized expression of known marker genes and candidate transcription factor genes across leiden clusters. *Otx2*, *Ascl1*, *Neurog2*, and *Olig2* highlighted for reference.

A

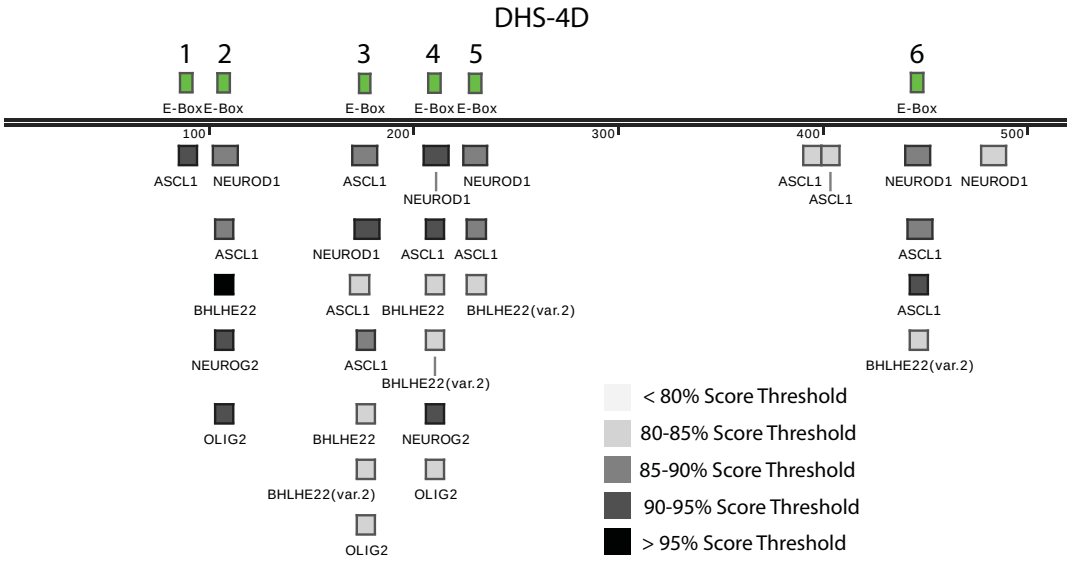

Figure S7: JASPAR transcription factor binding site analysis of DHS-4D. (A) Results of JASPAR transcription factor binding site analysis for bHLH transcription factors on the DHS-4D sequence. E-box placement and numbering are shown for reference. Grayscale gradient of transcription factors represents the JASPAR score threshold.

