
Figures and figure supplements

Lhx2 is a progenitor-intrinsic modulator of Sonic Hedgehog signaling during early retinal neurogenesis

Xiaodong Li et al.

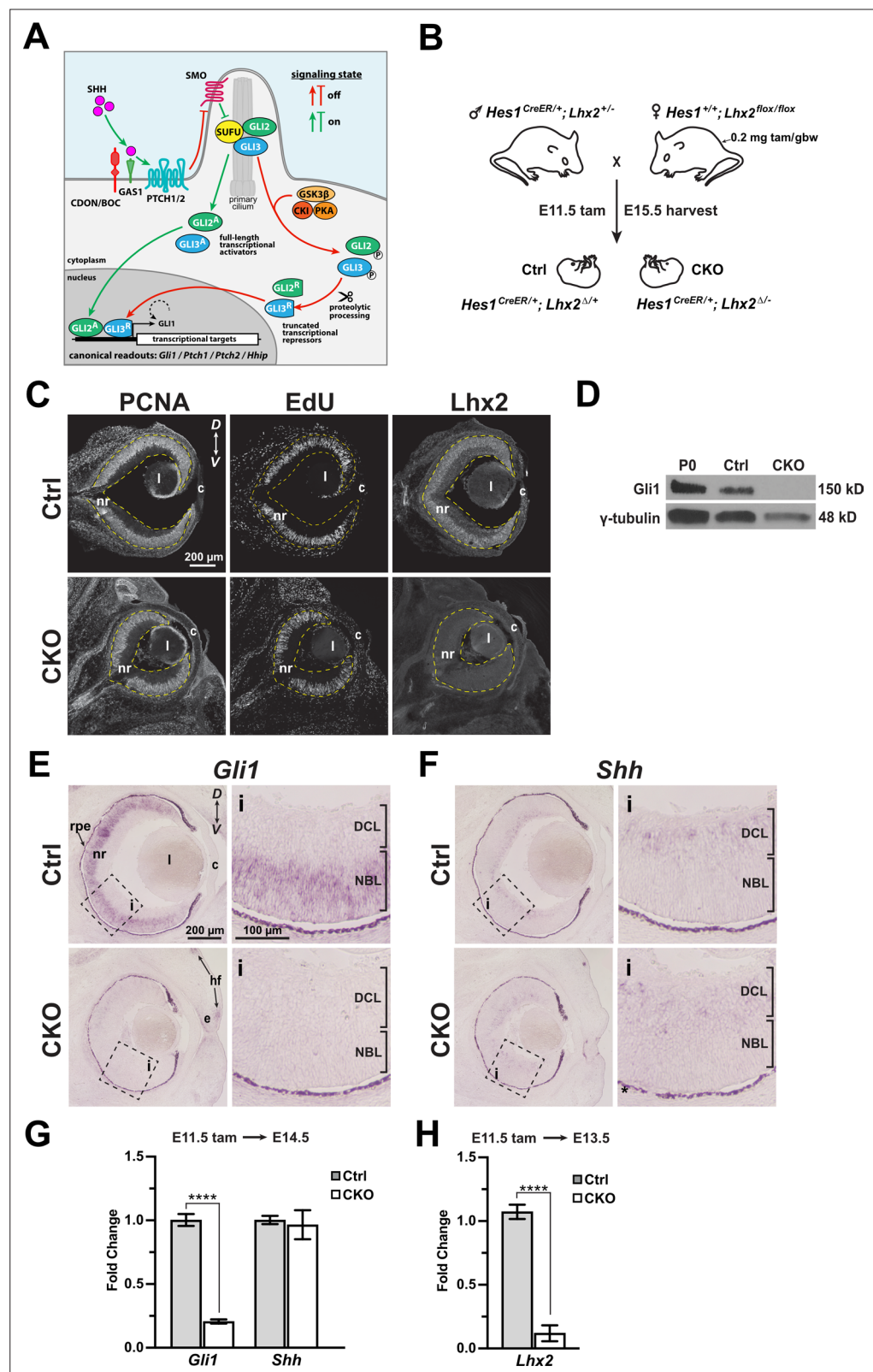


Figure 1. *Lhx2* is required for *Gli1* expression in RPCs. (A) Overview of Hh signaling. See text for details and [Briscoe and Thérond, 2013](#); [Kong et al., 2019](#); [Ramsbottom and Pownall, 2016](#) for more comprehensive pathway illustrations and descriptions. (B) Genetics and tamoxifen treatment paradigm. Female breeders are also homozygous for *Rosa*^{ai14/ai14} and all embryos are *Rosa*^{ai14/+}, allowing rapid screening for recombined embryos

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with tdTomato expression. Recombined *flox* alleles are indicated by Δ . **(C)** Immunohistology for markers of RPC proliferation (PCNA, EdU) and *Lhx2* expression in the E15.5 control and CKO eyes after tamoxifen treatment at E11.5. The retina is contained within the yellow dashed lines. **(D)** Western blot for *Gli1* protein expression in P0 wild type, E15.5 Ctrl and CKO retinas following tamoxifen treatment at E11.5. γ -Tubulin served as an internal loading control. **(E, F)** in situ hybridizations for *Gli1* expression in E15.5 Ctrl eyes following tamoxifen treatment at E11.5. **(F)** In situ hybridizations for *Shh* expression in E15.5 Ctrl and CKO eyes following tamoxifen treatment at E11.5. Dashed boxes reveal locations of close-up images **(i)**. Dark appearance of the rpe is due to natural pigmentation and does not indicate gene expression. **(G)** qPCR for *Gli1* and *Shh* expression in Ctrl and CKO retinas at E14.5 following tamoxifen at E11.5 (mean \pm /-S.E.M.; n=4 (Ctrl: *Gli1*); n=6 (CKO: *Gli1*); n=6 (Ctrl: *Shh*); n=8 (CKO: *Shh*); ****, $p_{\text{adj}}=5\times 10^{-6}$; unpaired t-tests with multiple comparisons correction; see **Supplementary file 3** for statistics). **(H)** qPCR for *Lhx2* expression in Ctrl and CKO retinas at E13.5 following tamoxifen at E11.5 (mean \pm /-S.E.M.; n=4 (Ctrl); n=4 (CKO); ****, $p<0.0001$; unpaired t-test; see **Supplementary file 3** for statistics). Abbreviations: D, dorsal; V, ventral; nr, neural retina; rpe, retinal pigment epithelium; l, lens; c, cornea; e, eyelid; hf, hair follicle; DCL, differentiated cell layer; NBL, neuroblast layer.

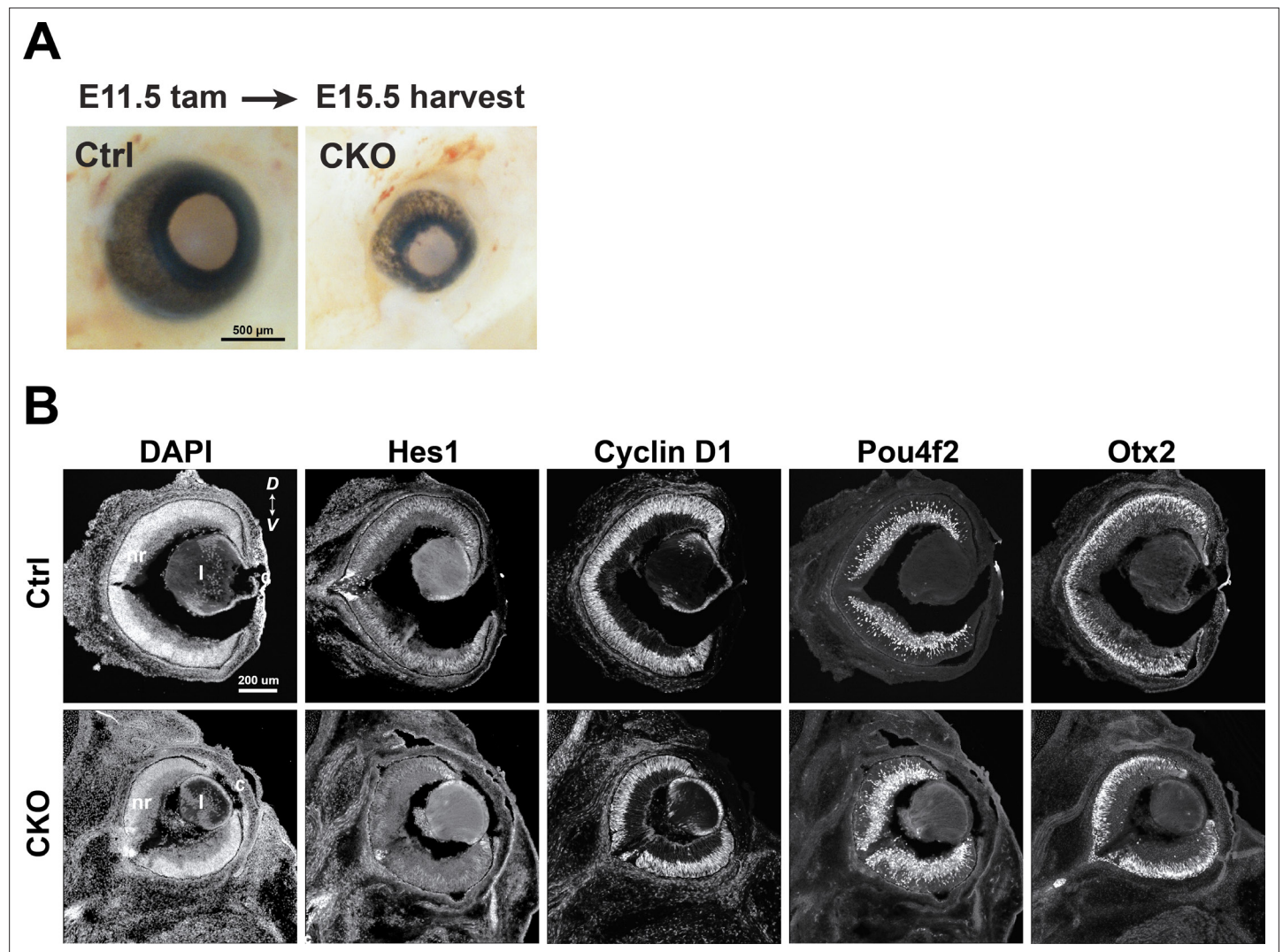


Figure 1—figure supplement 1. Immunohistology for *Lhx2* CKO retina at E15.5 following tamoxifen treatment at E11.5. **(A)** Relative eye sizes from control (left) and CKO (right) embryos. **(B)** Comparative immunohistology for control (top row) and CKO eyes (bottom row) showing tissue organization with DAPI, the RPC marker and progenitor maintenance factor *Hes1*, the RPC marker *Cyclin D1*, the RGC marker *Pou4f2*, and the photoreceptor precursor marker *Otx2*. Although the retina is noticeably smaller in the CKO compared to control, only *Hes1* shows a qualitative change in expression at E15.5, 4 days after tamoxifen treatment.

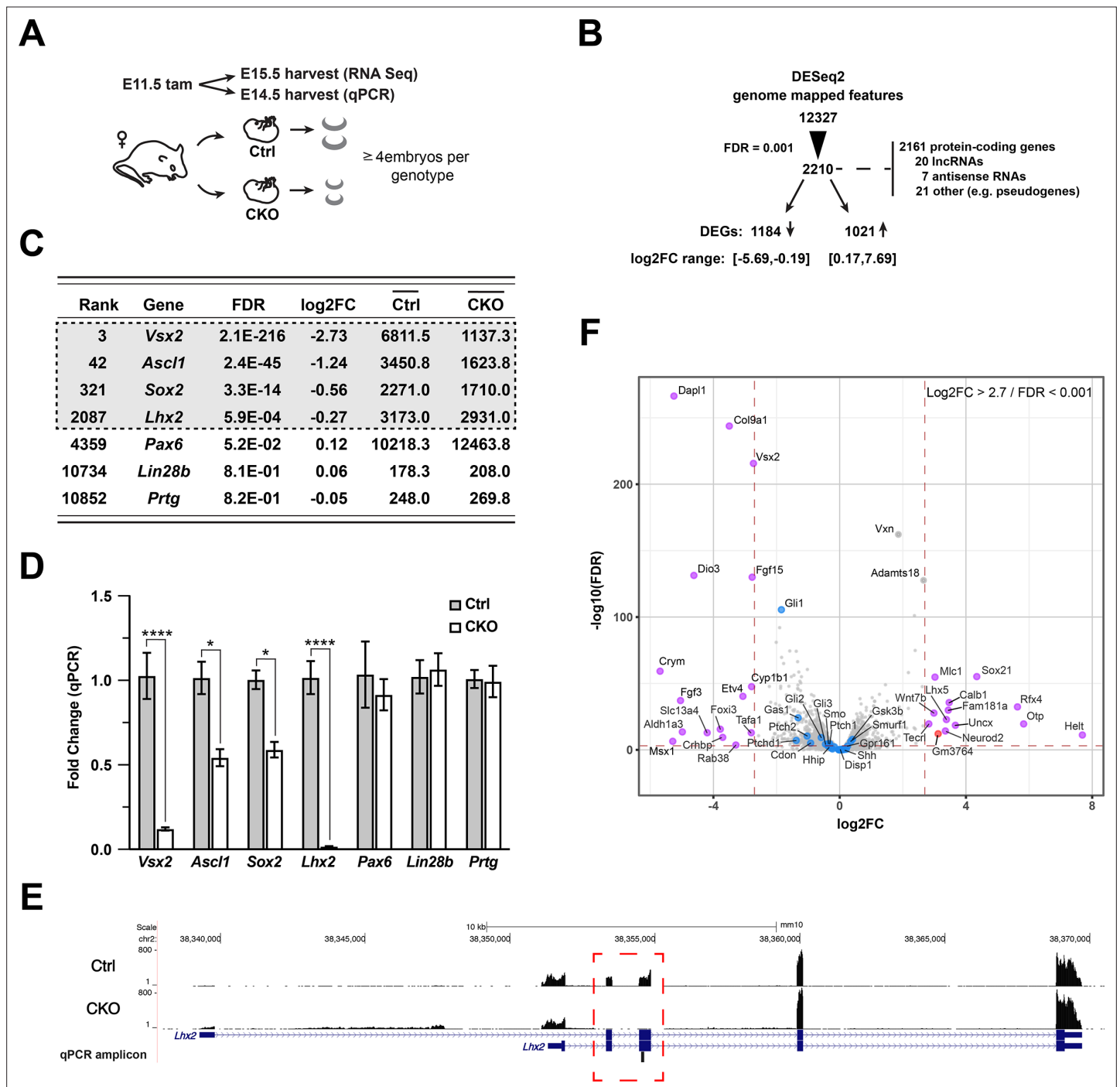


Figure 2. Gene expression changes due to *Lhx2* inactivation. **(A)** Schematic of experimental design for RNA sequencing and qPCR. **(B)** Summary of DESeq2 analysis of RNA sequencing datasets. **(C)** DESeq2-derived statistics for progenitor genes with requirements during early retinal neurogenesis. Genes in gray box were within the 0.001 FDR cutoff for differential expression. Averaged counts per gene are shown in the last two columns. **(D)** Relative expression for progenitor genes at E14.5 by qPCR as a function of the fold change from the mean of control for each gene. Only significant comparisons are noted (mean \pm S.E.M.; *, $p_{adj} < 0.05$; ****, $p_{adj} < 0.0001$; n=4 (Ctrl: *Vsx2*, *Ascl1*, *Lhx2*); n=3 (Ctrl: *Sox2*, *Pax6*); n=6 (Ctrl: *Lin28b*, *Prtg*); n=6 (CKO: *Vsx2*, *Ascl1*, *Lhx2*); n=4 (CKO: *Sox2*, *Pax6*); n=8 (CKO: *Lin28b*, *Prtg*); unpaired t-tests with multiple comparisons correction; see **Supplementary file 3** for statistics). **(E)** Coverage plot showing the mutant transcript is expressed and detected by RNA sequencing, indicating that nonsense mediated decay of mutant transcript is not occurring. The lack of reads in exons 2 and 3 (red box) of the CKO reveal the high degree of conditional deletion. Plots are the mean of counts for all samples. The qPCR amplicon located in the deleted exon 3, making the mutant transcript undetectable by qPCR with the Taqman probe used in the study. **(F)** Volcano plot showing selected Shh pathway genes (blue dots) relative to other DEGs. The most divergent DEGs gated on FDR (0.001) and absolute log2FC (2.7) cutoffs (red dashed lines) are highlighted (purple: mRNAs; red: lncRNA; gray: filtered out GMFs).

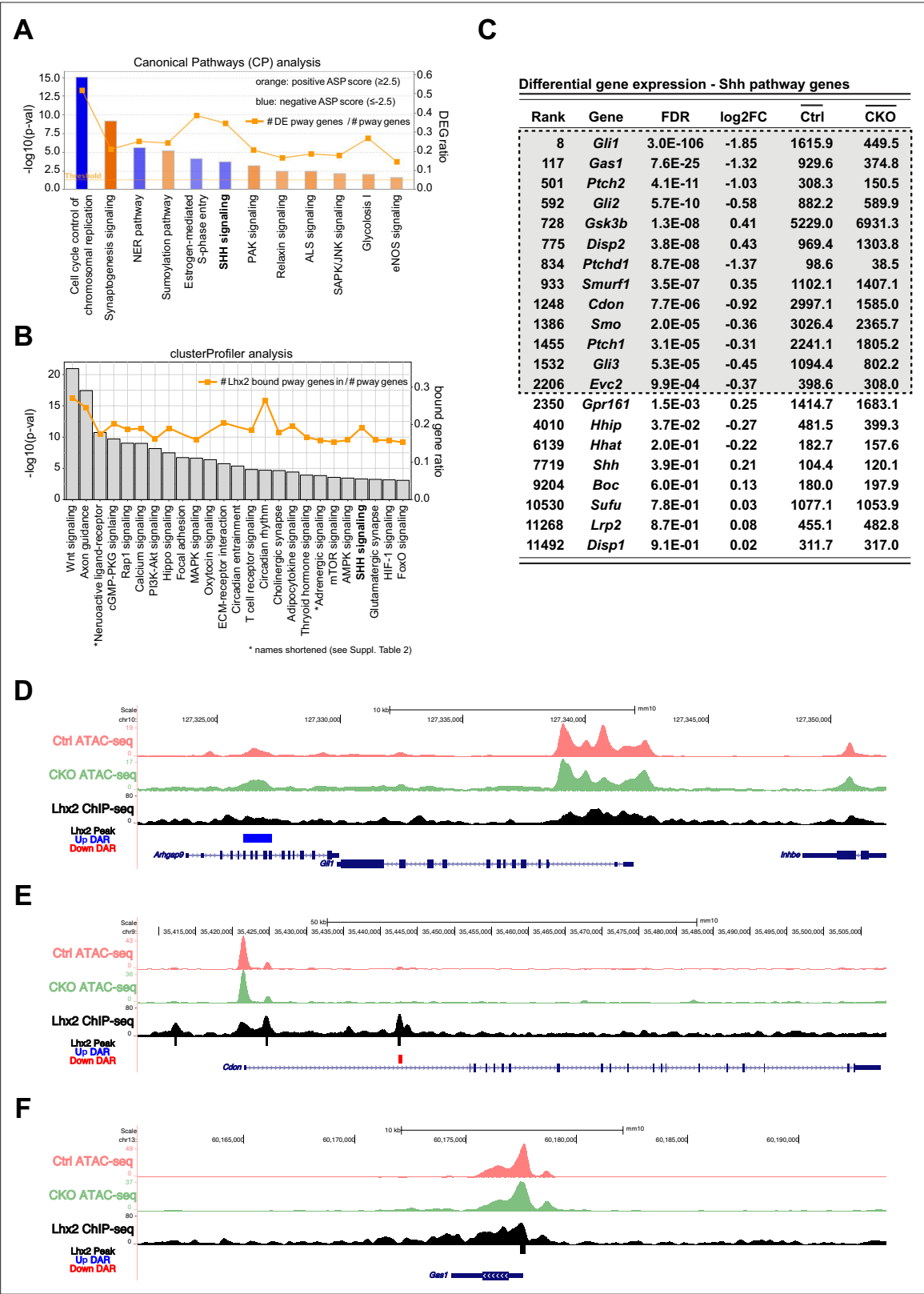
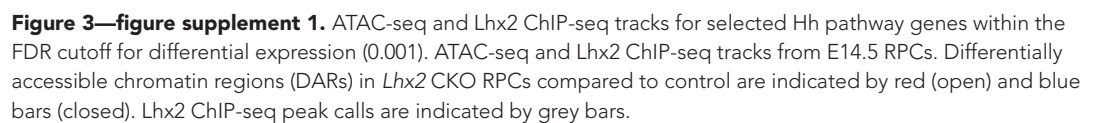