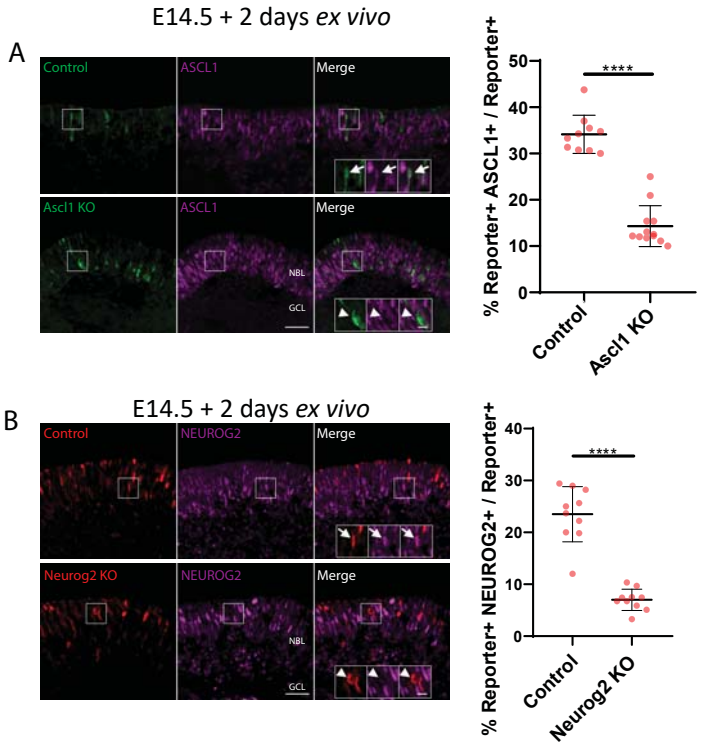

Figure S8: Ascl1 and Neurog2 CRISPR/Cas9 targeting efficiency. (A, B) Representative histology and quantification of non-targeting Controls and either Ascl1 or Neurog2 targeting CRISPR/Cas9 constructs. E14.5 retinas were electroporated with the constructs, grown in culture for two days, and stained for GFP, RFP, ASCL1 or NEUROG2. Arrows mark double labeled cells and arrowheads indicate singly labeled cells. Ascl1 and Neurog2 targeting constructs strongly reduce co-expression with ASCL1 and NEUROG2 proteins compared to Controls. Dots represent quantified images from N=3 retinas. NBL = neuroblastic layer, GCL = ganglion cell layer. Significance determined by two-tailed unpaired t-test. ****P<0.0001. Bars show the mean and s.d. Scale bars: 100μm; 25μm for insets.

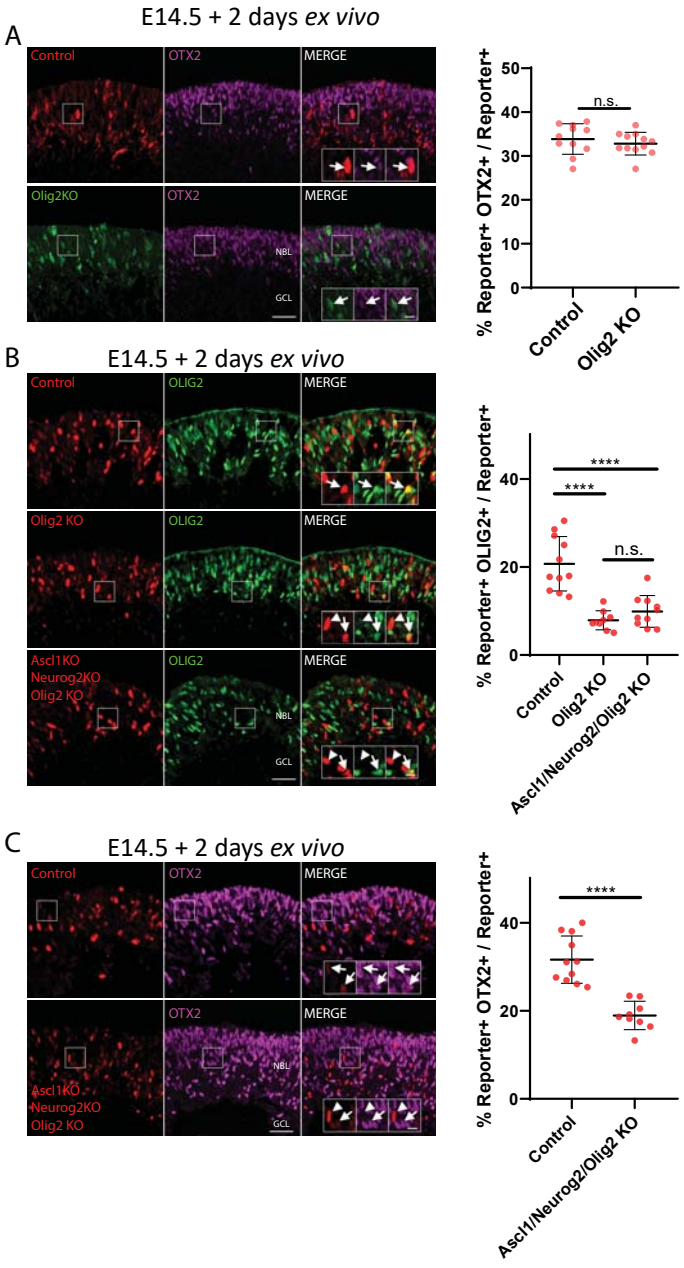

Figure S9: CRISPR/Cas9 knockout of *Olig2* does not inhibit OTX2 expression. (A) Representative histology and quantification of non-targeting Control and *Olig2* targeting CRISPR/Cas9 constructs electroporated into E14.5 retinal explants and cultured for two days. Sections are stained for GFP, RFP, or OTX2. Arrows mark electroporated cells that co-express OTX2. Dots represent quantified images from N=3 retinas. Comparison by two-tailed unpaired t-test. n.s = not significant. (B) Representative histology and quantification of E14.5 retinal explants electroporated with non-targeting Control, *Olig2* targeting, and triple *Ascl1/ Neurog2/Olig2* targeting constructs on OLIG2 expression. Sections are stained for RFP and OLIG2. Comparison by one-way ANOVA. n.s = not significant, ****P<0.0001. Targeting *Olig2* in isolation or as a combination has the same effect on reducing in the number of electroporated OLIG2+ cells. (C) Representative histology and quantification of E14.5 retinal explants electroporated with non-targeting Control and triple *Ascl1/Neurog2/Olig2* targeting constructs on OTX2 expression. Comparison by two-tailed unpaired t-test. n.s = not significant, ****P<0.0001. The triple targeting reduces OTX2 expression in electroporated cells as expected. This reduction is no greater than when *Ascl1* and *Neurog2* are co-targeted (see Figure 5B). Bars show the mean and s.d. NBL = neuroblastic layer, GCL = ganglion cell layer. Scale bars: 100μm; 25μm for insets.

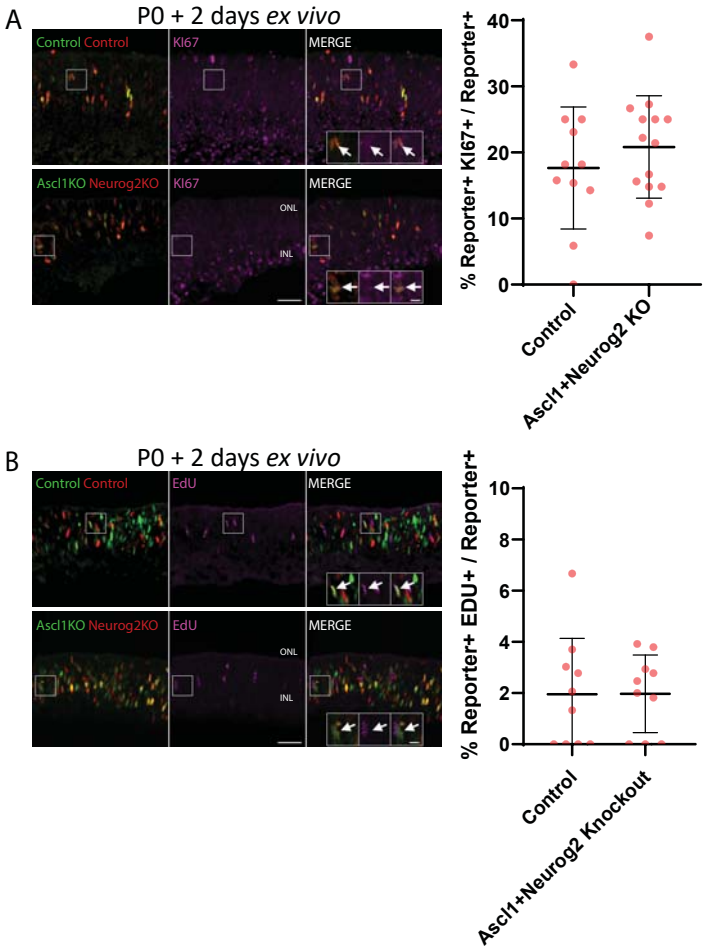

Figure S10: KI67 and EdU labeling is unchanged following Ascl1/Neurog2 double knockout. (A, B) Representative histology and quantification of non-targeting Controls and Ascl1/Neurog2 double targeting CRISPR/Cas9 constructs electroporated at E14.5 and collected after two days of culture. Sections are stained for GFP, RFP, Ki67 or EdU (given 30 minutes before collection). Arrows mark electroporated cells that co-express Ki67 or EdU. Dots represent quantified images from N=3 retinas. Bars show the mean and s.d. ONL = outer nuclear layer, INL = inner nuclear layer. Scale bars: 100μm; 25μm for insets.