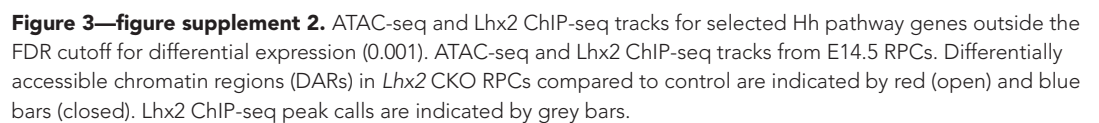
Figure 3. Lhx2 is required for the expression of multiple Hedgehog pathway genes. **(A)** Canonical Pathways (CP) analysis for DEGs with an FDR of 0.001 or smaller. Pathways that surpassed the significance cutoff of 1.3 (x-axis) and have an absolute ASP score of 2.5 or higher are shown. Orange bars predict pathway activation and blue bars predict pathway inhibition. Color intensity is directly correlated to ASP score (scores are listed in **Supplementary file 2**). The line indicates the number of DEGs found in each pathway as a fraction of pathway genes (DEG ratio; right y-axis). **(B)** KEGG pathways associated

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with Lhx2 ChIP-seq peak distribution from E14.5 mouse RPCs using ClusterProfiler. The line indicates the number of genes associated with Lhx2 chromatin binding in each pathway as a fraction of pathway genes (bound gene ratio; right y-axis). **(C)** Differential gene expression values for canonical genes in the Shh pathway. Genes in the gray box passed the cutoff for DEG designation. **(D–F)** ATAC-seq and Lhx2 ChIP-seq genomic DNA tracks at *Gli1* **(D)**, *Cdon* **(E)**, and *Gas1* **(F)** loci from E14.5 RPCs. DARs identified in *Lhx2* CKO RPCs are indicated by red and blue bars. Sites of Lhx2 chromatin binding are indicated by black bars from the ChIP-seq data.





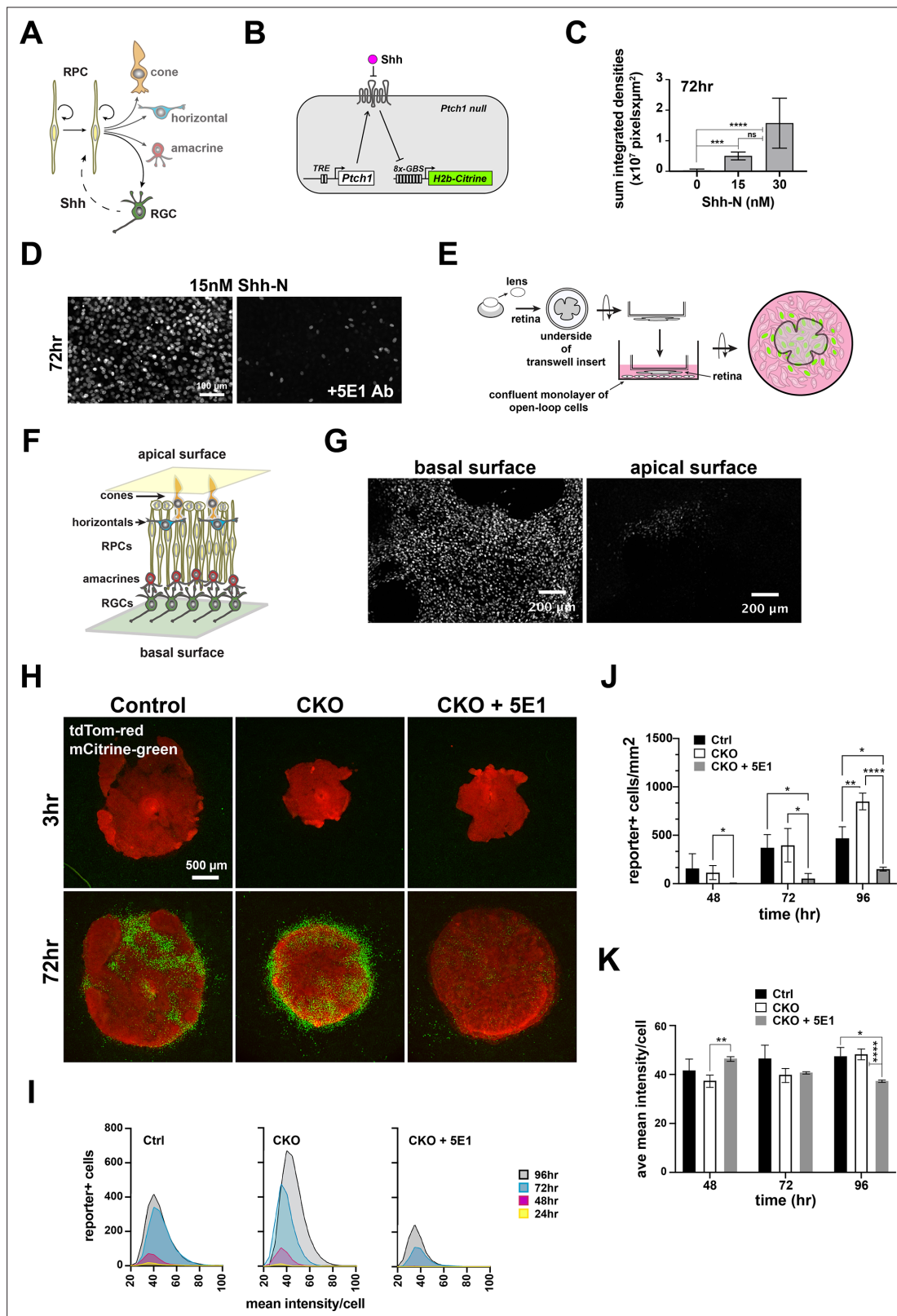


Figure 4. Evaluation of a live cell reporter system to test Shh bioavailability. (A) Intra-lineage architecture of Hh signaling at the start of retinal neurogenesis. RPCs initiate neurogenesis and begin generating RGCs, the Shh producing cells. RPCs are the responder cells, placing Shh upstream and downstream of RPCs. (B) Configuration of the *open-loop* circuit in the responder NIH3T3 *Ptch1*-null cell line (*open-loop* cells). *Ptch1* is produced from a doxycycline regulated transgene. Shh binds *Ptch1*, activating intrinsic signaling as well as Gli-dependent expression of mCitrine fused to

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Histone H2b through 8-multimerized Gli1 binding sites. **(C)** Dose response at 72 hr as a function of the total accumulation of signal intensities and area coverage of mCitrine + open loop cells (mean \pm S.D.; n=4 per condition; ns, not significant; ***, $p_{\text{adj}} < 0.001$; ****, $p_{\text{adj}} < 0.0001$; ANOVA followed by Tukey's multiple comparisons; see **Supplementary file 4** for statistics). **(D)** Addition of the Shh ligand-blocking monoclonal antibody, 5E1, diminishes mCitrine expression. **(E)** Design of coculture experiment. Freshly dissected embryonic retina is flat mounted to the underside of a transwell insert and placed into direct contact with a confluent monolayer of open-loop cells. mCitrine expression accumulates in open-loop cells that receive Shh from the retina. **(F)** Schematic cross-section of embryonic retina shows that the apical surface is comprised mainly of RPCs and developing photoreceptors (cones until ~E15.5, and a mix of rods and cones thereafter) and the basal surface is comprised of RGCs, the source of retinal SHH. Astrocytes and endothelial also reside on the basal surface (not shown). **(G)** E18.5 wild type retinas were cultured in opposite orientations such that the open-loop cells contacted the basal or apical surfaces of the retina. mCitrine expression was robustly induced in responder cells in close proximity to the basal surface but not the apical surface of the retina. **(H)** Cocultures for control (left), CKO (middle), and CKO incubated with 5E1 antibody (right) at 3 and 72 hr. Retinal tissues are tdTomato positive (red) and mCitrine positive nuclei are green. **(I)** Lowess-smoothed histograms showing the accumulation and fluorescence intensity distributions of mCitrine + responder cells at each timepoint during the co-culture period. The histograms are for the cocultures shown in A. **(J)** Quantification of mCitrine + cells at 48, 72, and 96 hr. Comparisons were done within timepoints only and the significant differences are shown (mean \pm S.D.; n=4 (Ctrl, all timepoints); n=6 (CKO, all timepoints); n=2 (CKO+5E1, all timepoints); *, $p_{\text{adj}} < 0.05$; **, $p_{\text{adj}} < 0.01$; ****, $p_{\text{adj}} < 0.0001$; 2-way repeated measures ANOVA followed by Tukey's multiple comparisons test; see **Supplementary file 4** for statistics). **(K)** Quantification of the average mCitrine fluorescence intensities per cell at 48, 72, and 96 hr. Comparisons were done within timepoints only and the significant differences are shown (mean \pm S.D.; n=4 (Ctrl, all timepoints); n=6 (CKO, all timepoints); n=2 (CKO, all timepoints); *, $p_{\text{adj}} < 0.05$; **, $p_{\text{adj}} < 0.01$; ****, $p_{\text{adj}} < 0.0001$; 2-way repeated measures ANOVA followed by Tukey's multiple comparisons test; see **Supplementary file 4** for statistics).