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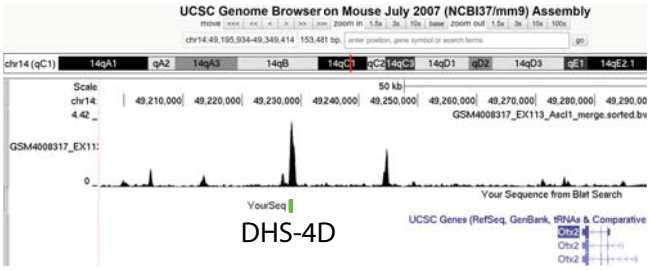

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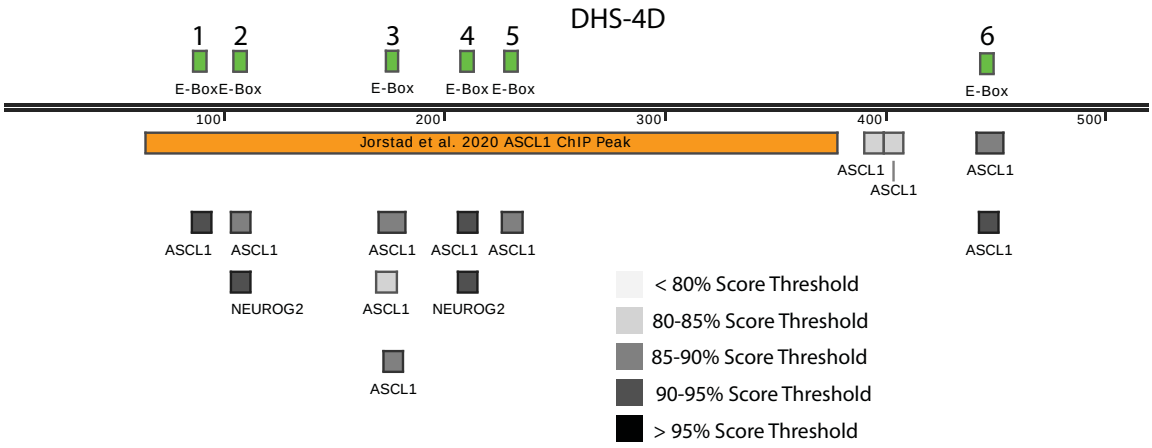

Figure S11: ASCL1 likely binds DHS-4D in the newborn retina. (A) Screenshot of the UCSC Genome Browser displaying the P0 retina ASCL1 ChIP-seq track from Jorstad et al. with DHS-4D highlighted (YourSeq) in green. There is a prominent ASCL1 peak centered on DHS-4D. (B) The DHS-4D sequence with JASPAR identified ASCL1 and NEUROG2 binding sites, numbered CANNTG E-Boxes, and the footprint of the Jorstad et al. ASCL1 ChIP-seq peak in orange. It is likely that ASCL1 binds one or more of the E-boxes in DHS-4D.

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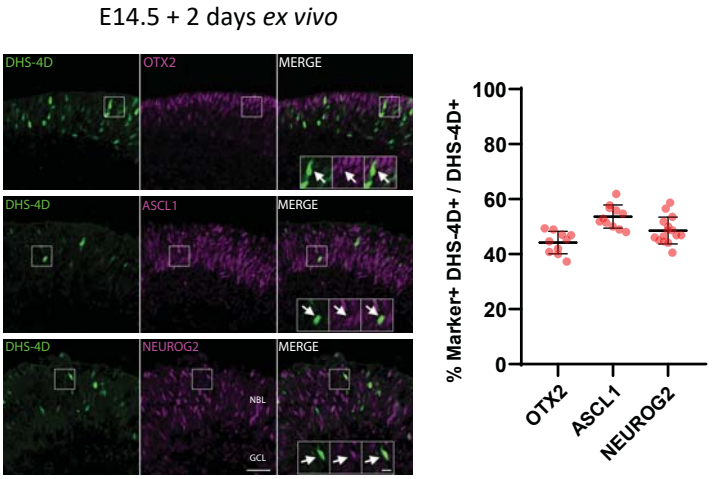

Figure S12: DHS-4D overlaps with ASCL1 and NEUROG2. (A) Histology and quantification of E14.5 retinal explants electroporated with DHS-4D TATA GFP constructs collected after 2 days of culture. Panels show co-staining of GFP with OTX2, ASCL1, or NEUROG2. About half of the GFP+ cells co-express ASCL1 or NEUROG2. N=3 retinas. Bars show the mean and s.d. NBL = neuroblastic layer, GCL = ganglion cell layer. Scale bars: 100μm; 25μm for insets.