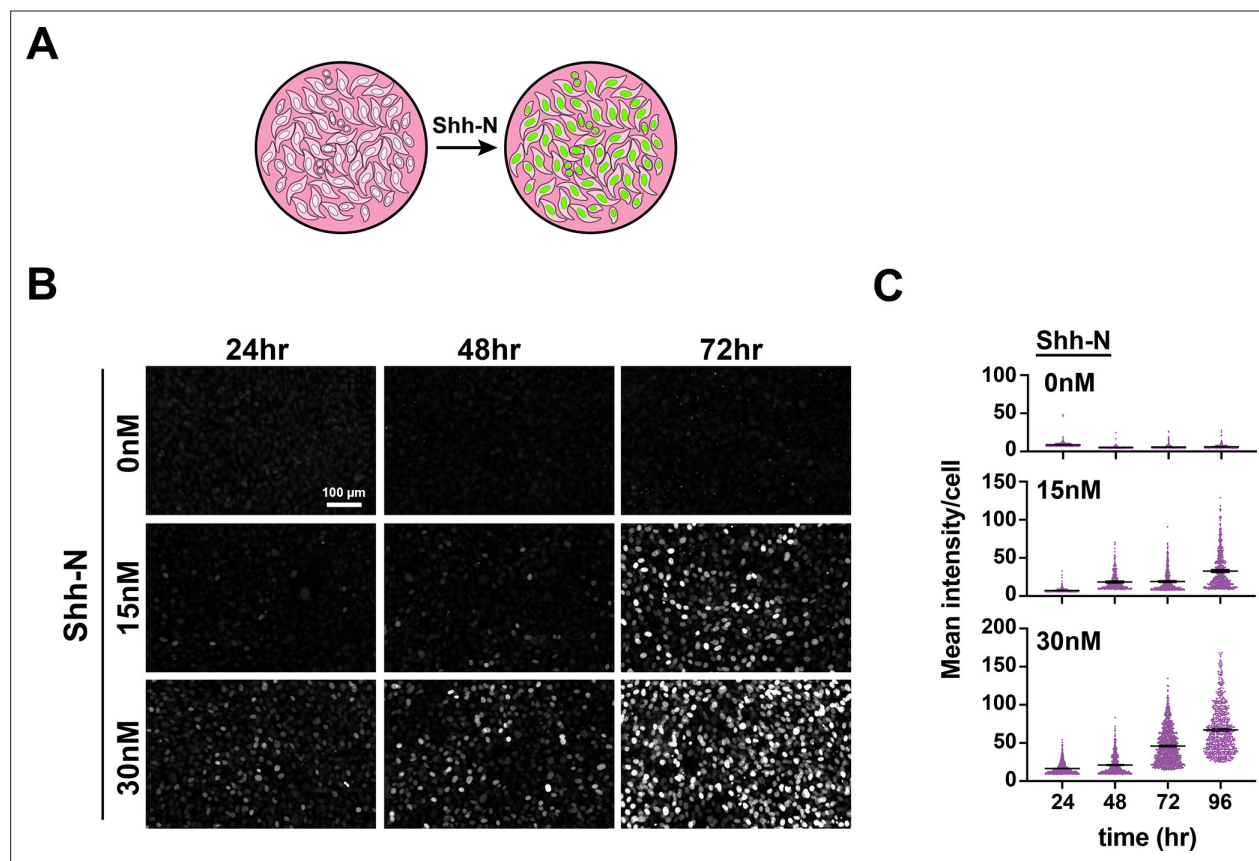


Figure 4—figure supplement 1. Responder cells express mCitrine near the basal surface of the retina. **(A)** Schematic showing that addition of recombinant Shh-N to a confluent monolayer of responder cells activates nuclear mCitrine expression. **(B)** Dose response and time course for accumulation of mCitrine expression in *open-loop* responder cells. Fields were randomly selected from the same cultures for each timepoint. **(C)** Scatter plots showing the accumulation of mCitrine+ cells over time for each dose tested as a function of average mean fluorescence intensity per cell. Means are shown for each condition (lines).

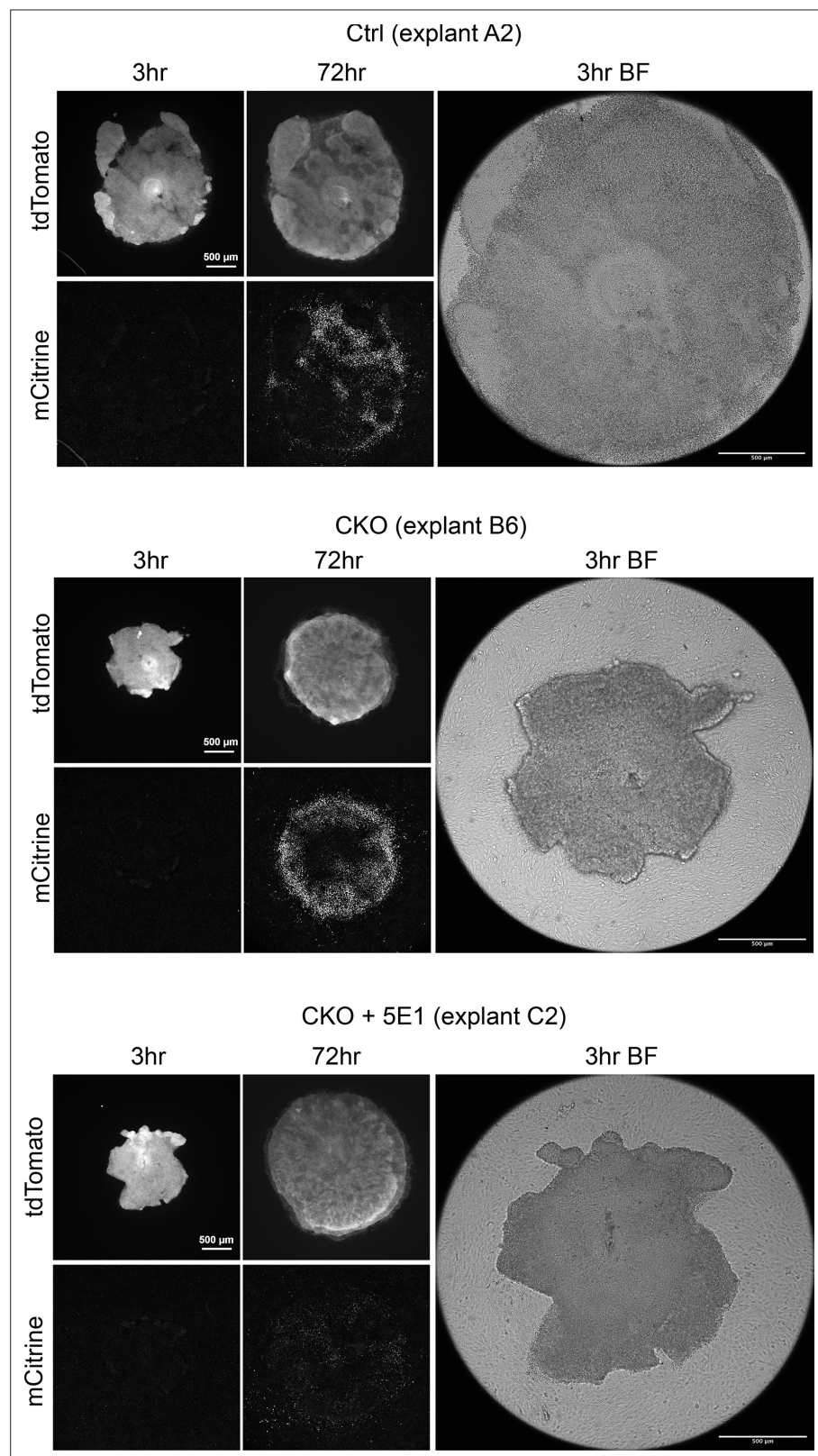


Figure 4—figure supplement 2. Single channel images Single channel fluorescence and bright field images for the cocultures shown in **Figure 5B**. .

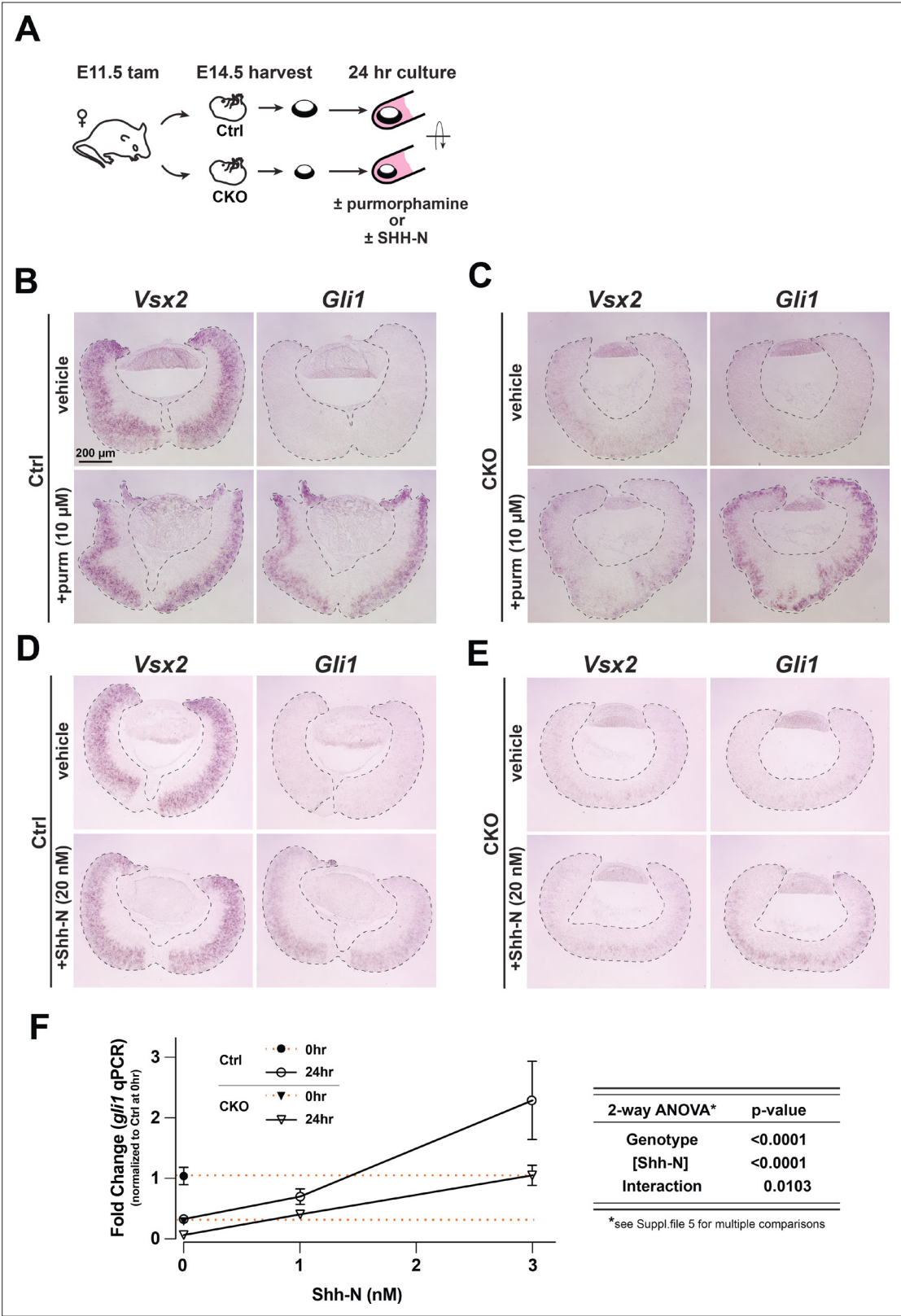


Figure 5. Purmorphamine and recombinant Shh-N stimulate signaling in *Lhx2* CKO retinal explants. **(A)** Experimental design of ex vivo retina-lens explant cultures. **(B–E)** in situ hybridizations of *Vsx2* and *Gli1* expression after 24 hr in culture to test *Smo* agonist purmorphamine or recombinant Shh-N. **(B)** *Gli1* expression declines in the absence of purmorphamine (vehicle) whereas *Vsx2* is maintained. *Gli1* expression is restored with purmorphamine. **(C)** *Vsx2* declines due to *Lhx2* inactivation. *Gli1* is expressed in response to purmorphamine. **(D)** *Gli1* expression declines in the absence of Shh-N

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(vehicle) whereas *Vsx2* is maintained. *Gli1* is restored with Shh-N. (E) *Vsx2* declines due to *Lhx2* inactivation. Similar to purmorphamine, *Gli1* expression is upregulated with Shh-N. (F) qPCR-based *Gli1* expression in control and CKO retinal explants at the start of the culture (t=0) and after 24 hr at different concentrations of Shh-N, as determined from a pilot dose response with wild type retinal explants (**Figure 5—figure supplement 1C, Supplementary file 5**). Expression values are relative to the mean control value at t=0 (closed circle). Orange lines extend from t=0 values for the control (upper line) and the CKO (closed triangle, lower line). Note that the value for the 24 hr control in 0 nM Shh-N overlaps with the CKO at t=0. To the right of the graph is the summary table for two-way ANOVA showing that the main effects (genotype and Shh-N concentration) are significant and interact. mean+/-S.E.M.; n=4 all conditions except CKO, 1nM Shh-N (n=3); Two-way ANOVA followed by Tukey's multiple comparisons test; See **Supplementary file 5** for statistics including p_{adj} values for multiple comparisons.

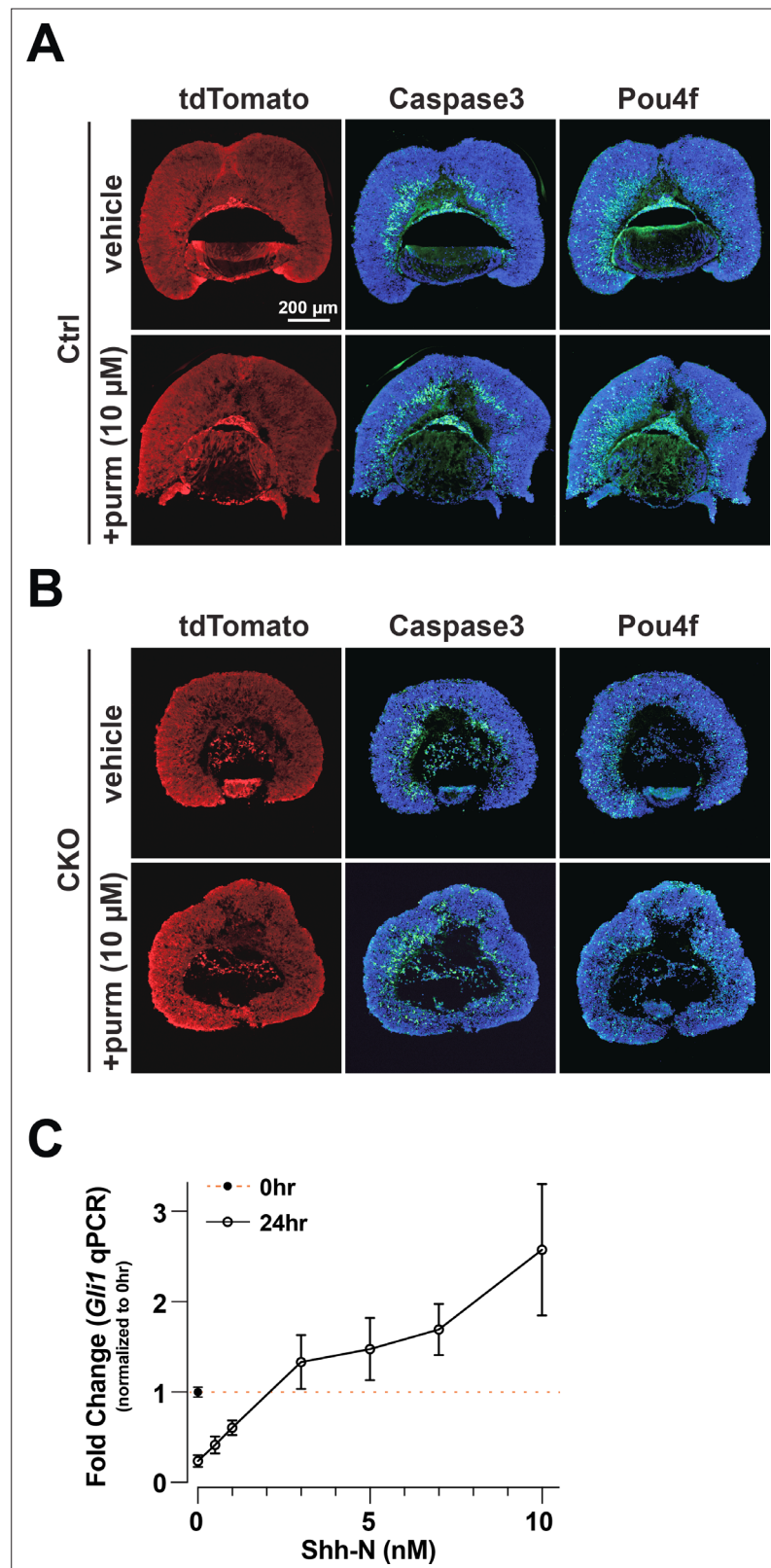


Figure 5—figure supplement 1. Markers of recombination, apoptosis, and RGCs after 24 hr, and dose response to estimate physiological range for recombinant Shh-N(C24II) protein. (A) Control and (B) CKO explants treated with vehicle or 10 nM purmorphamine for 24 hr. Left column: tdTomato expression from heterozygous *ai14* allele reveals CreER activity throughout the explants. Middle column: Caspase 3 immunofluorescence (green) reveals

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extent of apoptotic cells. Nuclei are stained with DAPI (blue). Right column: Pou4f2 immunofluorescence (green) marks RGCs. **(C)** Relative *Gli1* expression in wild type E15.5 retinas treated with increasing concentrations of recombinant Shh-N. Data points are relative to the mean expression value from retinas harvested at the start of the experiment (t=0; orange line). Means and standard deviation are shown, and results are from a pilot experiment (n=3 for all conditions; One way ANOVA followed by Dunnett's multiple comparisons test; see **Supplementary file 5** for statistics including p_{adj} values for multiple comparisons).

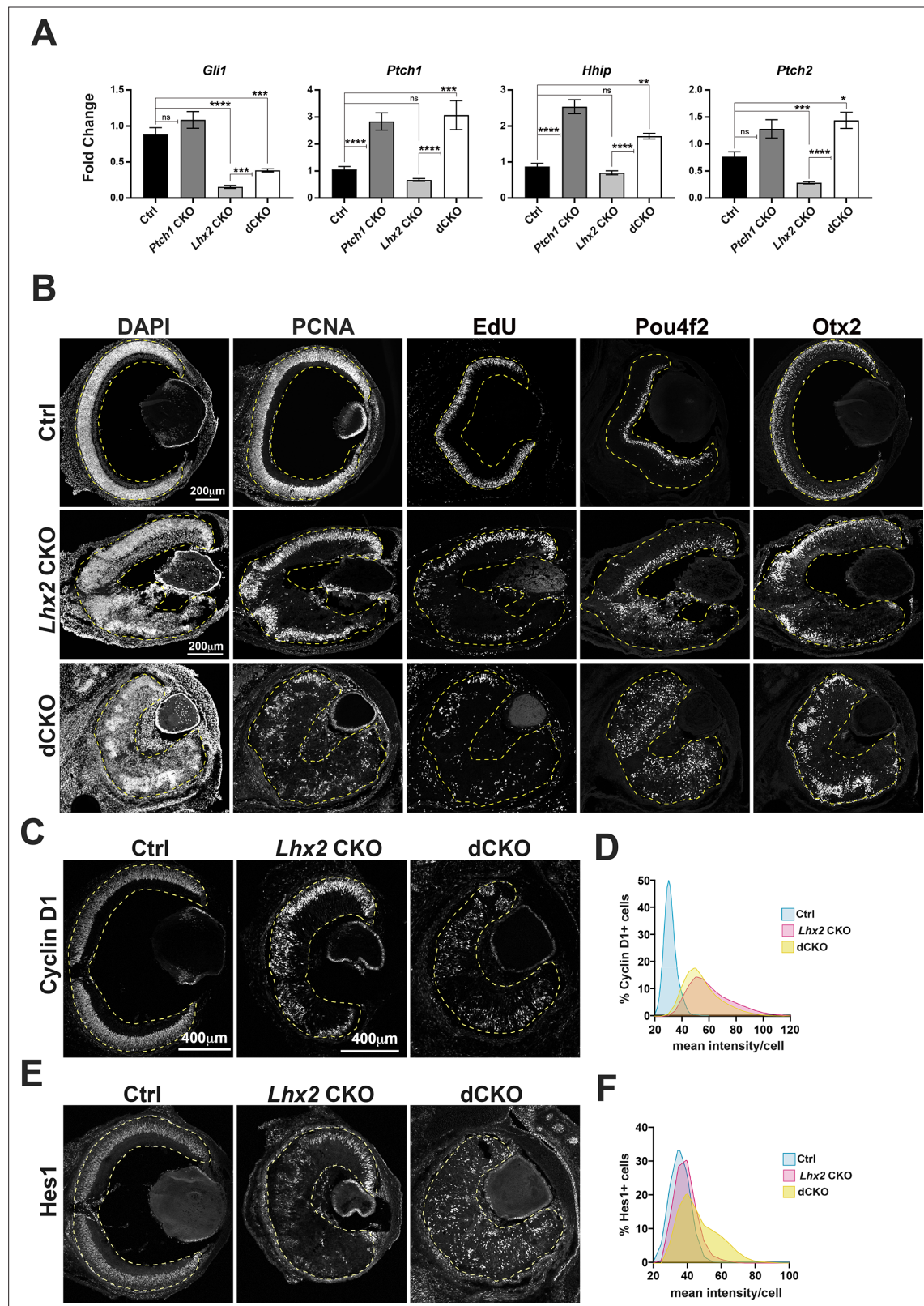


Figure 6. Hh signaling is enhanced in *Lhx2*-deficient RPCs by *Ptch1* inactivation in vivo. **(A)** Relative expression of *Gli1*, *Ptch1*, *Hhip*, and *Ptch2* at E15.5 following tamoxifen treatment at E11.5 in retinas with the following genotypes: control, *Ptch1* CKO, *Lhx2* CKO and *Lhx2*; *Ptch1* double CKO (dCKO). See **Figure 6—figure supplement 1A** for breeding scheme and genotypes assigned to control. For each gene, fold change values are relative to a specific control sample set as a reference. Shown are the comparisons for the three mutant genotypes compared to control and for the dCKO compared to the