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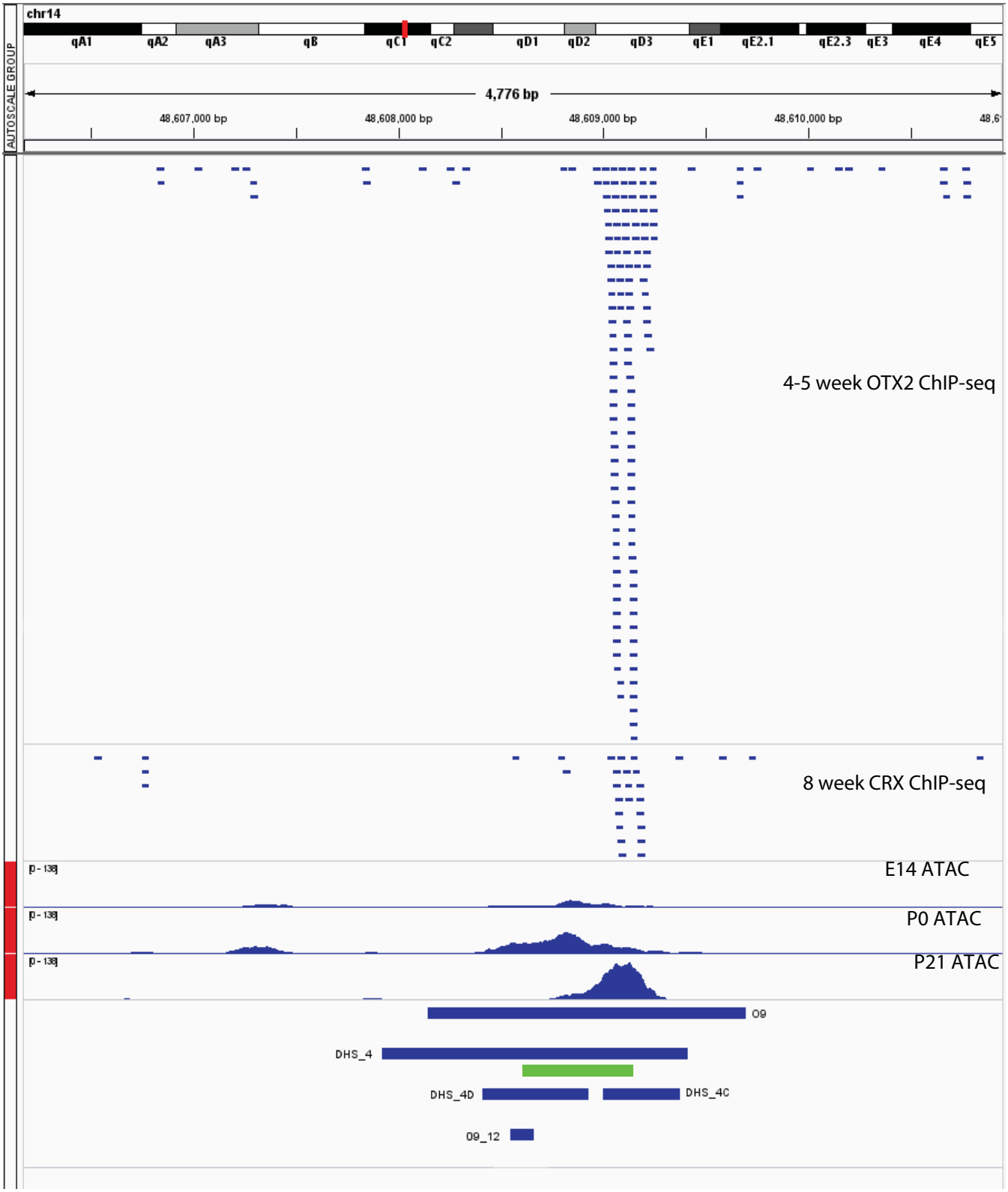

Figure S13: ATAC-seq and ChIP-seq peaks near the DHS-4 enhancer sequence region. (A) Screenshot of the IGV Genome Browser at the DHS-4 enhancer sequence. Tracks include 4-5 week old mouse whole retina OTX2 ChIP-seq, 8 week old mouse whole retina CRX ChIP-seq, and E14/P0/P21 mouse whole retina ATAC-seq. Below are the DHS-4, DHS-4D, DHS-4C, 09, and 09_12 enhancer elements for reference. OTX2 and CRX do not appear to bind in the DHS-4D region.

Table S1: Relevant sequences. Sequence list for DHS-4, sub-elements and CRISPR/ Cas9 targeting guide RNAs.

Click here to download Table S1