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Lhx2 CKO (means \pm S.E.M.; n=6 (Ctrl, all genes); n=9 (*Ptch1* CKO, all genes); n=7 (*Lhx2* CKO, all genes); n=4 (dCKO, all genes); ns, not significant; * $p_{\text{adj}} < 0.05$; ** $p_{\text{adj}} < 0.01$; *** $p_{\text{adj}} < 0.001$; **** $p_{\text{adj}} < 0.0001$; ANOVA followed by Tukey's multiple comparisons test; see **Supplementary file 6** for statistics and complete multiple comparisons list). **(B)** DAPI staining and expression patterns for PCNA and EdU incorporation to identify RPCs and Pou4f2 and Otx2 to identify nascent RGCs and photoreceptors at E18.5 following tamoxifen treatment at E11.5 in retinas from control (top row), *Lhx2* CKO (middle row) and dCKO (bottom row). **(C)** Cyclin D1 expression at E18.5 following tamoxifen treatment at E11.5 in control, *Lhx2* CKO and dCKO retinas. **(D)** Lowess-smoothed histograms showing the distribution of Cyclin D1 + cells as a function of the mean fluorescence intensity per cell. Each histogram is normalized to the number of Cyclin D1 + cells within the respective genotype. **(E)** Hes1 expression at E18.5 following tamoxifen treatment at E11.5 in control, *Lhx2* CKO and dCKO retinas. **(F)** Lowess-smoothed histograms showing the distribution of Hes1 + cells as a function of the mean fluorescence intensity per cell. Each histogram is normalized to the number of Hes1 + cells within the respective genotype.

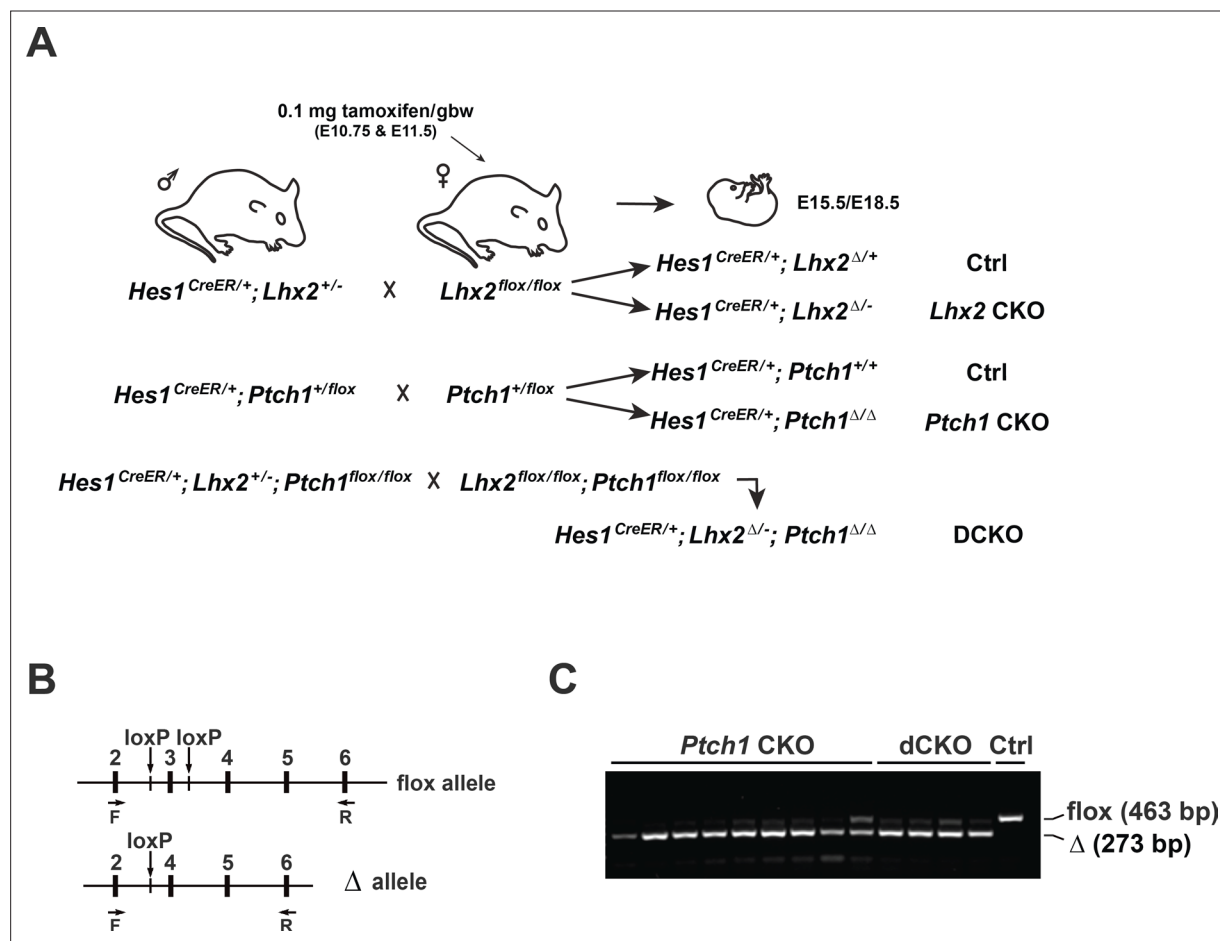


Figure 6—figure supplement 1. Genetics of *Ptch1* and *Lhx2* inactivation and validation of *Ptch1* recombination. **(A)** Overview of genetics and tamoxifen treatment. **(B)** Schematic of *Ptch1* locus encompassing the regions containing exons 2–6. Exon 2 forward (ex2F) and Exon 6 reverse (ex6R) were used for RT-PCR to detect unrecombined and recombined transcripts from the flox and CKO alleles, respectively. **(C)** Agarose gel shows most samples were recombined (CKO) although some unrecombined (flox) was detected. All samples were used for qPCR.

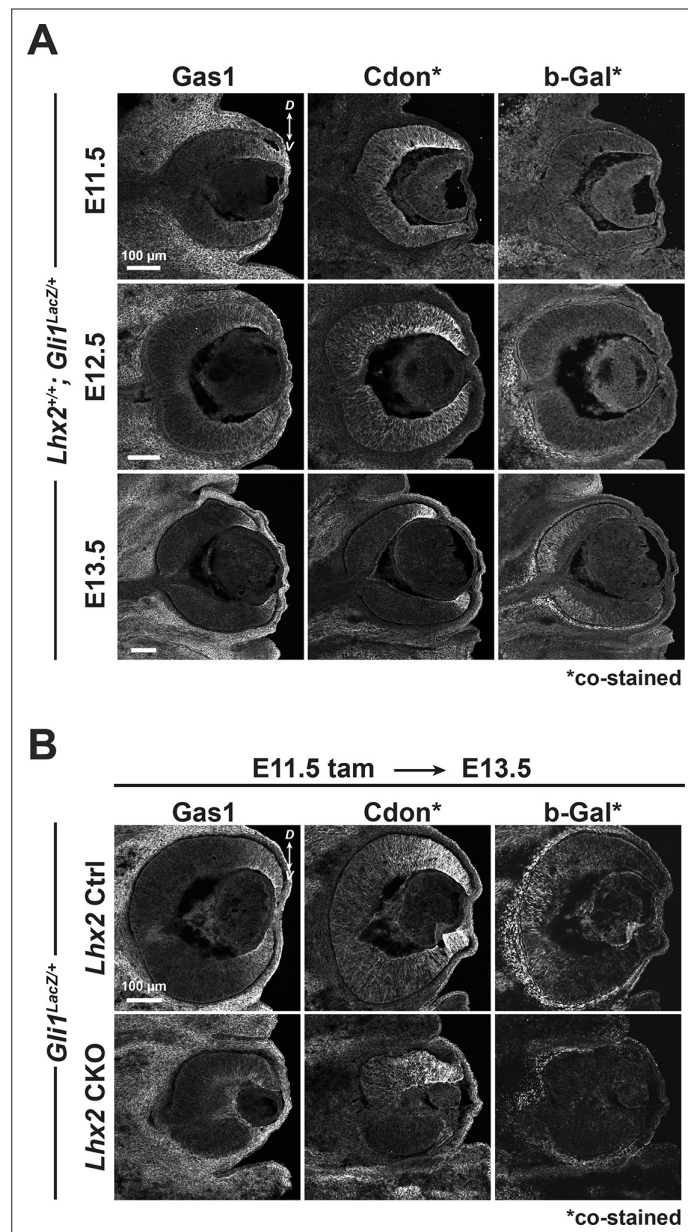


Figure 7. Cdon and Gas1 expression are dependent on Lhx2 prior to their downregulation at the start of Shh signaling. **(A)** Temporal expression patterns of Gas1, Cdon, and β -Gal at E11.5, E12.5, and E13.5 in *Gli1*^{LacZ/+} mice. Cdon and β -Gal were detected on the same tissue sections, Gas1 on adjacent sections (also in B). **(B)** Expression of Gas1, Cdon, and β -Gal in Ctrl and *Lhx2* CKO; *Gli1*^{LacZ/+} eyes at E13.5 following tamoxifen treatment at E11.5.

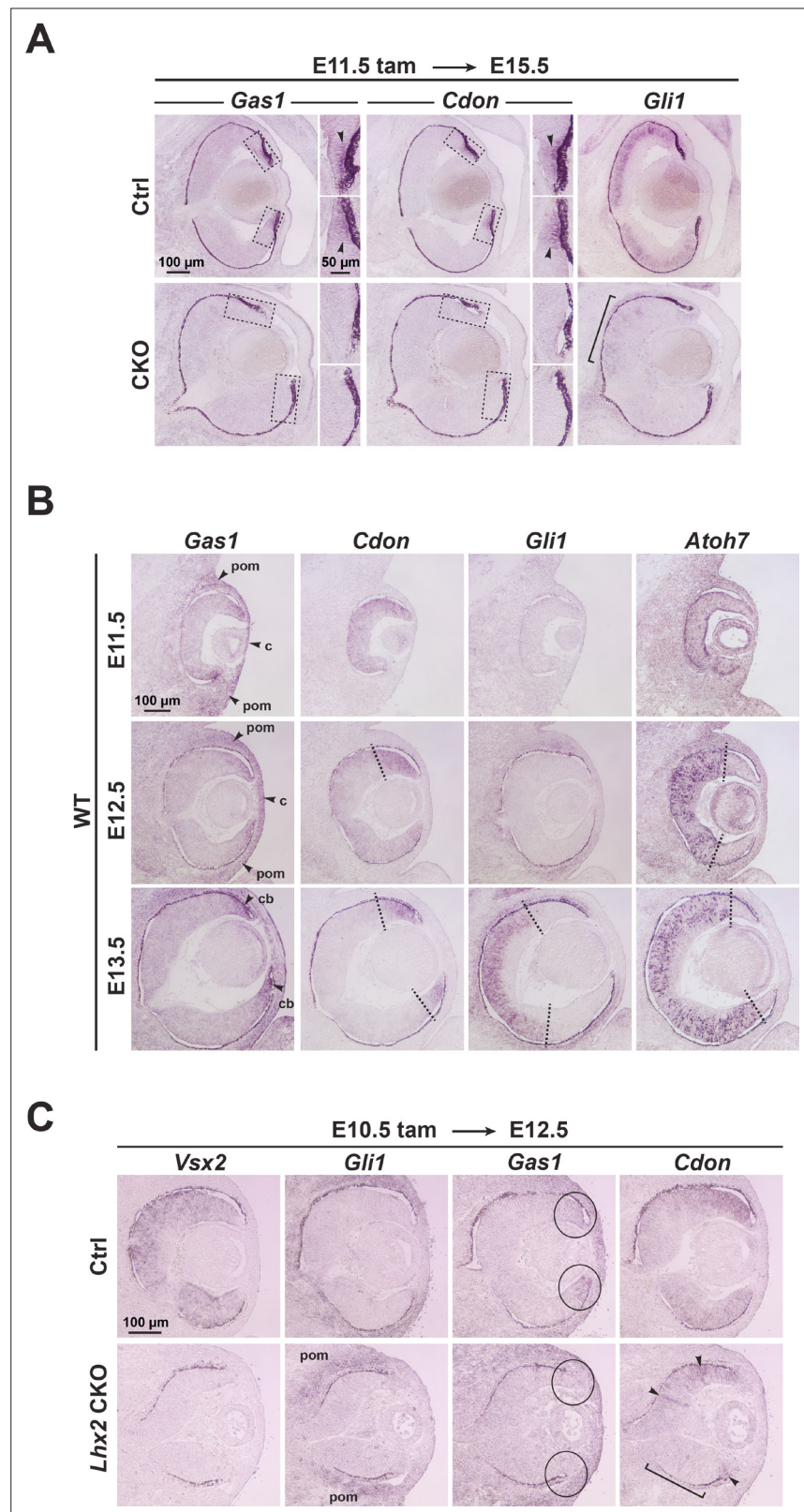


Figure 7—figure supplement 1. in situ hybridizations. (A) Expression of *Gas1*, *Cdon*, and *Gli1* at E15.5 after tamoxifen treatment at E11.5. Boxes in *Gas1* and *Cdon* panels mark locations of insets, which correspond to the retinal periphery, where *Gas1* and *Cdon* are expressed. Arrowheads in Ctrl panels show the restricted peripheral expression that is absent in the CKO. Bracket in CKO panel for *Gli1* expression reveals low, but persistent, Figure 7—figure supplement 1 continued on next page

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expression in the dorsal retina. **(B)** Temporal expression patterns of *Gas1*, *Cdon*, *Gli1*, and *Atoh7* from E11.5 to E13.5. Dashed lines in E12.5 and E13.5 images show that temporal downregulation of *Cdon* mRNA has a tighter spatial complementarity with *Atoh7* mRNA accumulation than *Gli1*. **(C)** Expression of *Vsx2*, *Gli1*, *Gas1*, and *Cdon* at E12.5 after tamoxifen treatment at E10.5. The presence of *Gli1* in the periocular mesenchyme indicates that the staining protocol was sufficient to detect expression in other tissues. Circles in *Gas1* images show regions of *Gas1* expression in peripheral retina that is lost upon *Lhx2* inactivation. Bracket in image of *Cdon* for the CKO image shows absence of *Cdon* in ventral domain, and arrowheads point to persistent *Cdon* expression. Abbreviations: c, cornea; cb, ciliary body; le, lens; onh, optic nerve head; nr, neural retina; pom, periocular mesenchyme.

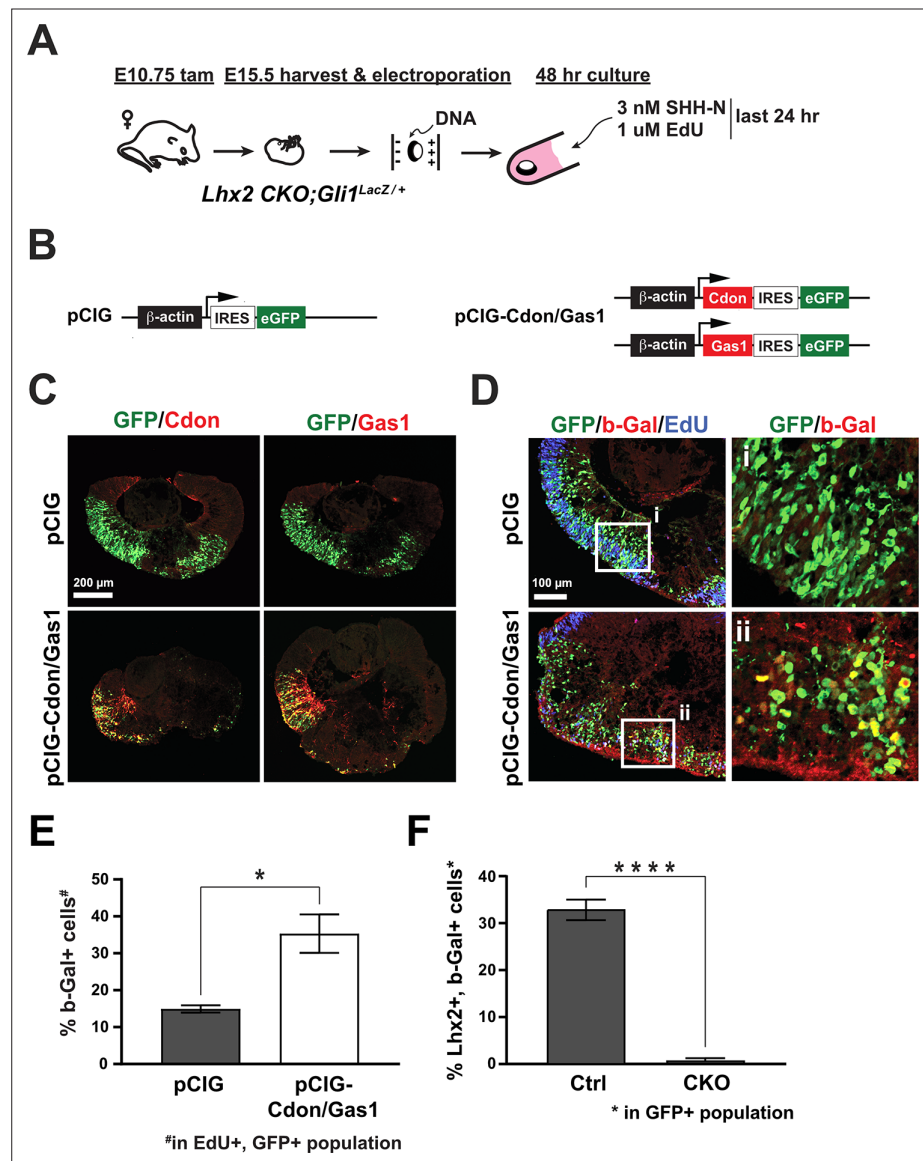


Figure 8. Cdon and Gas1 overexpression is sufficient to stimulate Shh signaling in the absence of Lhx2. (A) Experimental design for ex vivo electroporation and explant culture. $Lhx2$ CKO; $Gli1^{LacZ/+}$ explants were electroporated at the beginning of the culture. 3 nM Shh-N and 1 μ M EdU were added after 24 hr and cultured for an additional 24 hr. (B) DNA constructs used for electroporation. pCIG served as the control and pCIG-Cdon and pCIG-Gas1 were co-electroporated. (C) Upper panels: explants were electroporated with pCIG and co-stained for GFP and Cdon (left panel) or Gas1 (right panel). Lower panels: explants were co-electroporated with pCIG-Cdon and pCIG-Gas1 and co-stained for GFP and Cdon (left panel) or Gas1 (right panel). (D). Electroporated explants were co-stained for GFP, β -Gal, and EdU. Insets (i and ii) show GFP and β -Gal staining only. (E) Quantification of the percentage β -Gal+ cells in the EdU+, GFP+ cell populations from GFP (control) and Cdon/Gas1 electroporated $Lhx2$ CKO; $Gli1^{LacZ/+}$ explants (mean \pm S.E.M.; n=3, both conditions; *, p<0.05; n=3, both conditions; unpaired t-test). (F) Quantification of the percentage of Lhx2+, β -Gal+ cells in the GFP+ cell populations from control ($Lhx2^{\Delta/+}$; $Gli1^{LacZ/+}$) and $Lhx2$ CKO; $Gli1^{LacZ/+}$ explants electroporated with pCIG (mean \pm S.D.; n=7 (Ctrl); n=4 (CKO); *, p<0.0001; unpaired t-test) See **Supplementary file 6** for statistics. Single channel images for C and D and representative images for Ctrl ($Lhx2^{\Delta/+}$; $Gli1^{LacZ/+}$) and $Lhx2$ CKO; $Gli1^{LacZ/+}$ explants used for quantification in F are presented in **Figure 8—figure supplement 1**.

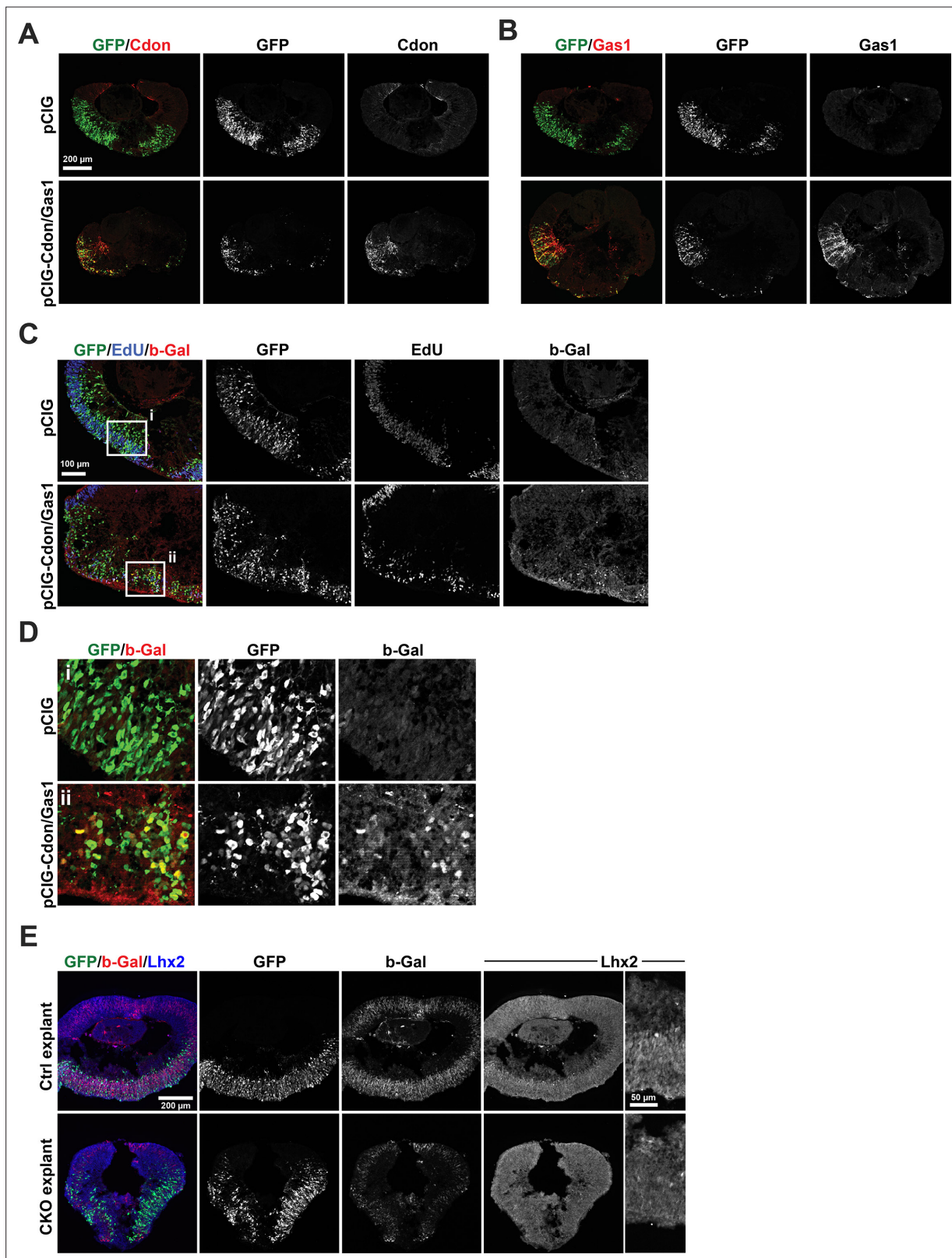


Figure 8—figure supplement 1. Single channel images for **Figure 8C and D**. **(A)** Merged and single channel images for left panels in 8C. **(B)** Merged and single channel images for right panels in 8C. **(C)** Merged and single channel images for left panels in 8D. **(D)** Merged and single channel images for right panels in 8D. b-Gal expression in C and D detects LacZ expression from the *Gli1^{LacZ}* allele. **(E)** Merged and single channel images for representative control and CKO explants electroporated with pCIG and stained for GFP, b-Gal, and Lhx2. Zoomed in views for Lhx2 are shown to emphasize the loss

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of Lhx2 protein in the CKO. The low signal to noise ratio for Lhx2 is due to the use of a secondary antibody conjugated to fluorophore in the 405 nm channel, which yields weak signal and elevated tissue autofluorescence and was necessary to perform the co-expression analysis (tdTomato occupied the 568 nm channel).

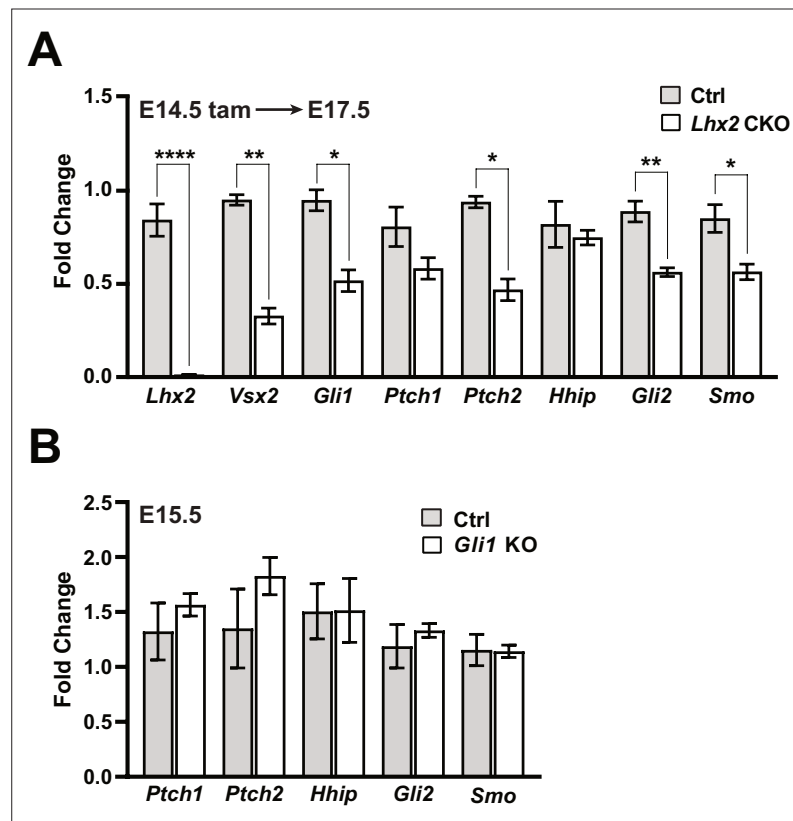


Figure 9. Lhx2 is required for sustained Shh signaling. **(A)** qPCR-based expression for *Lhx2*, *Vsx2*, *Gli1*, *Ptch1*, *Ptch2*, *Hhip*, *Gli2*, and *Smo* from E17.5 control and *Lhx2* CKO retinas following tamoxifen treatment at E14.5. For each gene, fold change values are relative to a specific control sample set as a reference. Only significant comparisons are noted (mean \pm S.E.M.; n=3 (Ctrl, all genes); n=4 (CKO, all genes); * $p_{adj} < 0.05$; ** $p_{adj} < 0.01$; **** $p_{adj} < 0.0001$; unpaired t-tests with correction for multiple comparisons; see **Supplementary file 3** for statistics) **(B)** qPCR-based expression for *Ptch1*, *Ptch2*, *Hhip*, *Gli2*, and *Smo* from E15.5 control (*Gli1^{LacZ/+}*) and *Gli1* KO retinas. For each gene, fold change values are relative to a specific control sample set as a reference. None of the comparisons were significant (mean \pm S.E.M; n=4 (both conditions, all genes); unpaired t-tests with correction for multiple comparisons; see **Supplementary file 3** for statistics).

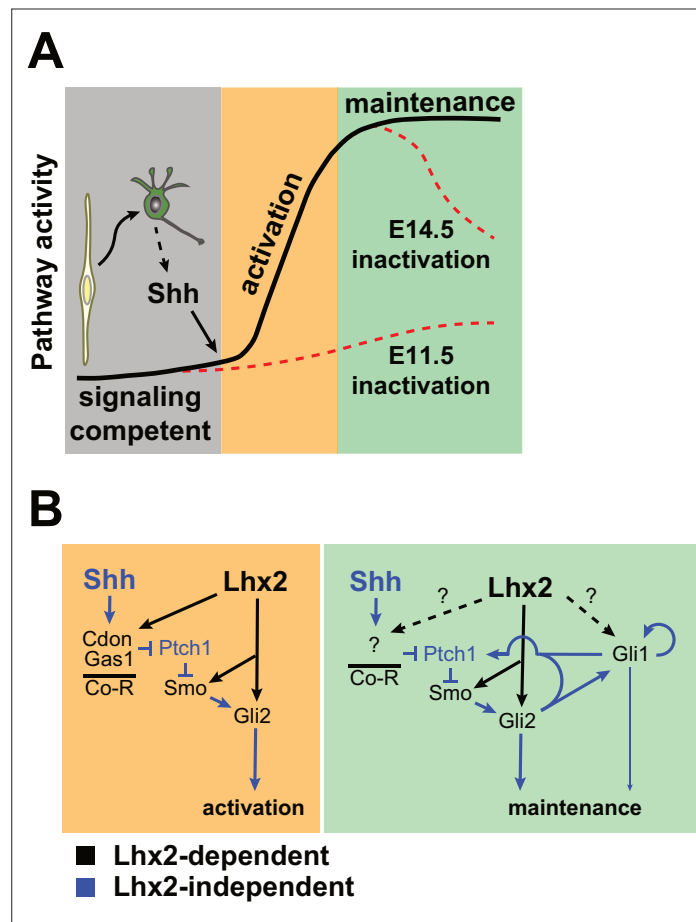


Figure 10. Models of interaction between *Lhx2* and Shh signaling during embryonic retinal neurogenesis. **(A)** At the cellular level, *Lhx2* promotes signaling competence in RPCs and once neurogenesis begins, RGCs produce Shh leading to pathway activation. This is revealed by *Lhx2* inactivation at E11.5. *Lhx2* also promotes the correct level of signaling in RPCs as evidenced by the drop in pathway readout gene expression when *Lhx2* is inactivated at E14.5. **(B)** Mechanistically, *Lhx2* promotes signaling competence and efficient activation by promoting the expression of the coreceptors (Co-R) for ligand reception, *Smo* for signal transduction, and *Gli2* for target gene activation. During the maintenance phase, *Lhx2* promotes signaling again by promoting *Smo* and *Gli2*. *Lhx2* may also regulate other coreceptors, *Gli1*, or other factors to promote efficient signaling.